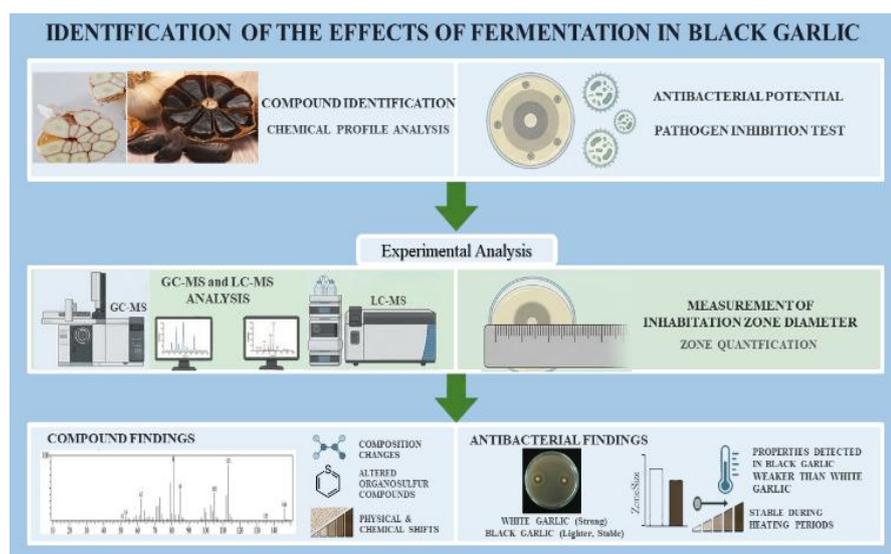


Effect of fermentation on the organosulfur compound profile and antibacterial activity of black garlic (*Allium sativum*) extract

Adela Syiddah Putri¹, Siti Nur Mufalla¹, Hanumi Oktiyani Rusdi¹, Surjani Wonorahardjo^{1*}, Suharti Suharti¹

Abstract

Black garlic is a derivative product of garlic that offers even more health benefits. During fermentation, various chemical reactions alter the chemical composition of garlic. In this study, the organosulfur compound profile of black garlic was analysed using GC-MS and LC-MS, and its antibacterial activity was also tested. In the initial stage, garlic was fermented at a constant temperature of 60 °C for 24 days. The GC-MS analysis revealed four organosulfur compounds. Meanwhile, the LC-MS analysis detected 16 distinct organosulfur compounds and their quantities at each fermentation stage. One organosulfur compound was consistently detected in LC-MS analysis across all fermentation stages. Antibacterial activity was tested using the paper disc diffusion method against *S. aureus* and *E. coli* bacteria. The research findings showed that the most effective black garlic in killing bacteria was fermented for 18 days. This was evident from the largest and most stable diameter of inhibition zones.



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Highlights

- New insights into the changes in organosulfur compounds in black garlic were obtained.
- GC-MS and LC-MS methods found changes in S-compounds during fermentation.
- *E. coli* and *S. aureus* antibacterial activity depends on the fermentation stages.
- The most effective black garlic extract in inhibiting bacterial growth was known.

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1. Introduction

Garlic (*Allium sativum*) is one of the types of bulb vegetables that has been used worldwide, primarily as a culinary additive to enhance the flavour of food (Hernawan and Setyawan, 2003). In addition to its culinary use, garlic also has applications as a traditional medicinal plant. Garlic has health benefits, including its ability to lower cholesterol levels and act as a diabetes-preventive agent (Moreno-Ortega *et al.*, 2020). The health benefits of garlic are attributed to its abundant secondary metabolites, including saponins, flavonoids, and organosulfur compounds. Among these metabolites, numerous studies have indicated that organosulfur compounds can provide benefits in addressing various metabolic disorders (Akhtar *et al.*, 2019).

One well-known benefit of garlic is its natural antibacterial ability. Garlic exhibits broad-spectrum antibiotic properties against both gram-positive and gram-negative bacteria (Purwantiningsih *et al.*, 2019). Allicin, a bioactive compound found in garlic, plays a significant role as an antibacterial agent. Previous research has demonstrated that Allicin can effectively eliminate antibiotic-resistant bacteria (Pajan *et al.*, 2016; Purwantiningsih *et al.*, 2019; Yuniasari *et al.*, 2021).

In its application as a remedy, garlic has been transformed into black garlic. This transformation occurs through a fermentation process that spans several days, accompanied by specific treatments (Dampati and Veronica, 2020; Qiu *et al.*, 2020; Zhafira, 2018). Fermentation leads to changes in the colour, aroma, and taste of garlic (Veronica *et al.*, 2021). Black garlic products exhibit a dark brown colour and a sweet, fresh taste (Agustina *et al.*, 2020; Lu *et al.*, 2017). During the fermentation process, the Allicin content in garlic and other typical components in garlic degrade into water-soluble antioxidant compounds, resulting in black garlic possessing higher antioxidant properties compared to regular garlic (Ammelia *et al.*, 2020; Herdyastuti *et al.*, 2021). More complex mixtures emerged in the profiles of black garlic over time, and their antioxidative properties were measured and reported (Wonorahardjo *et al.*, 2023). Therefore, the antibacterial properties must also be evaluated, as it is more likely that black garlic's functions can result from the blending of beneficial components.

