Antibacterial Activity of Garlic Added to Tempeh against Bacillus sp. and Escherichia coli

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ABSTRACT
Tempeh (tempe) has been known to have antibacterial properties due to the presence of glycoprotein compounds, antimicrobial peptides, and flavonoids produced by the fungus Rhizopus spp. during the fermentation process. The addition of garlic powder is expected to increase the antibacterial activity of tempeh against contaminant bacteria. This research aimed to analyze the antibacterial activity, water content, and organoleptic properties (taste, aroma, and color) of tempeh with garlic powder supplementation. Garlic powder was added to the tempeh fermentation process with various concentrations of 0.15, 0.25, 0.50, and 0.75%. Antibacterial activity, water content, and qualitative organoleptic tests were analyzed using paper disk diffusion, gravimetric, and questionnaire methods. The results showed that the addition of garlic powder in all treatments for Bacillus sp. had shown an inhibitory activity with moderate strength with an inhibitory power of 0.15; 0.25; 0.50; and 0.75% respectively 8±1.71; 10±1.71; 7±0.96; 9±0.58. Meanwhile, Escherichia coli showed no inhibitory activity. The addition of garlic powder at a concentration of 0.25% reduced the water content to 58.25±0.03. In addition, the addition of garlic powder was not able to affect the color but was able to influence the aroma and taste of tempeh. Garlic powder was able to inhibit Bacillus sp with the best concentration of 0.25%; the addition of garlic powder can reduce the water content and affect the aroma and taste of tempeh.

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INTRODUCTION
Tempeh (tempe) is a fermented food that is highly demanded among Indonesia's due to its high protein. According to Banobe et al. (2019), Tempeh made from soybeans has a protein content of 27.42%, fat content of 15.94%, carbohydrate content of 49.38%, and water content of 5.43%. A fermentation process using Rhizopus spp. is required to make tempeh. The fermentation process will result the formation of white hyphae that resemble thread strands that will envelop soybeans. The number of hyphae will be affected by the duration of the fermentation, and if there are more, they can bind soybeans into a single unit. Because of the dense mycelium, the tempeh structure will become compact and dense (Barus et al., 2019).

During the fermentation process, various types of natural additives can be added to tempeh, such as spices to improve the product's nutrition, taste, function, and attractiveness. According to Rahmi et al. (2018), garlic (Allium sativum) is one of the sources of natural additive. Allicin, alliinase, S-allyl cysteine, diallyl sulfide, and allyl methyl trisulfide are all found in garlic bulbs. When compared to other types of thiosulfate compounds, Allicin have the highest composition, approximately 70-80%. This allicin compound is responsible for the flavor and aroma of garlic. The allinase enzyme converts the aline compound into Allicin when the garlic
bulb is destroyed (Moula et al., 2018). In addition, Allicin has antimicrobial properties for both gram-positive and gram-negative bacteria, as well as antifungal properties (Salima, 2015).

Basically, *Rhizopus* spp. can produce antibacterial compounds such as glycoproteins, antimicrobial peptides, and flavonoids on their own during the tempeh fermentation process (Noviana et al., 2018). The addition of garlic powder is expected to be a solution for increasing the inhibitory power on the growth of unwanted gram-negative and gram-positive bacteria. The addition of garlic to the tempeh fermentation process can achieve the proper concentration of allicin compounds to inhibit contaminant bacteria in tempeh. However, another potential finding is that the identification process of excessive concentrations has the potential to inhibit the metabolic process and growth of *Rhizopus* spp. This antibacterial property can be tested by looking at a bright inhibition zone around the colony’s growth. Because the active compounds in the extract inhibit the bacterial growth, this zone indicates the absence of it (Mawaddah et al., 2018).

In this study, garlic bulbs were powdered and added to the tempeh fermentation process at various concentrations to increase the nutritional value and antibacterial properties tempeh. Through this process, allicin compounds from garlic bulbs that are antibacterial are not removed to determine the level of antibacterial activity or strength produced by adding garlic bulbs during the tempeh fermentation process. Rahmi et al. (2018) experimented with the addition of garlic during the tempeh fermentation process, and it has been shown to improve the quality of tempeh's taste, aroma, color, and texture. But the difference is the research conducted by (Rahmi et al., 2018) only reviewed the nutritional content, even though the compounds contained in garlic have the potential to not only increase nutritional content but also have the potential to inhibit contaminant bacteria if tempeh is made in an unhygienic way.

