Antimicrobial effect of *Zingiber officinale* var. officinale essential oil and nisin against pathogenic and spoilage microorganisms

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**Abstract.** This study was conducted to investigate the antimicrobial effect of *Zingiber officinale* var. officinale essential oil (EO), nisin, and their combination against some pathogenic and spoilage microorganisms. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Fungicidal Concentration (MFC) and the synergism effect were assayed. The MIC values of nisin on *Aspergillus niger*, *Eschericia coli* and *Pseudomonas fluorescens*; *Bacillus cereus*; and *Salmonella typhimurium* and *Staphylococcus aureus* were 250; 500; 2000; and >2000 IU, respectively. On the other hand, MIC values of EO on *E. coli*; *B. cereus* and *S. aureus*; *Pseudomonas fluorescens* and *A. niger*; and *Salmonella typhimurium* were 0.125; 0.25; 0.5; and 4%, respectively. 62.5 IU of nisin combined with 2% of EO could inhibit the growth of *Salmonella typhimurium*, *S. aureus*, and *E. coli*. In addition, the combination of nisin and EO had synergistic effect against *B. cereus*, *A. niger*, and *Salmonella typhimurium*. The combination of nisin and EO had no bactericidal effect against all five bacteria but it had fungicidal effect against *A. niger* at concentration 62.5 IU of nisin and 1% of EO. Sabinene (16.88%), Z-citral (11.25%), and camphene (10.05%) were the major components in *Zingiber officinale* var. officinale EO which contributed on its antimicrobial activity.

1. Introduction

Microbial contamination was one of the biggest cases found on food, this kind of contamination spoils the food and/or causing foodborne disease. In 2014, suspected foodborne outbreaks caused by microbial contamination in Indonesia reached 51.06%, that number was higher than the outbreaks caused by chemical and physical contamination [1]. The data decreased in 2015 and reached 42.62%, eventhough it was still the highest amongst others [2]. Data from *Center for Disease Control and Prevention* also showed the same trend. From 2011 until 2014, microbial contamination, caused by for example *Salmonella*, *Eschericia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, was the biggest outbreaks in America [3,4,5,6]. Natural antimicrobial and preservative agents are needed to be investigated.

Nisin, is a bacteriocin which ribosomally synthesised by *Lactococcus lactis* subs. *Lactis*. It has had classified by FDA as a safe food preservative agent/Generally Recognizes as Safe (GRAS) [7]. On several previous studies, nisin was identified as an effective agent on inhibiting the growth of microorganisms. Some microorganisms were inhibited by nisin, such as *Micrococcus luteus* ATCC...
10240 [8] Listeria monocytogenes [9], Bacillus cereus, Lactobacillus sakei, and Listeria monocytogenes HPB 2812 [10]. 100 IU/ml and 500 IU/ml of nisin could protect the serro cheese from the development of Staphylococcus aureus [11]. Nisin also produced low impact on sensory value, only 1/40 of nisin was detected by saliva. In addition, WHO recommends nisin as an additive with Acceptable Daily Intake/ADI is 33000 IU [12], it makes the use of nisin becomes more beneficial than the use of other antimicrobial agents. Unfortunately, nisin is less effective against Gram negative and fungi and it is also expensive [13,14].

On the other hand, the essential oil from Zingiber officinale var. affinicale or usually known as ginger, is one of the most potential antimicrobial agent to be developed in Indonesia. Data from Central Bureau of Statistics/Badan Pusat Statistik (BPS) Indonesia showed that in 2014 the production of ginger (as the raw material of ginger EO) reached 226,114.8 ton [15]. In addition, ginger EO also had effectiveness against several microorganisms. 2.0 mg/ml inhibited the growth of E. aurogenes and S. marcescens, 4.0 and 8.0 mg/ml inhibited the growth of E. coli and S. typhi respectively[16]. But, higher concentration of essential oil may disturb the sensory value of food when it was added [17,18].