The fermentation process in garlic, although seemingly simple, involves highly complex changes, including alterations in its chemical composition (Lestari *et al.*, 2021). Several analytical methods and instruments are commonly employed to identify components in natural substances, such as garlic, depending on their characterization and application (Wonorahardjo *et al.*, 2019). The components isolated from garlic are determined by methods and instrumentation capable of both separating and analysing the sample, such as gas chromatography and liquid chromatography equipped with mass spectrometry (GC-MS) (Wonorahardjo *et al.*, 2018; 2019).

The methods employed in this research are gas chromatography and liquid chromatography. These methods enable the calculation of changes in compound concentrations resulting from variations in heating time for the components present in black garlic. Previous researchers have explored changes in black garlic content using NMR and GC-MS methods during fermentation. While NMR can identify certain compound classes, it has limitations regarding dynamic range and sensitivity (Liang *et al.*, 2015). On the other hand, GC-MS analysis successfully demonstrated that the compound responsible for the pungent odour in garlic diminishes during fermentation and that the

antioxidant properties of black garlic increase (Ammelia *et al.*, 2020).

Prior research has also examined garlic's antibacterial properties, showing that garlic can inhibit the growth of bacteria such as *Escherichia coli* and *Staphylococcus aureus* at specific concentrations (Prihandani, 2015; Purwantiningsih *et al.*, 2019). However, there is a lack of research examining the antibacterial properties of black garlic with varying heating times against *Escherichia coli* and *Staphylococcus aureus*.

Given the existing issues, the objective of this research is to characterise the profile of organosulfur compounds using GC-MS and LC-MS analyses and to determine the antibacterial activity of black garlic extract against *Escherichia coli* and *Staphylococcus aureus* under varying heating times. The research outcomes are expected to provide valuable data on the molecular structure (MS) characteristics of compounds present in garlic, primarily organosulfur compounds, within the scope of analytical chemistry. Furthermore, the study aims to investigate changes in the content of organosulfur compounds in garlic resulting from varying fermentation durations. Additionally, it aims to explore the potential of black garlic extract to inhibit both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

2. Experimental

2.1. Tools and materials

Fresh garlic bulbs from a local supermarket were carefully selected and sorted for fermentation. The garlic plants were approximately 6 months old after being harvested. Heating at 60 °C was applied during this process. Fermentation occurs over 24 days using a rice cooker equipped with a TC4S temperature controller, which is connected via a solid-state relay to an FT-K-M6 thermocouple. No additional ingredients are added during the fermentation process. On days 6, 12, and 18, several cloves of black garlic are collected and used as samples with varying heating durations. The chromatography used was Shimadzu QP2010S. LC-HRMS was performed using Thermo Scientific Dionex Ultimate 3000 RSLCnano with a micro flow meter.

2.2. Antibacterial test

2.2.1. Preparation of black garlic extract

Black garlic samples are taken on days 0, 6, 12, and 18 for antibacterial activity testing. The garlic is crushed and squeezed to obtain a pure extract.

2.2.2. Antibacterial test

The antibacterial activity of black garlic extract is determined using the paper disc diffusion method. First, paper discs are inserted into each sample to be tested. Then, a liquid LB medium containing bacterial culture is placed into a solid LB medium. The solid LB medium is poured into Petri dishes, and once it starts to solidify, the paper discs soaked in the test solution are placed according to the prior sample labelling. Each antibacterial activity test is conducted aseptically in a laminar airflow cabinet, with two repetitions. After testing, the Petri dishes are incubated for 24 h in a controlled environment incubator. The overall diameter of the inhibition zone is measured when the inhibition zone around the disc is visible and devoid of colony growth.

2.3. Analysis of organosulfur compounds using GC-MS and LC-MS

2.3.1. Extraction of the back garlic sample

Black garlic samples are crushed and macerated in methanol. The resulting mixture is then filtered using Whatman filter paper. After filtration, the mixture is centrifuged and placed in a refrigerator for 1 day before being injected into the GC-MS and LC-MS columns.

2.3.2. GC-MS analysis

Prepared samples are taken and injected into the chromatography column. The gas chromatography column temperature is set to 50-250 °C at a rate of 10 °C/min, and methanol is used as the solvent.

2.3.3. LC-MS analysis

The prepared samples are diluted with methanol to a final volume of 1500 µL. The sample is vortex mixed. For the analysis of organosulfur compounds in garlic using LC, heat treatment was applied. The separation process is conducted on a C18 column (2.1 x 100 mm; 1.7 µm). Chromatographic elution is performed using a mobile phase consisting of 0.1% formic acid in distilled water (A) and 0.1% formic acid in acetonitrile (B). The column flow rate is 100 µL/min, and the injection volume is 5 µL. After entering the chromatography column, a high-resolution mass spectrometer is used.

3. Results and discussion

3.1. Black garlic fermentation

Garlic is a commonly used bulb vegetable and a base ingredient in many recipes. The fermentation process that occurs in garlic results in noticeable physical changes in terms of colour, texture, and aroma. The colour of garlic, initially pale white, gradually transforms into a deep black during the heating process on days 6, 12, 18, and 24. The colour change in garlic is evident as early as day 6 of heating, and as the heating time prolongs, the colour transformation becomes more intense.

The texture of the garlic, initially firm with a high moisture content, softens during heating on day 6. By day 12, the texture of black garlic becomes even softer and more elastic, resembling jelly. By day 18 of heating, the texture of black garlic becomes even softer and crumbles easily. Alongside changes in texture, black garlic also undergoes a shift in taste compared to raw garlic. Black garlic tends to have a sweeter taste and a less pungent aroma.

