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Tempeh flour as an excellent source of paraprobiotics

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Abstract. *Jonesti WP, Prihatna C, Natadiputri GH, Suwanto A, Meryandini A. 2023. Tempeh flour as an excellent source of paraprobiotics. Biodiversitas 24: 1817-1823.* Tempeh, a functional fermented food from Indonesia, has many benefits for human health. The presence of oligosaccharides, live and dead microbes in tempeh can function as prebiotics, probiotics, or paraprobiotics. This study aimed to determine the composition of live and dead lactic acid bacteria (LAB) and Enterobacteriaceae in tempeh flour using microbiological and molecular quantifications. Two samples of tempeh flour based on the drying process, i.e., sun-dried and oven-dried, were analyzed. Bacteria were quantified using standard plate count and qPCR, while bacterial cells damaged by the drying process were quantified using Propidium Monoazide-Quantitative Polymerase Chain Reaction (PMA-qPCR). Tempeh flours contained live bacteria as many as 4-5 log CFU/g and intact dead bacterial cells as many as 9 log CFU/g. The number of bacteria only differed slightly between the two drying processes. Based on qPCR analysis, the LAB number was higher than Enterobacteriaceae in tempeh flour. In addition, the bacterial population by qPCR was higher than by cultured analysis. These results indicated that sun-dried or oven-dried tempeh flour could be a functional food because it contained high amounts of live and dead bacteria as candidates for probiotics and paraprobiotics based on PMA-qPCR analysis.

Keywords: Paraprobiotic, PMA-qPCR, probiotic, tempeh flour

INTRODUCTION

In recent years, vegetable protein from soybeans (*Glycine max*) has been in great demand due to its health claims. However, soybeans' amino acid content remains lower than animal products (Vliet et al. 2015). Fermentation is the solution to increasing soybeans' amino acid content (Ali et al. 2016). Microbial catabolic reactions in the fermentation process cause biochemical and nutritional changes in soybean seeds. This process will increase the nutritional value and digestibility of food ingredients. One example of fermented food made from soybeans is tempeh.

Tempeh is a functional food from Indonesia made through fermentation and has many benefits for human health (Nout and Kiers 2005). Living microorganisms in tempeh can act as probiotics that have a good effect on digestive health. Tempeh contains non-digestible-galactooligosaccharides that can act as prebiotics to stimulate microbiota growth in the digestive tract (Stephanie et al. 2019). Microbial bodies that die in processing can become paraprobiotics to stimulate the body's biological response (Stephanie et al. 2017).

Tempeh has a relatively short shelf life due to its high-water content and mold that continues to grow, causing spoilage (Romulo and Surya 2021). The usefulness of tempeh can be extended by making tempeh flour. Generally, the process of making tempeh flour goes through the stages of cutting, drying, milling, and sifting. Tempeh flour has better protein quality when compared to boiled soybean flour and casein, so it can be used as a formulation of baby porridge and as an ingredient in making bread (Tampubolon et al. 2014; Astawan et al. 2015; Huang et al. 2019).

The fermentation process can create a consortium of microorganisms in tempeh (Yulandi et al. 2020). These microorganisms are likely carried into the tempeh flour because some bacteria are thermos-tolerant (Efriwati et al. 2013). Therefore, the presence of bacteria is advantageous as candidates for probiotics and paraprobiotics suitable for the digestive system. Furthermore, to analyze the bacterial contents, the cultured method can determine the composition with a standard plate count. While the uncultured method by metagenomic analysis through Quantitative Polymerase Chain Reaction (qPCR) analysis. The qPCR method is very well used to count bacteria in the food matrix and has been used to determine the number of LAB and Enterobacteriaceae in tempeh by other researchers (Erdiansyah et al. 2021; Ilham et al. 2021).

One of the limitations of qPCR analyses is that it has not been able to distinguish between live and dead cells because all DNA that matches the primer will be amplified simultaneously. Developing the qPCR method with propidium monoazide (PMA-qPCR) succeeds in overcoming this problem. Moreover, adding PMA before DNA extraction only enters dead bacterial cells, causes cross-linking with DNA bands, and inhibits the DNA amplification process (Li and Chen 2012). PMA-qPCR has

been proven suitable for quantifying probiotics in fermented products (Scariot et al. 2018).