The combination of nisin and ginger EO may reduce the concentration of nisin and/or essential oil, increase the effectivity of their antimicrobial activity, and it also protect the sensory value of food. On the previous research, the combination of nisin and Zingiber officinale var. rubrum essential oil showed an effectivity on Bacillus cereus, it also rose up the effectivity of nisin on Salmonella typhimurium, Pseudomonas fluorescens, and Aspergillus niger [19]. The aim of this research was to investigate the antimicrobial effect of the combination between nisin and ginger (Zingiber officinale var. officinale) essential oil on Bacillus cereus FNCC 0057, Salmonella typhimurium FNCC 0050, Staphylococcus aureus FNCC 0047, Eschericia coli FNCC 0091, Pseudomonas fluorescens FNCC 0070 and Aspergillus niger FNCC 6080.

2. Material and Method
2.1 Essential oil preparation
Fresh ginger was obtained from Pasar Legi, Surakarta, Indonesia. Then, it was sort and washed to remove the soil. After that, it was sliced into 2-3 mm thickness and dried under the air-drying condition for 4-6 days. After the simplicia reached 15-16% of moisture content, it was distillated. The distillation process was using the Stahl apparatus to obtain the essential oil [20].

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis
GC-MS Shimadzu QP 2010 (Tokyo, Japan) was used to analyse the chemical components of the ginger EO. 1 μl of EO was injected into the GC-MS. The oven temperature was setted on 50°C and it increased 5°C/minute until it reached its final temperature on 240°C. The injection temperature was 300°C. Helium as the carrier gas, flowed on 0.55 ml/minute flow rate and the pressure was 14.0 kPa [21].

2.3 Inoculum preparation
Bacillus cereus FNCC 0057, Salmonella typhimurium FNCC 0050, Staphylococcus aureus FNCC 0047, Eschericia coli FNCC 0091, Pseudomonas fluorescens FNCC 0070, and Aspergillus niger FNCC 6080 were obtained from Pusat Studi Pangan dan Gizi, Gadjah Mada University, Indonesia. All of the inoculum stocks were refreshed and incubated at 35±2°C. The bacteria cultures were growth on Nutrient Agar (NA) for 24 hours while the fungi culture was growth on Potato Dextrose Agar (PDA) for 3 days. The inoculum was suspended into saline water and its turbidity was adjusted with 0.5 McFarland [22].

2.4 Minimum Inhibitory Concentration (MIC) test
MIC test was conducted using microdilution method [21]. 1000 IU, 500 IU, 250 IU, 125 IU and 62.5 IU of nisin were made from nisin (10⁶ IU) that was diluted in 10 ml of media (Mueller Hinton Broth or RPMI 1640 2% of glucose without bicarbonate). The suspension of 4%, 2%, 1%, 0.5% and 0.25%
(v/v) of EO were made by using the same method as making nisin dilution. 0.5% (v/v) of Tween 20 was added into the suspension to make the dilution becomes more stable.

The 96-wells microplate was filled with 50 µl of nisin solution and ginger EO solution, then 10 µl of bacteria suspension or 100 µl of fungi suspension were added. For bacteria inhibition test, the microplates should be incubated for 20 hours while for fungi inhibition test was 48 hours. The MIC were determined from the minimum concentration that could inhibit the growth of the microorganisms.

2.5 Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) test
The suspension that showed MIC value were regrowth on Mueller Hinton Agar (MHA) at 35±2°C for 24 hours to determine the MBC while 20 µl from fungi MIC test were used and regrowth on Saboraud Dextrose Agar (SDA) at 35±2°C for 48 hours to determine the MFC [23,24]. MBC and MFC were identified as the minimum concentration that could eliminate the growth of the microorganisms.

2.6 Synergism Effect Analysis
FIC index were calculated to analyse the synergism effect of the combination. Fractional inhibitory concentration of the first and the second antimicrobial agents were added (FICA and FICB). A is identified as the MIC of the first agent that inhibited the microorganism when it is combined with the second agent. While MICA is described as the MIC of the first agent without combination. Whereas B and MICB have the same description as A and MICA but on the second agent.