3.2. Analysis of organosulfur compounds using GC-MS and LC-MS

During the fermentation process, in addition to physical changes, the chemical compounds present in black garlic also undergo transformations, altering both their composition and types. Among the numerous active compounds found in garlic, this research focuses on organosulfur compounds, given their substantial presence. Gas chromatography was employed to detect and analyse volatile organosulfur compounds, while liquid chromatography was used to analyse non-volatile organosulfur compounds. As a result, this analysis provides insights into the

overall changes in the profile of organosulfur compounds in garlic due to the heating process.

The GC-MS analysis yielded chromatograms for each sample, as shown in Fig. 1. Each peak was then identified by comparing the mass spectrum with the Wiley 8 library to determine the types of compounds present in the sample.

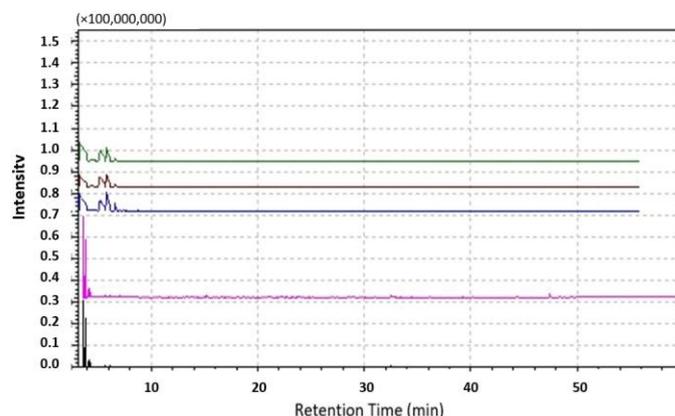


Figure 1. GC chromatograms of garlic with varying heating times (black=garlic, pink=black garlic 6-day fermentation, blue= black garlic 12-day fermentation, red= black garlic 18-day fermentation, green= black garlic 24-day fermentation).

Source: Elaborated by the authors.

In the GC-MS chromatograms, numerous components were detected in garlic at longer retention times. However, in black garlic, certain components appeared at shorter retention times, and specific volatile compounds disappeared. Furthermore, the peaks between different compounds were more distinct.

Changes in chromatogram peaks were observed on days 6, 12, and 18 as the fermentation process progressed. This indicates that the compounds in black garlic undergo changes in both quantity and chemical composition (Table 1). On day 24, which marks the end of the heating process, the chromatogram's percentage area showed a decrease in volatile compounds. This is marked by the disappearance of the pungent odour associated with black garlic, which originates from the organosulfur compounds present. These organosulfur compounds are believed to have been reduced during the heating process, specifically between days 21 and 24 (Ammelia *et al.*, 2020).

Organosulfur compounds in garlic have relatively high boiling points, making their analysis challenging using GC-MS. However, increasing the sample injection temperature makes volatile organosulfur compounds easier to detect. Moreover, prolonged heating reduces the formation of volatile organosulfur compounds. In black garlic heated for 18 and 24 days, these organosulfur compounds were no longer detected by GC-MS.

The organosulfur compounds detected by GC-MS varied according to the duration of fermentation. This indicates that these compounds undergo degradation and form new organosulfur compounds because of heating. For instance, diallyl disulfide, a compound with an area percentage of 3.35%, was detected in garlic. One of the stable organosulfur compounds from garlic is S-allyl cysteine (SAC). S-allyl cysteine (SAC) is an organosulfur compound derived from the most important organosulfur compound in garlic bulbs, namely the compound γ -glutamyl-S-alk(en)yl L-cysteine (Hernawan and Setyawan, 2003). However, in the GC-MS results shown in Table 1, the organosulfur compound detected was another derivative of γ -glutamyl-S-alk(en)yl L-cysteine, namely diallyl disulfide, a compound with an area percentage of 3.35%, was detected in garlic. This compound

is one of the characteristic organosulfur compounds found in garlic, alongside Allicin. Diallyl disulfide has a structure like Allicin but lacks the double bond between the S and O atoms, as shown in Fig. 2. Allicin produces numerous derivatives or degradation products due to its instability. One of its derivatives is the allyl group compound, which was detected in significant

amounts in the GC-MS results of garlic. An example is (E)-1-Allyl-2-(prop-1-en-1-yl) disulfane with an area percentage of 3.47%. Furthermore, in black garlic heated for 6 days, an allyl group compound, Allyl (3-methylbutyl) sulfide, was detected at an area percentage of 0.38%.

Table 1. Organosulfur compounds were identified through GC analysis.

Organosulfur Compounds	SI	Garlic	Black garlic 6 th day	Black garlic 12 th day	R. Time (min)
		%Area			
Disulfide, methyl 2-propenyl	72	0.54	-	-	7.733
3H-1,2-Dithiole	80	1.47	-	-	8.320
2,2-Dimethylthiazolidine	71	1.04	-	-	8.367
Diallyl disulphide	91	3.35	-	-	9.682
(E)-1-Allyl-2-(prop-1-en-1-yl)disulfane	74	3.47	-	-	9.887
3-Methyl-3H-1,2-dithiole	85	2.92	-	-	9.943
8-Thiabicyclo[3.2.1]octane	68	5.22	-	-	10.345
4-Methyl-1,2,3-trithiolane	77	0.56	-	-	10.525
(Z)-1-Methyl-2-(prop-1-en-1-yl)disulfane	76	5.87	-	-	10.648
3-Vinyl-1,2-dithiacyclohex-4-ene	94	12.89	-	-	10.808
2-Vinyl-4H-1,3-dithiine	88	8.43	-	-	11.011
2-Methylmercaptoaniline	81	3.37	-	-	11.320
(Z)-1-Allyl-3-(prop-1-en-1-yl)trisulfane	86	1.59	-	-	12.001
5-Methyl-1,2,3,4-tetrathiane	74	2.08	-	-	12.388
Allyl (3-methylbutyl) sulfide	66	-	0.38	-	8.740
3-Isothiazolecarboxamide	100	-	-	0.00	10.766
5-Isothiazolecarboxamide	100	-	-	0.00	10.766
Benzene, 1-(butylthio)-4-methyl	62	-	-	0.02	9.640
Non-Organosulfur Compound		45.01	99.62	99.98	
Total		100	100	100	

Source: Elaborated by the authors.