Potential microbes in tempeh can function as probiotics when the cells are alive and paraprobiotics when the cells are dead, in both intact and damaged cells. Cells that have died and undergone lysis are broken down into cell components, or "postbiotics" (Cuevas-González et al. 2020). Probiotics are defined as living microbes with cells that are still intact and that provide health benefits to the host given in sufficient quantities. Health effects are impacted by preventing and treating digestive system diseases, such as diarrhea, colitis, irritable bowel syndrome, *Helicobacter pylori* infection, allergies, and lactose intolerance (Sánchez et al. 2017). Many probiotics come from *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Pediococcus*, and some yeast. The probiotic mechanisms have impacts on the gut microbiota and enhance immune function. Therefore, the effective probiotic criteria for health are tolerance to acids and bile, adhesion to the mucosal and epithelial surfaces, antimicrobial properties against pathogenic bacteria, and bile salt hydrolase activity (Kechagia et al. 2013).

Moreover, survival is only recognized to qualify the probiotics. Therefore, health effects can also be obtained from paraprobiotics or postbiotics. Tempeh flour made through a drying process by heat will kill many bacteria. Therefore, tempeh flour has the potential to be a nutraceutical product because it is rich in paraprobiotics and postbiotics with various functional and bioactive properties through direct or indirect mechanisms. The direct mechanism occurs due to the interaction between postbiotics or paraprobiotics with various molecules or receptors through inhibitory actions such as antimicrobials, antioxidants, and immunomodulators (de Almada et al. 2016). Indirect mechanisms occur through microbiota homeostasis and host metabolic and signaling mechanisms to affect specific physiological reactions (Sharma dan Shukla 2016). The absence of information regarding the number of live and dead bacteria candidates for probiotics and paraprobiotics in tempeh flour makes this research necessary because it relates to the great benefits after that.

MATERIALS AND METHODS

Sample preparation

Fresh tempeh samples were obtained from tempeh producers in Empang Village (Bogor Regency, West Java, Indonesia). First, tempeh flour was made through the cutting stages with a thickness of ±1 mm, then dried in the sun for one day or in a Memmert UM 400 oven at 60°C for 24 h, grinding using a blender, and sifting using an 80-mesh sieve. Next, the flour samples were differentiated based on the drying method, sun-dried tempeh flour (CMS), and oven-dried tempeh flour (OVS). Finally, the microbiological and molecular quantification of tempeh flour bacteria was conducted at the Biotechnology Research and Development Laboratory of PT. Wilmar Benih Indonesia (Bekasi Regency, West Java, Indonesia).

Procedures

Propidium monoazide (PMA) treatment validation

Single colonies of *Escherichia coli* TOP10 (collections of PT. Wilmar Benih Indonesia, Bekasi, West Java, Indonesia) were grown on 10 mL Luria-Bertani (LB) media. Then, an overnight incubation was carried out at 37°C at 200 rpm. As much as 200 µL of cultures were transferred to new LB media, and incubation was carried out until Optical Density (OD)=1.5. The OD was measured at 600 nm using the GENESYS 10S Series UV-Visible Spectrophotometers (Thermo Scientific, Madison, WI, USA). At the same time, cells were harvested by centrifugation 5,000×g for 2 min. Next, the pellets were resuspended in 0.85% NaCl (OD=1.5). A total of 500 µL aliquots were used for assays with-without PMA with live percentages of *E. coli* (0.1% and 100%). DNA was extracted and quantified for analysis using qPCR with primers 16S rRNA, 341F (5′-CCTACGGGAGGCAGCAG-3′), and 518R (5′-ATTACCGCGGCTGCTGG-3′). In addition, 100 µL of this suspension was also used for bacterial quantification by culturing on Luria-Bertani Agar (LA) medium overnight.

Propidium monoazide (PMA) treatment

First, the PMA (Biotium Inc., Hayward, CA, USA) concentration of 20 mM was prepared in 20% dimethyl sulfoxide (Merck, Darmstadt, Germany) and stored at -20 °C in the dark. Next, a 1.2 µL PMA of 20 mM was added to a 500 µL aliquot and incubated for 5 min in the dark with occasional mixing. Aliquots were exposed to LED Grow light 4229.67±134.29 lux for 2 min at 20 cm from the light source and placed horizontally on the ice. Aliquots were centrifuged at 16,000×g for 2 min. The pellets were resuspended in 500 µL of 0.85% NaCl and used for DNA extraction.