According to Rahmi et al. (2018), garlic can also increase protein levels in tempeh. However, it can also increase several types of essential and non-essential amino acids, even though not significantly. It might because of the soybeans was soaked in garlic and steamed for 15–20 minutes. This treatment causes the loss of allicin compounds attached to soybean seeds since these compounds are thermolabile. Allicin compounds have numerous health benefits, including being a source of antioxidants that can prevent the body producing nitrate dioxide (Sudjatini, 2020), having antidiabetic properties (Liswanti and Haryanto, 2017), and being able to lower blood pressure, reduce platelet aggregation, prevent hyperlipidemia and boost immunity (Rahman, 2014). This study aimed to determine the effect of garlic powder addition to the antibacterial activity, water content, and organoleptic properties of tempeh.

**MATERIALS AND METHODS**

This research was conducted in September – November 2021 at the Microbiology and Biochemistry Laboratory, Faculty of Biology, Satya Wacana Christian University. The materials used in this study included garlic bulbs (*Allium sativum*), boiled soybeans from tempeh maker in Gamasan, Bandungan, Central Java, tempeh yeast (RAPRIMA, Indonesia), ethyl acetate (Degrees of Analysis, Merck Millipore, Germany), nutrient agar (Merck Millipore, Germany), NaCl (Degrees of Analysis, Merck Millipore, Germany), tetracycline antibiotics 30 mcg (Oxoid, USA), filter paper, and disk paper (Oxoid, USA).

**Samples preparation**

Garlic powder was made by drying garlic bulbs in a dehydrator at 60°C for 1–3 hours. When the garlic bulbs have dried, then crushed using blender until turned into powder and ready to be added in tempeh fermentation process. The process of making tempeh begins by inoculating 2 grams of starter inoculum into 100 grams of boiled soybeans, then garlic powder was added with concentrations of 0%(control), 0.15, 0.25, 0.50, and 0.75%. The mixtures were put in a plastic ziplock and then punched with a toothpick to keep oxygen circulating during the tempeh fermentation. Then incubated at room temperature for 48 hours.

**Antibacterial activity assay**

An antibacterial test was carried out using a disk diffusion assay. The tempeh was extracted by maceration method using ethyl acetate. Fifty grams of mashed tempeh were mixed with ethyl acetate until all sample parts were submerged. The container was closed and stored at room temperature in the dark for three days under stirring conditions at a speed of 150 rpm. The ethyl acetate solvent was changed daily to maximize the obtained extract. The macerate was concentrated using a rotary vacuum evaporator at 45°C. The resulting thick section was aerated with nitrogen gas to evaporate the remaining solvent completely. The antibacterial test was carried out against
**RESULTS AND DISCUSSION**

**The effect of garlic powder addition to the antibacterial activity of tempeh**

Antibacterial activity is a test used to determine the strength or ability of antibacterial substances to inhibit the growth of bacteria (Afriani et al., 2017). The results of the One Way Anova test with a 5% level of antibacterial activity against *Bacillus sp* showed that each treatment with the addition of garlic powder showed a significant difference in the effect on the antibacterial activity of tempeh (*p*<0.05) (Table 1). The result shown inhibition of the growth of *Bacillus sp* that might be caused by an antibacterial compound from Allicin (Table 2, Figure 1). This compound has been known to have antimicrobial activity by blocking the synthesis of RNA, DNA, and protein (Purwantiningsih et al., 2019). Allicin compounds can also inhibit the synthesis of phospholipids in the bacterial cell wall, which results in increases the permeability of the cell wall (Purwantiningsih et al., 2019). Increases the permeability of cell wall also causes the sulphhydril and disulfide groups in cystine and cysteine to undergo lysis, as a result protease enzymes cannot synthesize and the cell proliferation process becomes inhibited (Purwantiningsih et al., 2019).