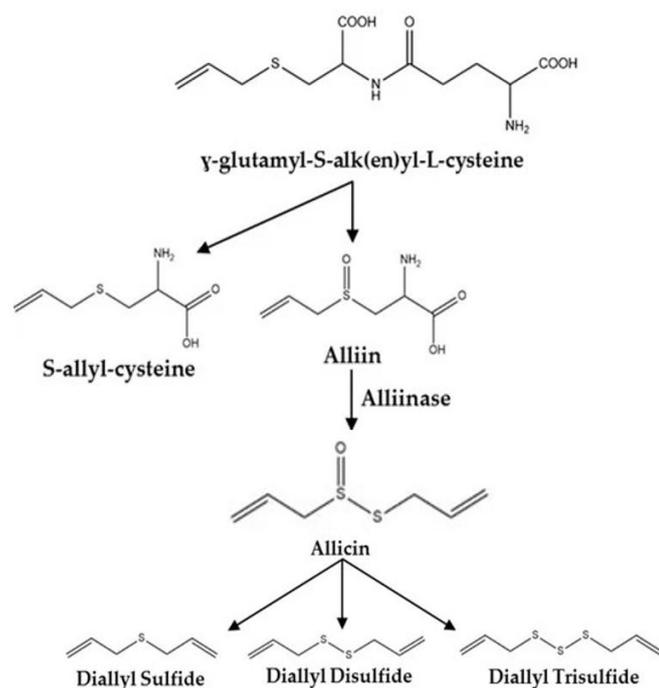


Figure 2. The main structure of organosulfur compounds and their derivatives.

Source: Elaborated by the authors.

Based on existing theories, garlic contains various organosulfur compounds, including allicin, allyl sulfide, and diallyl sulfide. However, in this testing, the presence of these compounds could not be detected. This might be due to several factors, such as using solvents with low boiling points, which can

cause other sulphur-containing components not to bind strongly as the solvent evaporates, given that organosulfur compounds usually have high boiling points. Additionally, unstable temperatures can lead to the degradation of organosulfur components into different forms.

The results of several GC-MS chromatograms indicate the presence of organosulfur compounds. Figure 3 illustrates one of these chromatograms, where each peak indicates the presence of characteristic organosulfur compounds found in garlic.

In the chromatogram, the compound suspected to be diallyl disulfide has a molecular weight of 146. There are several characteristic peaks that help identify this compound. Some notable peaks include m/z 62, 81, 85, 105, 113, and 146. The peak at m/z 81 has an intensity of 100% and serves as the base peak. The process of analysing the compound's structure in the GC-MS result involves the use of high-energy electrons to break the compound down. The goal is to generate fragments with varying masses. These fragments are then depicted graphically, with the height of each bar representing its mass. Compound fragmentation occurs when the rightmost fragment is subtracted by a specific number derived from other fragments that have been cleaved. The fragmentation data is presented in Fig. 4, illustrating various mass fragments derived from diallyl disulfide. For instance, the fragment at m/z 105 arises from the mass difference between 146 and 41, corresponding to a fragment of the compound $C_3H_5S_2$. The fragment at m/z 103 stems from the mass difference between 146 and 43, corresponding to a fragment of the compound $C_3H_5S_2$. The m/z 64 fragment arises from the mass difference between 146 and 82, representing a fragment of the compound S_2 .

The analysis results from the fragment pieces, supported by matching with the hit list and the NIST database, indicate that the detected organosulfur component is diallyl disulfide, with a molecular formula of $C_6H_{10}S_2$ and an m/z of 146.

Further analysis was conducted to determine the content of organosulfur compounds in garlic using LC-MS. Several larger components with non-volatile properties were detected using LC-

MS. The LC-MS analysis results reveal that the percentage area of organosulfur compounds increases progressively with fermentation duration, as illustrated in Fig. 5.

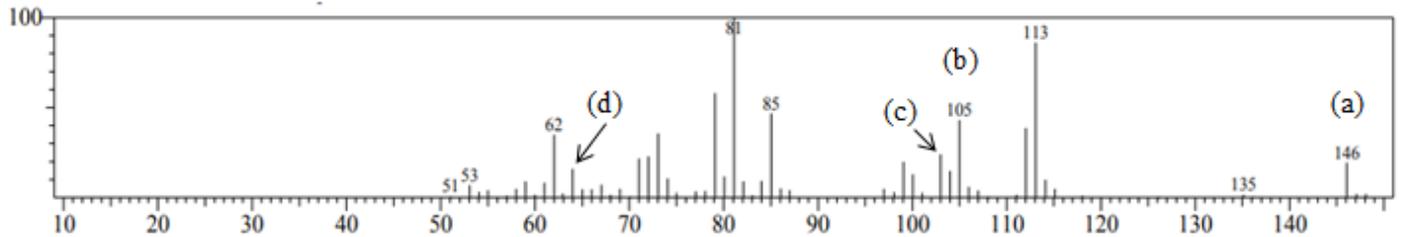


Figure 3. Chromatogram of diallyl disulfide compound (letter annotations on the image refer to the fragmentation presented in Fig. 4).
Source: Elaborated by the authors.

