Inhibition of Superbug Formation by Blocking Transmission of Bacterial Necrosignal Using Kimchi LAB Metabolites

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Abstract— **Multidrug resistance of superbugs has become one of the greatest threats to global health. Previously, it was understood that bacteria that survive direct exposure to antibiotics acquire antibiotic resistance traits and become superbugs. However, a 2020 study by Bhattacharyya et al. introduced a novel mechanism of superbug formation: dying bacteria transfer resistance-enhancing factor AcrA to living bacteria through a process called necrosignaling. In this study, we investigate whether the metabolites released by kimchi lactic acid bacteria (LAB) of various fermentation stages could block the transfer of necrosignals and therefore inhibit the creation of superbugs. To test this hypothesis, dying *E. coli* was applied on one side of LB agar plates and kimchi metabolites were injected into their borders. Agar diffusion tests were performed with *E. coli* on the other side of the agar plates. It was found that *E. coli* evolved into multi-resistant bacteria under necrosignal conditions, and kimchi LAB metabolites of all three fermentation stages blocked the transmission of these signals, resulting in a reduction in bacterial growth. Notably, LAB metabolites of kimchi in the second, or moderate, fermentation stage were the most effective. These results demonstrate that the LAB metabolites found in kimchi block the transmission of necrosignals, prompting further research into the use of kimchi postbiotics as a potential health food or medical treatment to counter multidrug resistance.**

I. Introduction

Superbugs, or multidrug resistant bacteria, pose severe threats to global health and food security, infecting 2.8 million people in the U.S. and resulting in 35,000 deaths in 2019 [1]. However, the survival of bacterial swarms and the rise of multidrug resistance remains relatively unfamiliar. Until now, it had been understood that bacteria that survive direct exposure to antibiotics acquire antibiotic resistance and become a superbug [2]. However, a 2020 study by Bhattacharyya et al. introduced a novel mechanism of superbug formation: dying bacteria release resistance-enhancing substance AcrA, a specific molecule found in a type of pump within the bacterial cell called RND efflux pump that interacts with the outer membrane of live cells (Figure 1) [3]. This interaction stimulates the live cells to expel or pump out drugs, such as antibiotics, from inside the cell through a process called necrosignaling [3].

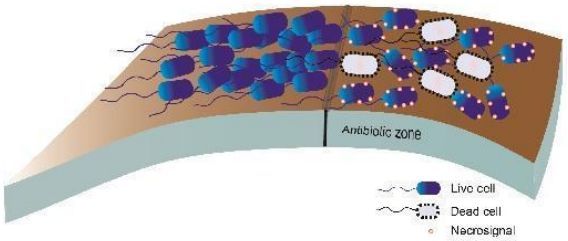

Figure 1. Dying bacteria cells releasing necrosignals to enhance antibiotic resistance in living cells

Necrosignaling occurs as a form of microbial communication called quorum sensing, which allows bacteria to coordinate responses through signaling molecules [3]. Thus, disrupting this communication could deter the transfer of resistance-enhancing signals.

Studies investigating promising compounds to prevent quorum sensing have discovered that bacteria in kimchi possess antimicrobial and anti-biofilm properties [4]. Specifically, during the process of fermentation, the bacteria release various byproducts known as lactic acid bacteria, or LAB, metabolites. Some of these LAB metabolites significantly inhibit the formation of biofilms, or communities of bacteria that stick together to form a protective shield against antibiotics, by disrupting quorum sensing. As the concentration of these metabolites increased, they became even more effective at interfering with quorum sensing, indicating their potential ability to target microbial communication pathways like necrosignaling [5].

In this study, we use agar diffusion tests to examine if kimchi LAB metabolites inhibit bacterial necrosignaling and determine the fermentation stage of kimchi that most effectively prevents these chemical signals, ultimately revealing a novel candidate for future treatment of multi-resistant superbugs. Through the examination of clear zone sizes formed when bacteria are exposed to antibiotics, we also aimed to analyze the antibacterial efficacy of kimchi LAB metabolites.

II. Methods

To prepare bacteria culture media MRS and LB agar, a mixture containing 27.5g of MRS powder, 7.5g of agar powder, and 500 mL of distilled water (DW), and another mixture with 10g of LB powder, 7.5g of agar powder, and 500 mL DW were autoclaved and left to cool. To make MRS and LB broth, one mixture containing 100 mL of DW and 27.5 g MRS broth powder and another mixture containing 500 mL of DW and 10g of LB broth powder were autoclaved and left to cool.