In addition, garlic contains several flavonoid compounds that can interfere with cell membrane permeability by forming hydrogen bonds with extracellular proteins and integral proteins on bacterial cell membranes. This bond involves absorption reactions and occurs in the hydrophilic part of the membrane. Furthermore, the phenol–protein complex will enter the cell membrane and cause protein precipitation, resulting in the inactivation of enzymes in bacteria, disruption of the nutrient transport process, and increased membrane permeability (Purwantiningsih et al., 2019). Increased membrane permeability can also cause metabolic enzymes to diffuse out of the cell so that the reaction for the formation of ATP as an energy source for cell growth cannot run properly (Mawaddah et al., 2018). Based on research conducted by Soraya et al. (2018), garlic also contains tannin compounds that can inhibit the performance of proteolytic enzymes so that protein macromolecules cannot be broken down into their constituent amino acids. As a result, bacteria cannot use protein molecules to support their growth.

The bacterial inhibition mechanism can also be caused by the presence of glycoprotein compounds,
### Table 1. One way anova test of antibacterial activity of garlic tempeh against *Bacillus* sp.

|                          | Anova                      |
|--------------------------|----------------------------|
|                          | Sum of Squares | df | Mean Square | F    | Sig.     |
| Between Groups           | 19.800          | 4  | 4.950       | 3.375| .037     |
| Within Groups            | 22.000          | 15 | 1.467       |      |          |
| Total                    | 41.800          | 19 |             |      |          |

### Table 2. Strength category of antibacterial activity of garlic tempeh against *Bacillus* sp.

| Treatments               | Inhibition Zone Diameter (mm) | Category*     |
|--------------------------|-------------------------------|---------------|
| Positive control (Tetracycline) | 25                            | Very strong   |
| Negative control (0 %)     | 8±0.50                        | Medium        |
| 0.15%                    | 8±1.71                        | Medium        |
| 0.25%                    | 10±1.71                       | Medium        |
| 0.50%                    | 7±0.96                        | Medium        |
| 0.75%                    | 9±0.58                        | Medium        |

Note: *Category based on (Mawaddah et al., 2018) ≤ 5 mm, weak; 5–10 mm, medium; 10–20 mm, strong; ≥ 20 mm, very strong

### Table 3. Strength category of antibacterial activity of garlic tempeh against *Escherichia coli*

| Treatments               | Inhibition Zone Diameter (mm) | Category*     |
|--------------------------|-------------------------------|---------------|
| Positive control (Tetracycline) | 27                            | Very strong   |
| Negative control (0%)     | 0±0                           | No inhibitory zone |
| 0.15%                    | 0±0                           | No inhibitory zone |
| 0.25%                    | 0±0                           | No inhibitory zone |
| 0.50%                    | 0±0                           | No inhibitory zone |
| 0.75%                    | 0±0                           | No inhibitory zone |

Note: *Category based on (Mawaddah et al., 2018) ≤ 5 mm, weak; 5–10 mm, medium; 10–20 mm, strong; ≥ 20 mm, very strong

Antimicrobial peptides, and flavonoids produced by the fungus *Rhizopus spp.* itself (Noviana et al., 2018). Based on Noviana et al. (2018), glycoprotein compounds can bind to iron which acts as a cofactor for various enzyme performances in the cellular respiration system and as a cytochrome component in bacterial cells, the presence of this bond will cause iron deficiency and inhibit the growth *Bacillus* sp. Glycoprotein compounds can only inhibit the growth of *Bacillus* sp. as a gram-positive bacteria because its cell structure is simple and it has a thin cell wall ranging from 25-30 μm. The cell wall consists of one layer composed of 90% peptidoglycan and 1–4% lipids, so it causes glycoproteins can penetrate cell walls more easily (Noviana et al., 2018). Then antimicrobial peptides are also able to act as antibacterial compounds by modifying the outer structure of the cell membrane, which causes depolarization and increased membrane permeability, that these peptides are also able to inhibit the synthesis of proteins, DNA, and RNA from bacteria (Noviana et al., 2018). Furthermore, flavonoid compounds act as antibacterial agents by disrupting cell membrane permeability (Noviana et al., 2018). The presence of natural antimicrobial compounds in tempeh caused the control treatment to show inhibitory activity against *Bacillus* sp.