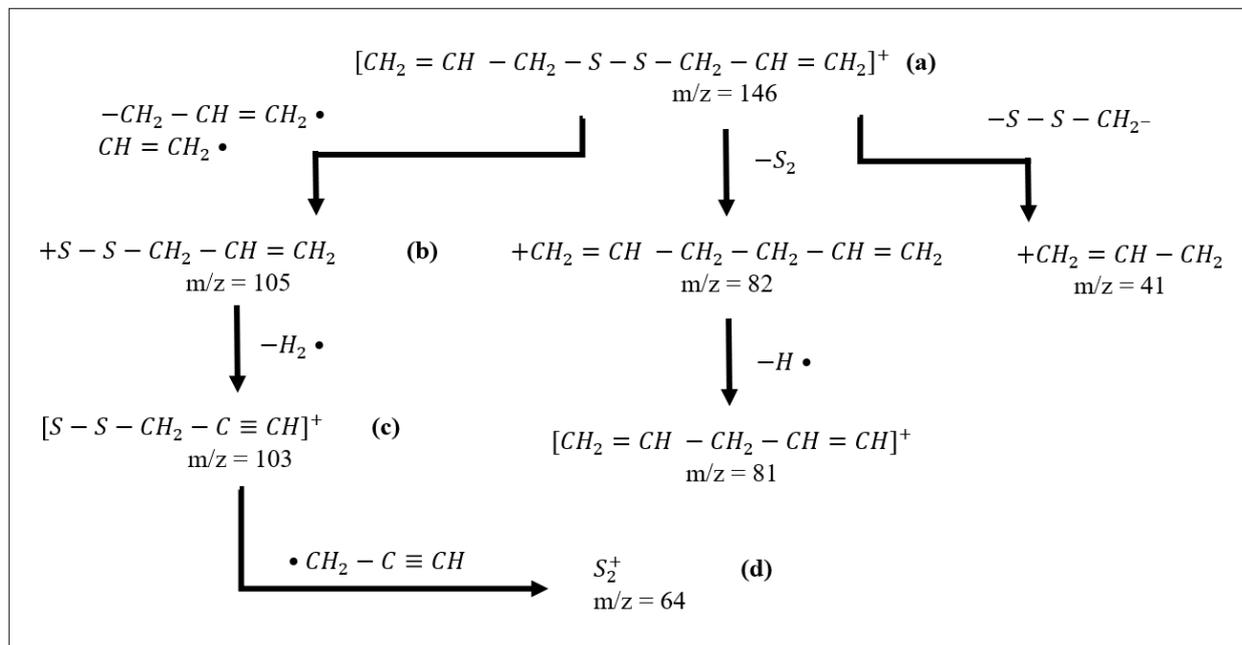


Figure 4. Fragmentation of diallyl disulfide identified by MS (letter annotations on the image refer to the chromatogram presented in Fig. 3).

Source: Elaborated by the authors.

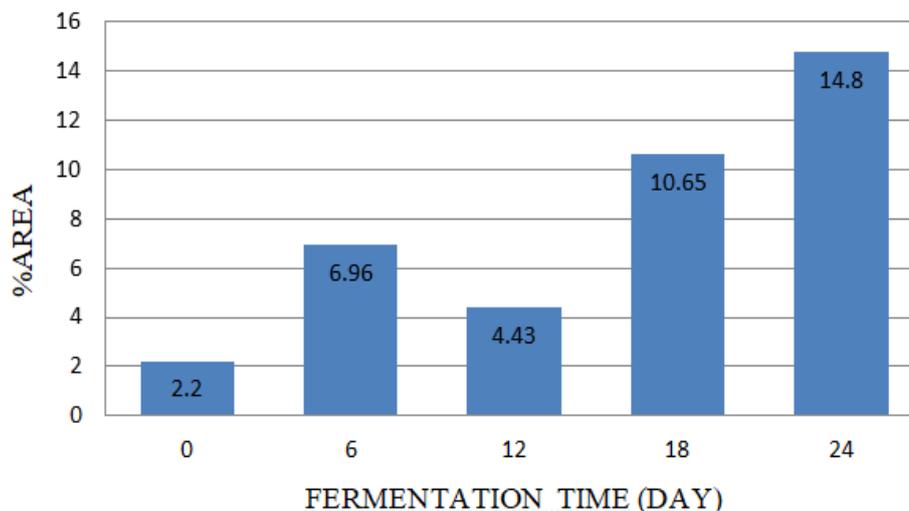


Figure 5. Percentage area of total organosulfur compounds from LC-MS analysis.

Source: Elaborated by the authors.

The results of the MS analysis indicate an increase in the percentage area of organosulfur compounds in garlic that have undergone the heating process compared to those that have not. This change becomes noticeable starting from the 6th day of garlic fermentation. However, on the 12th day, the percentage area decreases, possibly due to the decomposition of non-volatile molecules into smaller fragments. This observation is consistent with findings from the GC analysis, in which new compounds emerged with lower retention times. Subsequently, on the 18th day, there is a significant increase in the percentage area, which continues until the end of the heating process on the 24th day.