Three types of kimchi were prepared according to different fermentation levels: kimchi with fermentation grade 1 for shortest (24 hours), 2 for medium (30 days), and 3 for longest (1 year) period of fermentation. Samples of kimchi soup were incubated in both aerobic and anaerobic conditions (Figure 2). 30 uL of kimchi soup and MRS broth were cultured in conical tubes. After being removed from the incubator, bacterial colonies in MRS plates were

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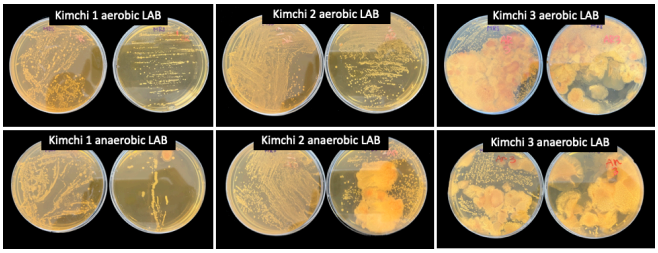


Figure 2. Kimchi LAB colonies on MRS agar plates after culture

Petri dishes were divided into three sections for antibiotic susceptibility tests and halves for border crossing assays. We note that if the size of the clear zones formed during any of these tests were too large, they could leak into neighboring sections of the petri dish and compromise experimental data. Thus, it was important that clear zones larger than half of the radius of the petri dish do not form. As the petri dish used in this study was 4.5 cm in radius, 2.25 cm would be the maximum diameter a clear zone could cover without spilling over to other sides. Since 0.01x dilutions of ampicillin and meropenem and 0.001x dilutions of ceftezole produced clear zones closest to 2 cm, these specific concentrations were selected for further use (Figure 3).

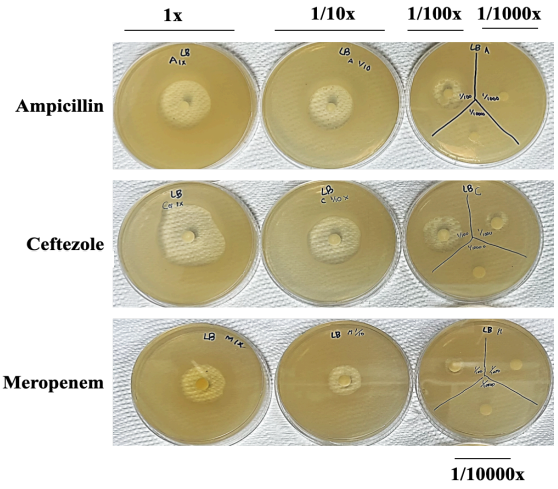


Figure 3. Clear zones formed by different dilutions of antibiotics

To test the antibiotic susceptibility of *E.* coli, three types of antibiotics, ampicillin, meropenem, and cephalosporin, were first diluted to various concentrations. *E. coli* stock solution was spread out onto agar plates, and paper discs were placed on each surface for antibiotic treatment (Figure 4). Each agar plate was incubated at 30°C for 48 hours.

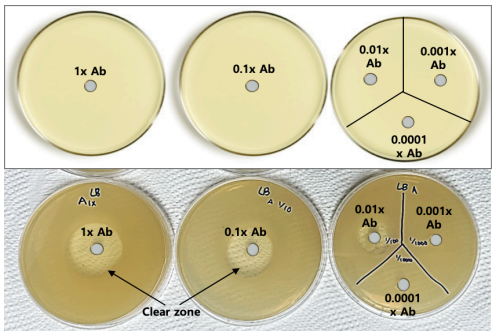


Figure 4. Placement of paper discs based on dilutions of antibiotic treatment

To test the antibiotic susceptibility of *E. coli* under the influence of necrosignals, LB agar plates with cultured *E. coli* were prepared according to different experimental conditions (Figure 5). Bacterial colonies were extracted from three locations (A, B, C) to be spread on new LB agar plates. After antibiotic treatment and incubation, the diameters of clear zones were measured as an indication of the inhibition of bacterial growth.