The inhibition category was classified as moderate because the diameter of the inhibition zone against *Bacillus* sp. was about 5-10 mm (Table 2). It might be caused by the concentration of garlic powder being too small so that the effect on
antibacterial properties was not significant. Furthermore, Allicin has unstable properties during storage at room temperature; therefore, the compound will be further metabolized into an ajoene compound, which has another name, diallyl disulfide or vinylidene (Purwantiningsih et al., 2019). The presence of ajoene compounds causes the low antibacterial properties of garlic tempeh because this compound has a antibacterial properties weaker than allicin (Purwantiningsih et al., 2019). Another factor might be caused by the extraction process, which cannot completely extract polar compounds. The solvent used in this study was ethyl acetate, which has semi-polar properties so that compounds such as flavonoids cannot be adequately extracted (Mawaddah et al., 2018). Based on research conducted by Kemit et al. (2016), flavonoids are polar compounds due to their structure being composed of hydroxyl groups, so this means only solvents that have polar properties are able to extract them completely.

The antibacterial compounds in tempeh have a bacteriostatic mechanism of action (Figure 3). That can only inhibit the growth of Bacillus sp. bacteria without killing it because when incubated for 24 hours at 37°C, it can show inhibitory activity, but when further incubated for up to 5 days. The clear zone, which indicates the inhibition zone, has begun to disappear (Mawaddah et al., 2018). Based on the strength test of antibacterial activity, the results showed that the addition of garlic powder at a concentration of 0.25% showed the best inhibitory power against Bacillus sp. bacteria. In contrast, the higher concentration of garlic powder showed decreased antibacterial activity. It might be due to the garlic powder inhibiting the growth of Rhizopus spp., hence natural antibacterial compounds in tempeh cannot be produced properly (Noviana et al., 2018).

Although the garlic tempeh extract showed antibacterial activity against Bacillus sp. (Table 3, Figure 2), its active compounds could not inhibit the growth of E. coli bacteria because there is no inhibition zone formed. It might be due to E. coli belonging to gram-negative bacteria, which has a more complex cell wall component. It consists of three layers lipoproteins, lipopolysaccharides, and peptidoglycan (Noviana et al., 2018).

The lipoprotein layer contains hydrophilic protein porin, making it difficult for antimicrobial compounds to enter bacterial cells (Noviana et al., 2018). The antibacterial activity of allicin against gram-negative bacteria occurs through 2 mechanisms, that is damaging the cell wall and inhibiting the synthesis of RNA molecules (Lestari et al., 2018). The process of cell wall destruction occurs by inhibiting the formation of the peptidoglycan layer, which gives strength to the bacterial cell wall. Allicin compound is able to penetrate the lipopolysaccharide layer of gram-negative bacteria and cause membran permeability to increase. Allicin is also able to attack the phospholipid layer and cause it to break down into glycerol and phosphoric acid then followed cell lysis (Lestari et al., 2018). Then the process of inhibiting RNA synthesis occurs by forming bond with the DNA dependent RNA polymerase enzym (Lestari et al., 2018).