During this final heating phase, non-volatile components experience an increase, causing the volatile components responsible for the garlic odour, namely the organosulfur compounds, to decrease and eventually be eliminated by the end of the heating process on the 24th day (Ammelia *et al.*, 2020).

During the initial stages of garlic fermentation, on the 6th day, the compound composition changes, with some compounds lost and new compounds emerging, as illustrated in Table 2. The newly formed compounds are non-volatile compounds generated during the heating process.

Table 2. Organosulfur compounds were identified through LC analysis.

Organosulfur Compounds	Garlic	Black Garlic			
		6 th day	12 th day	18 th day	24 th day
		% Area			
5-([3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl)thio-4-pentyl-4H-1,2,4-triazol-3-ol	1.60	2.84	1.58	0.83	3.02
4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	-	0.71	2.04	4.88	7.18
4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione	-	0.21	0.65	1.47	1.95
L-(-)-methionine	0.01	0.06	0.16	-	-
Biotin	-	-	-	0.58	0.33
ethyl 4-(2-chloro-6-fluorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate	0.19	-	-	-	-
N-Butylbenzenesulfonamide	0.06	0.06	-	-	-
N-((1S,2S,8S,8aS)-8-Hydroxy-1,4a-dimethyl-7-((2S)-1-oxo-1-(1-piperidinyl)-2-propanyl)decahydro-2-naphthalenyl)methanesulfonamide	-	-	-	-	0.77
2-[(2-chlorobenzyl)sulfanyl]-4,6-dimethylnicotinonitrile	0.39	0.28	-	-	-
2-[(2,3,4,5,6-Pentamethylbenzyl)thio]ethanohydrazide	-	-	-	0.63	-
N1-(2-amino-2-oxoethyl)-2-(isopropylthio)acetamide	-	-	-	-	0.01
(1R,9R)-5-Cyclohexyl-11-(propylsulfonyl)-7,11-diazatricyclo[7.3.1.0 _{2,7}]trideca-2,4-dien-6-one	-	0.08	-	-	-
N-2-(4-[(2,6-Dimethoxy-4-pyrimidinyl)amino]sulfonyl)phenyl)-2-furamide	-	-	-	-	0.41
N6-[(5-nitro-2-thienyl)methylidene]-2,3-dihydro-1,4-benzodioxin-6-amine	-	0.07	-	-	-
2-[3-methyl-2-(methylimino)-4-oxo-1,3-thiazolan-5-yl]acetic acid	-	2.64	-	2.27	-
ethyl 2-[2-(phenylsulfonyl)amino]-1,3-thiazol-4-yl]acetate	-	-	-	-	1.05
Non-Organosulfur Compounds	97.80	93.04	95.57	89.35	85.20
Total	100	100	100	100	100

Source: Elaborated by the authors.

Additionally, several organosulfur compounds are consistently detected during different heating periods, as depicted in Fig. 6. One of these compounds is 5-([3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl)thio-4-pentyl-4H-1,2,4-triazol-3-ol, which appears from garlic's initial state to the 24th day of heating and plays a crucial role as an antiviral agent (Seetaha *et*

al., 2022). Furthermore, there are compounds like 4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione with antidepressant properties and 4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione with antibacterial roles. Both compounds are present during black garlic formation from the 6th day to the 24th day.

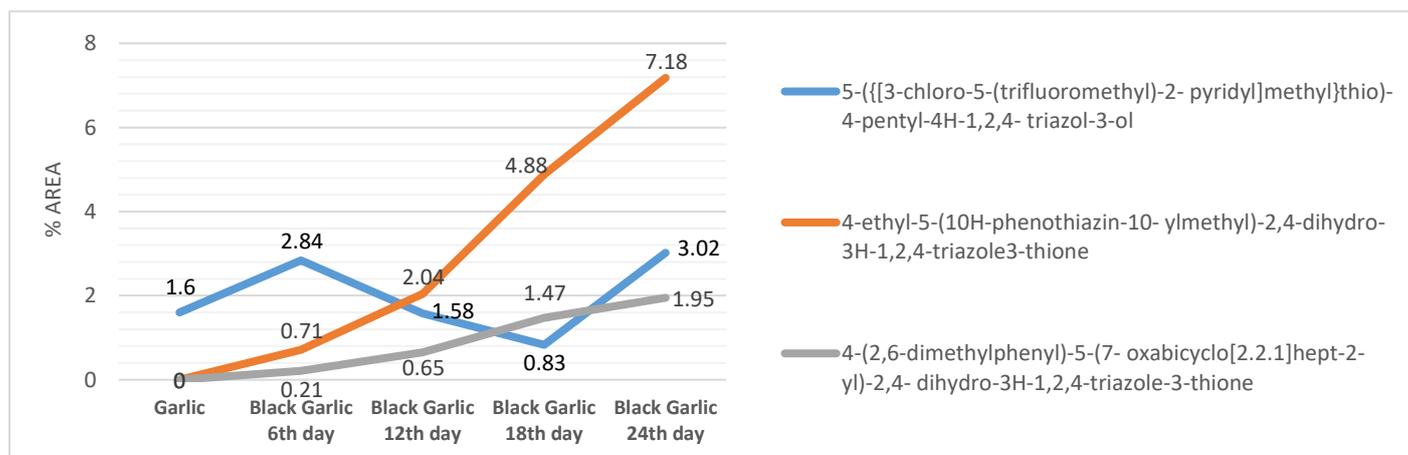


Figure 6. Consistently detected organosulfur compounds in LC-MS analysis.