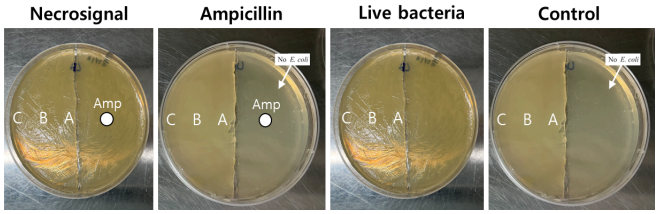


Figure 5. Method for testing antibiotic resistance of bacteria in different experimental conditions

The absorbance values of kimchi cultivated solutions were obtained, and 1 ABS per 1 mL was measured. Then, with a 0.2 um syringe filter, LAB was removed from cultivated solutions to recover the kimchi LAB metabolites.

In order to confirm if superbug formation would be inhibited, the midline of cultured LB agar plates were cut and saturated with kimchi LAB metabolites (Figure 6). Bacterial colonies from three locations were spread on new LB agar plates. After antibiotic treatment and incubation, the diameter of each clear zone was measured.

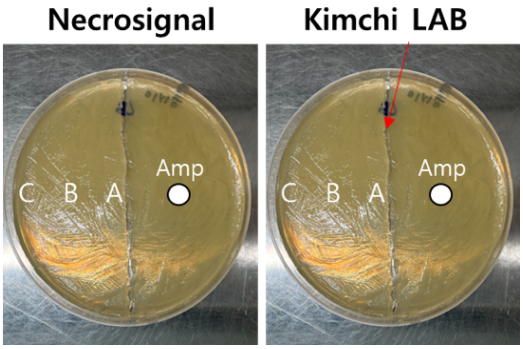


Figure 6. LB agar plates with and without kimchi LAB metabolites in border based on experimental conditions

Finally, kimchi samples with only LAB metabolites or together with LAB were prepared. Then, these samples were

autoclaved at 121°C, 1.2 atm., refrigerated at -75°C, and irradiated to simulate the packaging conditions of kimchi. These metabolites were injected into the cut midlines of cultured LB agar plates (Figure 7). Bacterial colonies from location B were applied on new LB agar plates. After treatment with antibiotics and incubation, the diameters of clear zones were measured to test the active range of kimchi LAB metabolites.

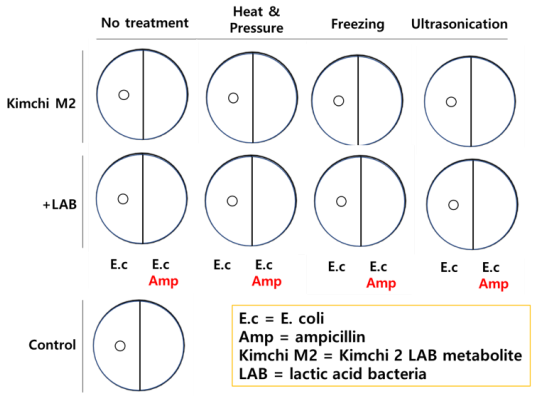


Figure 7. Method of detecting active range of kimchi LAB metabolites

III. Results

We first found that necrosignals induce antibiotic resistance of bacteria. The diameters of clear zones under necrosignal condition were shorter than that of ampicillin, live bacteria, or control conditions, especially in distance B, which represents a moderate distance between the isolated bacterial colonies and the line where kimchi LAB was injected. (Figure 8). Notably, it was found that ceftezole and meropenem under necrosignal conditions inhibited an area of bacterial growth of diameters 0.9 cm and 0 cm, respectively, in contrast to live bacteria condition, in which clear zones had diameters of 1.9 cm and 1.3 cm, respectively (Figure 9 B and C). The differences in these clear zones show that bacteria exposed to necrosignals under 'necrosignal conditions' developed resistance, as clear zones represent the extent to which antibiotics prevented the growth of visible bacterial colonies on agar plates.