Genomic microbial DNA extraction

First, 50 g of tempeh flour was homogenized in 200 mL of 0.85% NaCl and centrifuged at 1,000×g speed for 10 min at 4°C. Next, the supernatant was centrifuged at 17,000×g for 10 min at 4°C. Finally, the pellets were resuspended in 2 mL of 0.85% NaCl. An aliquot of 500 µL suspension was used for experiments with and without PMA addition. Aliquots of this treatment were extracted to obtain DNA using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Microbiological analysis

First, 100 µL of the bacterial suspension was serially diluted to 10^{-4}. Then, the suspension was spread on Man-Rogosa Sharpe Agar (MRSA) media with the addition of 0.5% (w/v) calcium carbonate ($CaCO_3$) (Merck, Darmstadt, Germany) and 5% (v/v) ProPlant C (an in-house peptide-based antifungal compound) (Prihatna et al. 2022) to determine the LAB population. Next, Eosin Methylene Blue Agar (EMBA) media (Merck) was used to grow bacteria from the Enterobacteriaceae family. Next, aerobic incubation at room temperature for two days was carried out to grow Enterobacteriaceae. Next, microaerophilic

incubation was carried out using a candle jar at 37°C for two days to grow LAB. Finally, the number of colonies growing on the dish was calculated as Colony Forming Units (CFU)/g.

Quantification of qPCR

Analysis of qPCR used primers SKfw (5′-GGGGATAACAYYTGGAAACAG-3′) and SKrw (5′-CTCGGCTACGTATCATTGTCTTG-3′) for quantification of LAB with 178 bp PCR product. In addition, Enterobacteriaceae were quantified using primer *rplP* 1F (5′-ATGTTACAACCAAAGCGTACA-3′) and primer *rplP* 185R (5′-TTACCYTGACGCTTAACTGC-3′) with 185 bp PCR product. The qPCR reactions were carried out in a total volume of 25 µL with the following composition, 12.5 µL SensiFAST™ HRM Kit (Bioline Inc. USA), each primer (10 pmol/µL), DNA template 2 µL (15 ng/µL), and NFW.

Moreover, the CFX96 Real-Time Detection System (Bio-Rad Laboratories Pte. Ltd. Singapore) was used to amplify the reaction. The amplification protocol for LAB was carried out under the following cycles: 5 min pre-denaturation at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 54°C, and 30 s elongation at 72°C. The amplification reaction conditions for Enterobacteriaceae were as follows; 5 min pre-denaturation at 95°C, followed by 39 cycles of 15 s denaturation at 95°C, 30 s annealing at 59°C, and 1 min elongation at 72°C. The fluorescence signal is read at each 72°C elongation step's end. Next, the Melt curve analysis was conducted at 65°C to 95°C. Finally, LAB and Enterobacteriaceae (CFU/g) were calculated by the following equation (Ilha et al. 2016);

$$Quantity\ of\ bacteria\ (\mathrm{CFU/gr}) = \frac{A \times B \times C}{D \times E}$$

where A is defined as the number of bacteria (CFU) obtained from the sample Ct using a standard curve equation, while B is defined as the concentration of extracted DNA (ng/µL). Next, C is defined as the total volume of extracted DNA (µL), D is the mass of the DNA template in the qPCR reaction (ng), and E is the sample mass for DNA extraction (g).

Standard curve construction

Standard curves were made using genomic DNA from *Lactobacillus fermentum* H.R_B7 and *Klebsiella pneumoniae* H.R_E10; Both were culture collections of Wilmar Benih, Indonesia. DNA has serially diluted to 10^9 to 10^3 gene copies per 3 µL. The number of bacterial gene copies was determined from calculations based on the genome size of the *L. fermentum* strain (CP025592.1) and *K. pneumoniae* strain (FO834906.1), Avogadro constant (6.023×10^{23}) and DNA molecular weight (660 Da/bp). The standard curve was made by plotting the logarithm of the number of bacterial gene copies against the cycle threshold (Ct) value. In addition, one gene copy represents only one bacterial CFU.

Data analysis

Statistical analysis and data visualization of LAB and Enterobacteriaceae populations were performed using R version 4.2.0 utilizing the ggplot2 and the ggpubr packages.