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FIC_{\text{Index}} = FICA + FICB
\]

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FIC_A = \frac{A}{MICA}
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\[
FIC_B = \frac{B}{MICB}
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Synergistic effect of the combination is determined with \( FIC < 0.5 \), partial synergistic is determined when \( 0.5 < FIC < 0.76 \). Additive effect is determined with \( 0.76 < FIC < 1 \), indifference effect is identified with \( 1 < FIC \leq 4 \), and antagonistic effect is identified when \( FIC > 4 \) [25].

3. Result and Discussion
3.1 Chemical composition
The ginger EO was analysed by using GC-MS. Sabinene (16.88%), Z-citral (11.25%), camphene (10.05%), citral (8.57%), and zingiberene (8.49%) were the major components of ginger EO.

| Chemical Components      | Ginger EO (%) |
|--------------------------|--------------|
| Sabinene                 | 16.88        |
| Z-citral                 | 11.25        |
| Camphene                 | 10.05        |
| Citral                   | 8.57         |
| Zingiberene              | 8.49         |
| Beta-sesquiphellandrene  | 4.21         |
| Alpha-pinene             | 4.03         |
| Alpha-farnesene          | 3.41         |
| Benzene                  | 2.61         |
| Beta-bisabolene          | 2.20         |
| Myrcene                  | 2.11         |
On the other hand, beta-sesquiphellandrene (4.21%), alpha-pinene (4.03%) and alpha-farnesene (3.41%) and other minor components also helped to determine the antimicrobial activity of ginger EO (Table 1). Environmental condition and genetic of the plant may contribute on the composition of EO [26].

3.2 MIC, Synergism effect, MBC, and MFC

The MIC value are shown on Table 2. MIC is described as the minimum concentration of the antimicrobial agent that inhibits the growth of microorganism [27,28]. Most of the target microorganisms needed no more than 2000 IU to inhibit their growth (Table 2). 250 IU inhibited the growth of Aspergillus niger FNCC 0057 and Pseudomonas fluorescens FNCC 0070, while the inhibition of Bacillus cereus FNCC 0057 needed 2000 IU of nisin. No activity was observed on Salmonella typhimurium FNCC 0050 and Staphylococcus aureus FNCC 0047 at 2000 IU of nisin. The sensitivity of E. coli and Staphylococcus aureus were different from the previous studies, which stated that Gram positive was more sensitive than Gram negative and fungi [10,13,14]. But, the growth of Staphylococcus aureus may also be affected by the capability of that microorganism to adapt with new habitat [29], and it becomes more resistant with nisin.

Ginger EO showed an effective activity. At the lowest concentration, 0.125% of ginger EO could inhibit the growth of Eschericia coli FNCC 0091. 0.25% inhibited Bacillus cereus FNCC 0057 and Staphylococcus aureus FNCC 0047 to grow normally. Pseudomonas fluorescens FNCC 0070 and Aspergillus niger FNCC 6080 were affected by 0.5% ginger EO, while 4% of EO was active against Salmonella typhimurium FNCC 0050. The EO activity had the same result with the previous study, where it showed inhibition activity on Gram positive, Gram negative and fungi [16].

Combination results are reported on Table 2. 62.5 IU of nisin and 0.125% ginger EO inhibited Bacillus cereus FNCC 0057 and Aspergillus niger FNCC 6080 to grow. 62.5 IU with 2% ginger EO was active against Salmonella typhimurium FNCC 0050. Pseudomonas fluorescens FNCC 0070 was inactive against 62.5 IU of nisin with 0.5% of ginger EO. To inhibit the growth of Staphylococcus aureus FNCC 0047 and Eschericia coli FNCC 0091 62.5 IU of nisin should be combined with 2% of ginger EO.