Source: Elaborated by the authors.

The graph indicates that several organosulfur compounds are consistently detected in black garlic, and their levels tend to increase as the heating duration progresses.

3.3. Antibacterial test of black garlic extracts at different fermentation times

Antibacterial testing of black garlic extract was conducted against the growth of *S. aureus* and *E. coli* (Table 3). The inhibition test was performed using the disc diffusion method, which involves placing paper discs soaked in the sample extract on the agar surface. These discs are then placed on an agar medium inoculated with bacteria. During the incubation period, the extract diffuses from the paper disc into the agar medium, and the formation of

clear zones around the paper discs indicates antibacterial activity. The size of the inoculated organism must be standardised according to the McFarland (1907) 0.5 standard or an approximate bacterial concentration of 1.5×10^8 CFU/mL (Bhakti *et al.*, 2023). This is done to avoid excessively large or small inhibition zones due to improper inoculum size.

The test bacteria are inoculated on the same testing medium, Luria Bertani agar. Luria Bertani (LB) medium is a commonly used bacterial growth medium suitable for various bacterial species, including *E. coli* and *S. aureus*. Bacterial microorganisms grow on the agar medium, while the sample on the paper disc inhibits their growth. The inhibition zone formed around the paper disc can be measured to determine the susceptibility of bacteria to the black garlic sample.

Table 3. Diameter of inhibition zones of black garlic extract with fermentation time variations.

Bacteria	Garlic day	Repetition I (mm)	Repetition II (mm)	Average (mm)	SD (mm)	Positive control (mm)
<i>E. coli</i>	0	18.4	18.7	18.55	0.15	18.95
	6	4.4	6.8	5.6	1.2	
	12	3.5	2.2	2.85	0.65	
	18	5.2	4.3	4.75	0.45	
<i>S. aureus</i>	0	29.6	31.3	30.45	0.85	28.9
	6	4.3	1.7	3	1.3	
	12	3.2	2.3	2.75	0.45	
	18	6	2.9	4.45	1.55	

Source: Elaborated by the authors.

The antibacterial assay of black garlic extract with varying heating times was conducted in 2 repetitions for each sample and bacterial strain. Based on the data in Table 3, it is evident that garlic (*Allium sativum*) has a larger inhibition zone diameter than black garlic. This discrepancy might be attributed to the heating process, which could remove certain compounds during black garlic production through evaporation at elevated temperatures or degradation during specific heating periods. New compounds with antibacterial properties may not have yet formed, resulting in decreased antibacterial efficacy from garlic to black garlic. However, on day 18, the inhibition zone diameter increased, suggesting that degraded compounds might be forming new molecules that enhance black garlic's antibacterial potential. This

phenomenon also agrees with the previous result of this group (Ammelia *et al.*, 2020), that the antioxidant properties reach their highest on the 18th day of fermentation.

In addition to the black garlic samples, antibiotic testing was also performed as a positive control, as indicated in Table 3 and 4. Tetracycline was used as the positive control. The inhibition zone for the positive control measured 18.95 mm against *E. coli* and 28.9 mm against *S. aureus*. Thus, to achieve an antibacterial effect equivalent to that of an antibiotic against *E. coli*, consuming several cloves of black garlic in a single meal is necessary. Similarly, for *S. aureus* bacteria. Alongside positive control, negative control using distilled water (aqueous) was also employed.

Table 4. Diameter of inhibition zone (mm) data with measurement time for 7 days.

Bacteria	Repetition	Type of Garlic	Day 1 (mm)	Day 2 (mm)	Day 3 (mm)	Day 4 (mm)	Day 5 (mm)	Day 6 (mm)	Day 7 (mm)
<i>E. coli</i>	First	Garlic	18.4	18.4	18.6	18.7	19.7	19.7	18.4
		6 th day	4.4	2.9	2.9	2.9	2.9	2.9	2.8
		12 th day	3.5	3.2	2.8	2.8	2.08	2.8	2.1
		18 th day	5.2	4.2	3.3	3.3	3.3	3.3	3.3
	Second	Garlic	18.7	18.7	18.7	20.1	20.9	20.5	20.1
		6 th day	6.8	3.5	3.5	3.5	3.5	2.1	1.7
		12 th day	2.2	2	2	2	2	2	2
		18 th day	4.3	4.3	4.3	3.4	2.9	2.7	2.7
<i>S. aureus</i>	First	Garlic	29.6	29.6	29.6	30.2	26.4	30.6	29.9
		6 th day	4.3	4.3	4.3	4.3	4.1	3.9	3.9
		12 th day	3.2	3	2.1	2.1	2.1	1.7	1.7
		18 th day	6	6	5.5	5.5	5.5	5.5	5.1
	Second	Garlic	31.3	31.3	31.3	33.8	33.3	31.8	31.1
		6 th day	1.7	1.7	1.7	1.7	1.7	1.6	1.7
		12 th day	2.3	2.3	2.3	1.2	1.2	1	1
		18 th day	2.9	2.9	2.9	2.6	2.5	1.8	1.5

Source: Elaborated by the authors.

In previous studies, it has been demonstrated that Allicin can enhance the permeability of bacterial cell walls. Additionally, allicin exhibits antibacterial activity by rapidly and comprehensively inhibiting RNA synthesis (Pajan *et al.*, 2016). However, Allicin is less stable; at room temperature, it is metabolised into diallyl disulfide (DADS) within a few hours. DADS also exhibits antibacterial properties, with a mechanism like Allicin, but its potency is relatively lower. Therefore, organosulfur compounds, including Allicin, play a significant role in influencing garlic's antibacterial properties.