RESULTS AND DISCUSSION

Validation of propidium monoazide treatment

The composition of intact and damaged cells was determined using qPCR with the addition of PMA. The Ct (ΔCt) value difference was quite clear between the PMA-induced and non-PMA-induced treatments, with a maximum value is about 3.24 at a percentage of 0.1% and a minimum value of 0.33 at 100% (Figure 1). The qPCR quantification results with the PMA combination were lower than qPCR alone. That followed the expectation that PCR efficiency would decrease because some cells had membranes damaged due to heating, and then PMA could enter and inhibit the PCR process. As a result, the threshold cycle (Ct) value became higher during the qPCR amplification process. Based on research from Erkus et al. (2016) stated that PMA treatment could provide a maximum difference in Ct values of around 6.6. In addition, according to Soejima et al. (2012), treatment with PMA usually underestimated the number of dead bacteria on qPCR results of up to 3.5 log. The Ct value was higher for the dead cell population due to PMA penetration and DNA modification, so the amplification process did not occur. PMA was selective and only entered dead bacteria with damaged cell membranes, not intact ones.

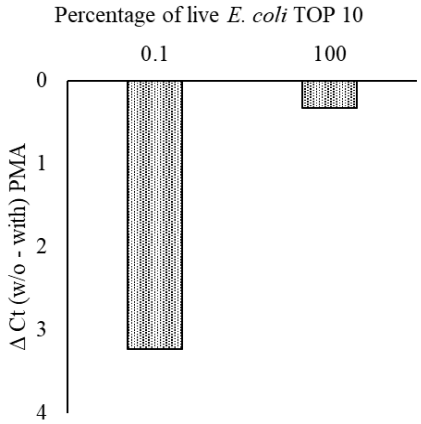

Figure 1. Validation of propidium monoazide (PMA) treatment based on the percentage of live *Escherichia coli* TOP 10. The cycle threshold (Ct) value was obtained from the difference in treatment with-without PMA

Standard curve and limit of detection

LAB and Enterobacteriaceae were calculated using a standard curve based on the gene copy numbers in *L. fermentum* and *K. pneumoniae.* The results of the qPCR reaction were reliable because several parameters, such as efficiency, correlation coefficient, and slope, were at optimum values. The efficiency of qPCR reactions in LAB using SK primers targeting the 16S rRNA region was 97.5%. The correlation coefficient (R^2) value was 0.9961, and the slope was -3.833 (Figure 2). That is aligned with previous studies, which resulted in a correlation coefficient (R^2) of 0.99 (Pontonio et al. 2017). The results of the Enterobacteriaceae qPCR reaction using a primer targeting the *rplP* gene in the L16 region of the ribosomal protein had an efficiency value of 106.2%, a correlation coefficient (R^2) of 0.9767, and a slope of -3.1749 (Figure 3). This result can be seen from the correlation coefficient's value, which follows the report of Takahashi et al. (2017). In addition, Broeders et al. (2014) stated that the optimum efficiency value is 90-120%, and the correlation value is 0.98.

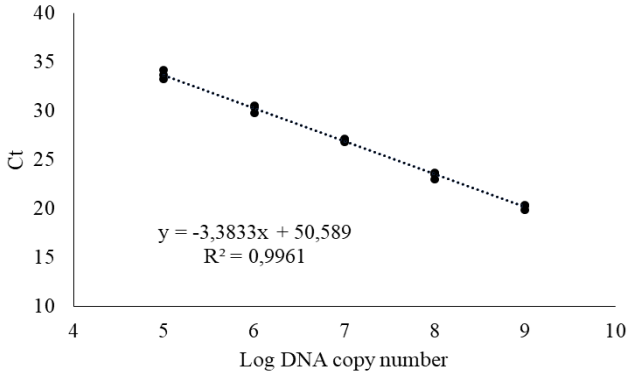

Figure 2. Standard curve for quantifying lactic acid bacteria (LAB) in qPCR analysis. The displayed value was the average result of three repetitions