The effect of garlic powder added to the water content of tempeh

Tempeh without garlic powder treatment gets the highest percentage of water content, which was 62.50±0.03% (Table 2). The addition of garlic powder reduces tempeh’s water content up to 58.25±0.03% at a concentration of 0.25%. It shows that the concentration of 0.25% garlic powder did not inhibit the growth of the Rhizopus spp. The decreasing of water content happen because of the water produced through the fermentation process can still be reused by Rhizopus spp. to carry out the respiration (Purwanto and Weliana, 2018). The respiration process often involves the performance of hydrolytic enzymes from Rhizopus spp. so, the decreasing water content indicates the fermentation activity of Rhizopus spp increase. This is due to in this hydrolysis process, the free water contained in the substrate will be reduced and used as a reagent and be converted into bound water (Budiono, 2016). This statement was also similar to Purwanto and Weliana (2018), the high rate of respiration and fermentation activity could reduce the percentage of the water content of tempeh. Meanwhile, the addition of garlic powder under concentrations of 0.50 and 0.75% increases the water content tempeh. This could be due to decreased respiration rate due to damage to hydrolytic enzymes from the fungus Rhizopus spp. due to thiosulfinate compounds (Allcin), so growth and fermentation activity decreased (Purwanto and Weliana, 2018). But beside on Table 4 all treatments did not show any difference with the control because the significance value was >0.05. Based on the results of all water content measurements obtained, it is possible to conclude that all treatments produced results that met the SNI standard of 65%. 
Figure 1. Antibacterial activity of garlic tempeh against Bacillus sp bacteria. Note: Negative control and Positive control (A), Treatment of garlic tempeh with concentration of 0.15, 0.25, 0.50, and 0.75% (B).

Figure 2. Antibacterial activity of garlic tempeh against E. coli bacteria. Note: Negative control and Positive control (A), Treatment of garlic tempeh with concentration of 0.15, 0.25, 0.50, and 0.75% (B).

Figure 3. Antibacterial activity after incubation for 5 days. Note: Bacillus sp (A), Escherichia coli (B).

Table 4. One way anova test of water content on tempeh added with garlic.

| Water_Content | Sum of Squares | df | Mean Square | F   | Sig. |
|---------------|----------------|----|-------------|-----|------|
| Between Groups | 47.300         | 4  | 11.825      | 1.513 | .248 |
| Within Groups  | 117.250        | 15 | 7.817       |      |      |
| Total          | 164.550        | 19 |             |      |      |
The effect of the addition of garlic powder to the organoleptic properties of tempeh

The organoleptic test aims to determine the level of presence of the panelists to tempeh added with garlic powder at different concentrations. Table 6 shows the results for the color parameters of all types of tempeh obtained a fairly good value, that met on a scale of 3, and for concentration of 0.15, 0.25, 0.50%, the same value was obtained, that is 3.4. hence it can conclude that all treatments were well received by the panelists. Then for the aroma, the results obtained for concentrations of 0.15, 0.25, 0.50% also obtained a good assessment and a concentration 0.25% which has the highest value. And the concentration of 0.75%, the lowest value was obtained because the addition of garlic powder that was too high could cause a pungent odor due to organosulfur compounds (Pratama, 2017). Then for the taste, all types of tempeh can be well received by the panelists and the concentration of 0.25% which has the highest value.

Based on Table 6, it shows that all treatments had no significant effect on color parameters (p > 0.05). It is because garlic powder only contained flavonoid, which has colorless characteristics, and flavone that gave yellowish-white color to garlic. According to Zhafira (2018), the addition of garlic powder tends not to contribute significantly to the color of tempeh (Zhafira, 2018). For the following parameter, garlic powder affected the aroma of tempeh (P<0.05) because garlic bulbs contained allicin compounds. Although allicin compounds are easily oxidized, the oxidation product still forms organosulfur compounds (Pratama, 2017). In terms of taste parameters, the results of adding garlic powder could affect tempeh's taste. The panelists recommended the highest concentration of 0.25 %. This could be because the application of garlic powder at that concentration does not inhibit the fermentation process by Rhizopus spp, so it does not affect the original taste of tempeh. On the other hand, the presence of organosulfur compounds in garlic such as allin, S-methyl-cysteine, allinase, S-propylcysteine, Allicin, S-allyl cysteine, S-ethyl–cysteine, dialill sulfide, allyl methyl trisulfide, dialill disulfide, dialill trisulfide, and methyl allyl trisulfide (Moulia et al., 2018).

**CONCLUSION**

Based on the findings, the addition of garlic powder to the tempeh fermentation process affected the antibacterial activity for Bacillus sp. and the concentration of 0.25%, which provided the most excellent inhibitory activity but had no effect on E. coli. Aside from that, the addition of 0.25 percent garlic powder reduced the water content to58.25±0.03. The addition of garlic powder can then affect the aroma and taste of tempeh in the organoleptic test.