**Table 2.** The MIC of Nisin, Essential Oil, Their Combination, and Their Synergism Effect.

| Microorganism                  | Minimum Inhibitory Concentration | FIC | Synergism effect | MBC or MFC |
|-------------------------------|----------------------------------|-----|------------------|------------|
| Bacillus cereus FNCC 0057     | Nisin (IU) 2000 Essential oil (0,25) Nisin (IU) + Essential Oil (%) 62,5 IU + 0,125% | 0,5312 P | - |
| Salmonella typhimurium FNCC 0050 | > 2000 4 Nisin (IU) + Essential Oil (%) 62,5 IU + 2% | 0,5312 P | - |
| Staphylococcus aureus FNCC 0047 | > 2000 0,25 Nisin (IU) + Essential Oil (%) 62,5 IU + 2% | 8,0312 A | - |
| Eschericia coli FNCC 0091     | Pseudomonas fluorescens FNCC 0070 500 0,125 Nisin (IU) + Essential Oil (%) 62,5 IU + 2% | 16,125 A | - |
| Aspergillus niger FNCC 6080   | 250 0,5 Nisin (IU) + Essential Oil (%) 62,5 IU + 0,125% | 0,5 S 62,5 IU + 1% |

S : Synergistic effect
P : Partial synergistic effect
I : Indifference effect
A : Antagonistic effect

Combination could rise the effectivity of both EO and nisin. From the previous research [10], to inhibit the growth of B. cereus LPSQ 2872, Salmonella typhimurium SL 1344, and Staphylococcus aureus ATCC 2913 172 ppm of nisin were needed, while on E. coli O157:H7 more than 172 ppm was needed.
needed, these results were higher than when nisin was combined with ginger EO on this assay. Those activity caused by the activity of both agents. The hydrophobic cation in nisin will bind the phosphate of Gram positive bacteria. It causes the disturbance of cell membrane transglycosylation, it also creates pores that causes lysis [30, 31, 12, 32]. The addition of ginger EO may increase that activity. EO which is also hydrophobic creates pores on the cell, so the antimicrobial activity increases [33, 34]. While the activity on the fungi, ginger EO may disrupts the ergosterol metabolism, that ergosterol, has function on keeping the strenght of the cell wall [35]. If the metabolism is disrupted than the cell is also deteriorated. Nisin helps the inhibition process after the cell wall was degraded [36].

The synergism effects between two antimicrobial agents were showed by their FIC index value. The synergism effect was greater when the FIC index value was lower. The combination of ginger EO and nisin showed a total synergistic effect against Aspergillus niger FNCC 6080. The combination effects against Bacillus cereus FNCC 0057 and Salmonella typhimurium FNCC 0050 showed partial synergistic effect with FIC index value was 0.5312. The combination indicated indifference effect when it was used for against the Pseudomonas fluorescens FNCC 0070. Whereas, when the combination was used on Staphylococcus aureus FNCC 0047 and Eschericia coli FNCC 0090, it showed antagonistic effect. Nisin may inhibit the EO penetration, it also makes the outer membrane more stable [28, 29].

Furthermore, the bactericidal and fungicidal effect of the combination were tested (Table 2). The combination did not show bactericidal effect on all targeted bacteria. Contrastly, it killed the Aspergillus niger FNCC 6080 and showed fungicidal effect at 62.5 IU of nisin and 1% of ginger EO. Optimum condition, including temperature, pH, water activity, redox potential, and nutrient, may affect on MBC test [14].

4. Conclusion
In conclusion, the combination of ginger EO and nisin increased the activity of both agents and showed total and partial synergistic effect against Bacillus cereus FNCC 0057, Salmonella typhimurium FNCC 0050 and Aspergillus niger FNCC 6080. 62.5 IU plus 2% of ginger EO were effective on Salmonella typhimurium FNCC 0050, Eschericia coli FNCC 0091, and Staphylococcus aureus FNCC 0047. While Pseudomonas fluorescens FNCC 0070; Bacillus cereus FNCC 0057 and Aspergillus niger FNCC 6080 needed lower concentration at 62.5 IU plus 0.5% and 62.5 IU plus 0.125%.

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