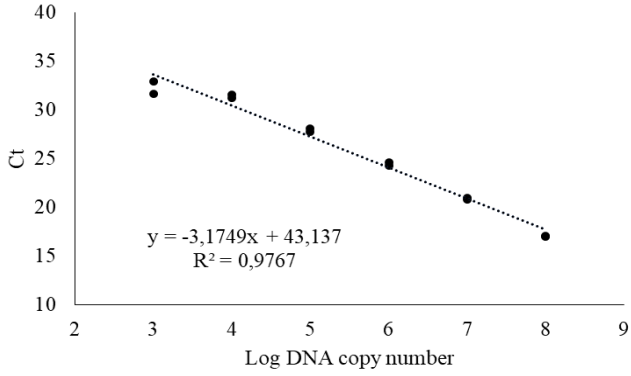

Figure 3. Standard curve for quantifying Enterobacteriaceae in qPCR analysis. The displayed value was the average result of two repetitions

The detection limits on the standard curves of LAB and Enterobacteriaceae was 5-9 log CFU and 3-8 log CFU, respectively. The detection limit can be defined as the smallest amount calculated on the sample based on the curve. The smallest amount that can be detected on the LAB curve at 5 log CFU was not quite low. However, this range limitation still permitted calculating the number of LAB in samples ranging from 8-9 log CFU. In the Enterobacteriaceae curve, the minor limit reached 3 log CFU, and the number of Enterobacteriaceae calculated in the sample ranged from 5-6 log CFU. The reports of previous research results with a detection limit value of 4 log CFU were used to analyze LAB and Enterobacteriaceae populations in tempeh (Erdiansyah et al. 2021; Ilham et al. 2021). In several reports, the LAB detection limit value obtained was 2.78 log CFU of *L. paracasei* from yogurt (Ilha et al. 2016), 2-3 log CFU of *L. plantarum* and *L. fermentum* in cocoa fruit (Schwendimann et al. 2015). Then a detection limit of 2-4 log CFU was reported by Takahashi et al. (2017) to count Enterobacteriaceae such as *Citrobacter freundii, Enterobacter aerogenes, E. coli, Proteus mirabilis,* and *K. pneumoniae*.

Lactic Acid Bacteria and Enterobacteriaceae populations

This study analyzed the number of lactic acid bacteria (LAB) and Enterobacteriaceae in tempeh flour. Those bacteria could be the candidates for probiotics and paraprobiotics suitable for the digestive and immune systems. The number of LAB and Enterobacteriaceae in the sun-dried and oven-dried tempeh flours did not significantly differ. LAB quantification resulted in approximately 9 log CFU/g by qPCR and 4-5 log CFU/g by cultured (Figure 4). This proportion was relatively high, similar to the total found in fresh tempeh at 10 log CFU/g (Erdiansyah et al. 2021). Moreover, tempeh contained thermophilic bacteria that could survive after heating (Ilham et al. 2021). Some bacteria could be in a "quiescent" stationary phase and did not experience morphological differentiation due to environmental stress, such as heating and drying (Rittershaus et al. 2013). The number of Enterobacteriaceae in tempeh flour was approximately 6 log CFU/g, and almost all were culturable (Figure 5); this total was lower than fresh tempeh, at 8 log CFU/g (Ilham et al. 2021). The drying process was the main factor in reducing the number of bacteria, but it did not eliminate them. In addition, some bacteria just simply more resistant to heat and desiccation (Everis 2001).

The PMA-qPCR method was successfully applied to count the number of intact and damaged bacterial cells in tempeh flour. On the other hand, the qPCR without PMA estimated total bacterial counts, both intact and damaged cells. In contrast, qPCR with PMA estimated the number of intact bacterial cells only (Nocker et al. 2007). The number of bacteria in qPCR and PMA-qPCR difference was insignificant, indicating that most bacterial cells in tempeh flour were intact. The result of PMA treatment validation was carried out and showed promising results. Therefore, more attention was conducted to the level of cell damage that was less severe in dead bacteria due to the drying process during the manufacture of tempeh flour. That made tempeh flour rich in intact dead bacterial cells, supposed to be a candidate for paraprobiotics.