The standard deviation measures the dispersion of data relative to its mean. When the standard deviation approaches zero, the variability of the data around the mean becomes smaller, indicating more stable data. Data instability can also affect the average results of the black garlic antibacterial testing, due to variations in heating time.

3.4. The difference in measurement time for antibacterial stability according to fermentation time

The inhibition zone diameter is measured at 1, 2, 3, 4, 5, 6, and 7 days during antibacterial testing. The first-day measurement is performed after the sample is placed on an agar medium inoculated with bacteria and incubated for 24 h. Periodic measurements were taken over 7 days to assess the ability and stability of black garlic extract to inhibit bacterial growth.

Repeating tests and conducting periodic measurements over several days during the antibacterial assay is intended to achieve more accurate and reliable results, as outlined in [Table 4](#). Additionally, it allows for the observation of trends in the inhibition zones of black garlic extract. As observed in the table, the inhibition zone diameter of white garlic against both *E. coli* and *S. aureus* initially increases until day 4-5 of measurement, after which it starts to decrease. This indicates that the antibacterial activity of white garlic peaks on days 4 to 5, after which it weakens, allowing bacteria to proliferate again and reducing the inhibition zone diameter.

In contrast, black garlic exhibits a different pattern, with the inhibition zone diameter remaining relatively stable from the first day of measurement to day 3 or 4. Subsequently, there is a gradual decline, although not overly drastic. This trend suggests that black garlic can gradually and steadily eliminate bacteria. While its antibacterial properties may not be as potent as those of white garlic, black garlic still holds potential as an antibacterial agent.

Data obtained through periodic antibacterial measurements over 7 days, with 2 repetitions, show that both white and black garlic, with variations in heating time, exhibit antibacterial properties. These antibacterial effects are observed to be effective for only 3-5 days after the samples are incubated for 24 h. Therefore, to reap the health benefits of garlic, it should be consumed regularly and periodically. By maintaining a consistent consumption pattern, garlic's antibacterial properties can provide tangible benefits to the body.

4. Conclusions

The physical properties of black garlic, such as colour and texture, change during the fermentation process. Black garlic turns dark and becomes more concentrated as fermentation time increases. The colour change is accompanied by a change in texture, making the garlic softer. The duration of heating also influences the reduction of volatile organosulfur compounds, which contribute to the characteristic odour of garlic.

The GC-MS analysis detected 4 organosulfur compounds in black garlic. On the 6th day, Allyl (3-methylbutyl) sulfide was detected, followed by the detection of 3 compounds on the 12th day: 3-isothiazolecarboxamide, 5-isothiazolecarboxamide, and benzene, 1-(butylthio)-4-methyl. Meanwhile, LC-MS detected 16 organosulfur compounds in black garlic. In the black garlic heated for 6 days, 9 compounds were detected. On the 12th day, 4 compounds were detected, and on the 18th day, 6 compounds were detected. Compounds such as 5-([3-chloro-5-(trifluoromethyl)-2-pyridyl]methyl)thio)-4-pentyl-4H-1,2,4-triazol-3-ol, 4-ethyl-5-(10H-phenothiazin-10-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione, and 4-(2,6-dimethylphenyl)-5-(7-oxabicyclo[2.2.1]hept-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione are some examples of organosulfur compounds identified through LC-MS analysis.

Pure black garlic extract also exhibits potential as an antibacterial agent against the bacteria *Escherichia coli* and *Staphylococcus aureus*. The duration of heating also affects the size of the inhibition zone. The research results indicate that the most effective black garlic in killing bacteria is heated for 18 days. This is because black garlic heated for 18 days exhibits the largest and most stable inhibition zone diameter against *E. coli* and *S. aureus* bacteria.

Authors' contribution

Conceptualization: Surjani Wonorahardjo; Hanumi Oktiyani Rusdi; Suharti Suharti; **Data curation:** Adela Syiddah Putri; Siti Nur Mufalla; Surjani Wonorahardjo; **Formal Analysis:** Surjani Wonorahardjo; Hanumi Oktiyani Rusdi; **Funding acquisition:** Surjani Wonorahardjo; **Investigation:** Adela Syiddah Putri; Hanumi Oktiyani Rusdi; Siti Nur Mufalla; Surjani Wonorahardjo; **Methodology:** Hanumi Oktiyani Rusdi; Suharti; Suharti, Surjani Wonorahardjo; **Project administration:** Surjani Wonorahardjo; **Resources:** Not applicable; **Software:** Adela Syiddah Putri; **Supervision:** Hanumi Oktiyani Rusdi; Surjani Wonorahardjo; Suharti; **Validation:** Surjani Wonorahardjo; **Visualization:** Adela Syiddah Putri; Siti Nur Mufalla; Hanumi Oktiyani Rusdi; **Writing – original draft:** Adela Syiddah Putri; Hanumi Oktiyani Rusdi; **Writing – review & editing:** Adela Syiddah Putri; Surjani Wonorahardjo.

Conflict of interest

The authors declare that there is no conflict of interest.

Data availability statement

The data will be available upon request.

Artificial Intelligence usage statement

We confirm that Artificial Intelligence (AI) tools were utilized in preparing parts of the manuscript, specifically for revising grammar and enhancing the English quality. The tools employed include Grammarly, which were used to analyze and improve sentence structure, grammar accuracy, and overall readability throughout the manuscript.

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