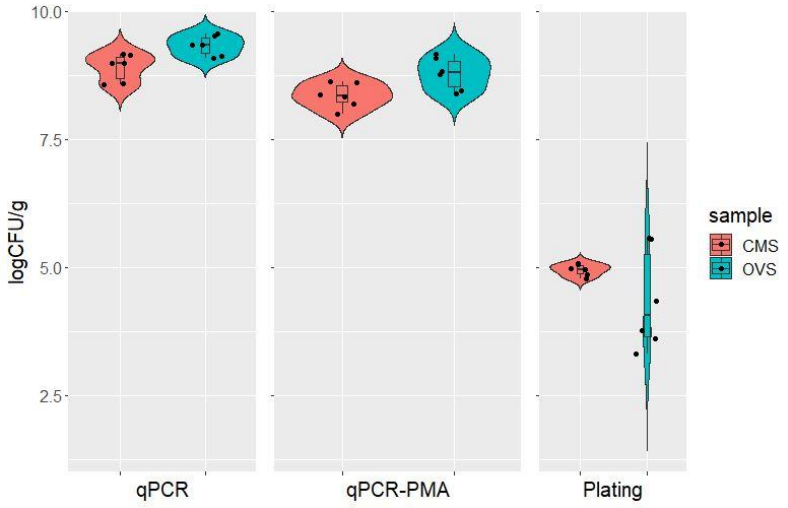

Figure 4. Comparison of the number of Lactic Acid Bacteria (LAB) using cultured and uncultured analysis (qPCR and PMA-qPCR). The displayed value was the average result of six repetitions

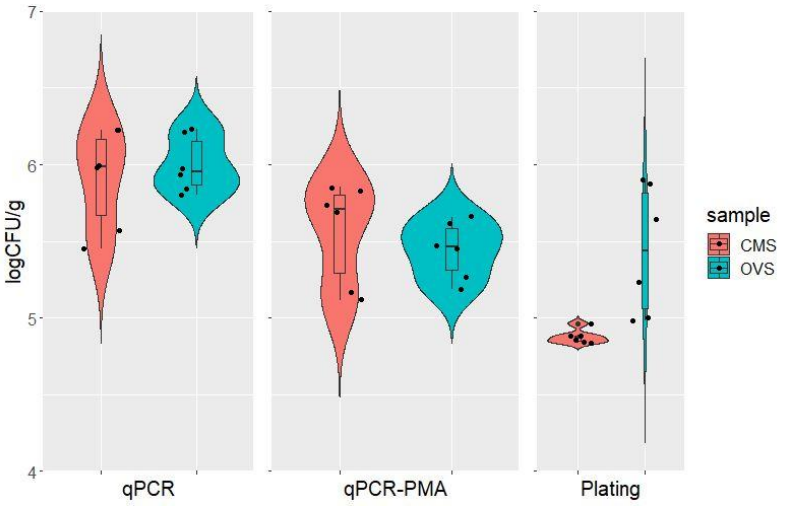

Figure 5. Comparison of the number of Enterobacteriaceae using cultured and uncultured analysis (qPCR and PMA-qPCR). The displayed value was the average result of six repetitions

Foods containing paraprobiotics could be the natural vaccines to increase the body's immune system. Furthermore, in the health sector, tempeh flour formula could be used as an antidiarrheal (Soenarto et al. 2001). Then the bacterial cells found in tempeh, even though cooked, could increase the secretion of IgA protein (Soka et al. 2015). Therefore, paraprobiotics in food ingredients were considered safer and better for health even though they were given to immunocompromised people (Siciliano et al. 2021). Other several benefits that could be obtained were increased resistance to common cold symptoms and mood control (Murata et al. 2018), regulation of intestinal function (Sawada et al. 2016), and prevention and treatment of intestinal diseases (Buckley et al. 2018). Based on the research that has been done, it could be observed that the benefits of paraprobiotics could be taken from consuming tempeh flour. This result follows the promised benefits of tempeh products as a functional food.

In conclusion, the PMA-qPCR combination was successfully used to selectively analyze live and dead LAB and Enterobacteriaceae in tempeh flour. PMA increased the value of Ct in the sample and indicated many bacterial cells that had died but were still intact with data comparing the total number of bacteria and the number of culturable bacteria. Tempeh flour still contained live bacteria as many as 4-5 log CFU/g based on cultured analysis and had intact dead bacterial cells as many as 9 log CFU/g based on PMA-qPCR analysis. The drying type used only produced a slight difference in the number of bacteria. The number of LAB was higher than Enterobacteriaceae in both samples of tempeh flour through qPCR analysis and less in cultured analysis. Therefore, tempeh flour could be used as a functional food and expand its use of tempeh.

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