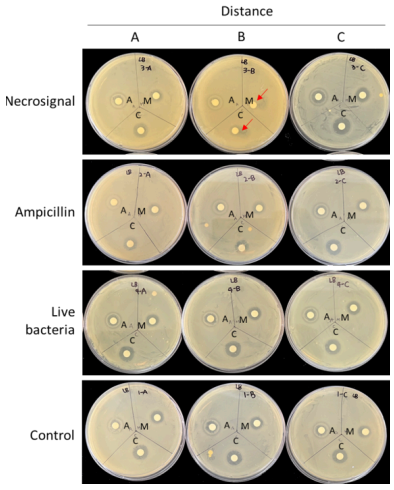


Figure 8. Qualitative effect of necrosignal on multi-drug resistance of bacteria

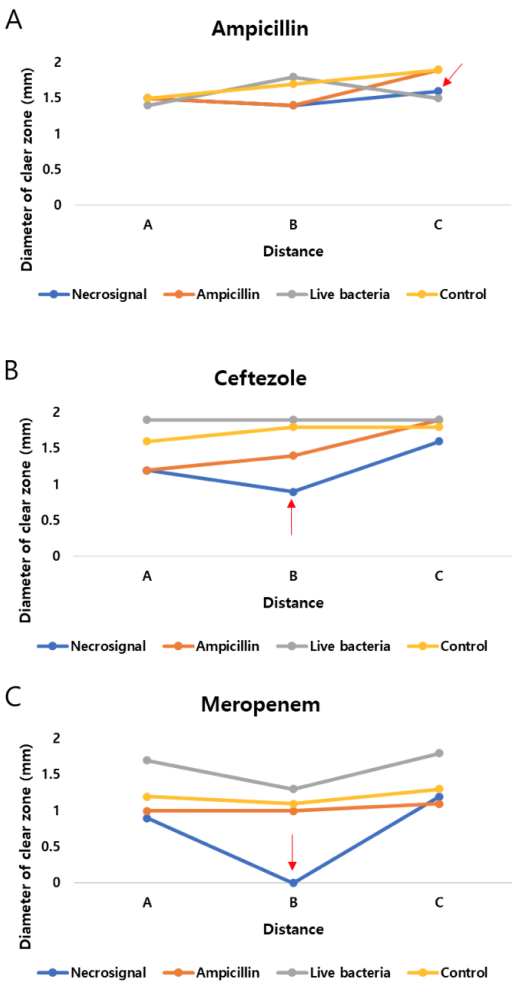


Figure 9. Diameters of clear zones formed by antibiotics depending on distance from border and experimental conditions

Koreans enjoy various types of kimchi with different fermentation stages: freshly made (24 hours), moderately fermented (30 days), and highly fermented (1 year) kimchi. Classification of kimchi LAB colonies and analysis of LAB proliferation revealed that the type and amount of kimchi LAB colonies varied widely based on fermentation stage and culture conditions (Figure 10 and 11).

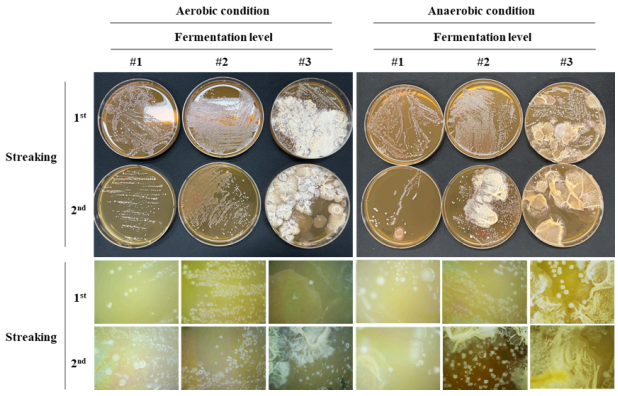


Figure 10. Kimchi LAB culture in aerobic and anaerobic conditions

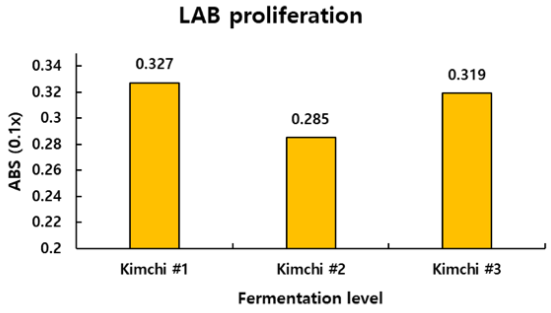


Figure 11. LAB proliferation (ABS, or absorbance, per 1 mL) of kimchi with different fermentation periods

Most importantly, in the antibiotic susceptibility test of *E. coli* by necrosignal, we concluded that kimchi LAB metabolites inhibit transmission of necrosignals. In comparison to necrosignal conditions, larger clear zones appeared in the presence of kimchi LAB metabolites (Figure 12). For example, in distance B, the diameters of clear zones under necrosignal conditions (control) were lowest, 1.4, 0.9, and 0 cm for ampicillin, ceftezole, and meropenem, respectively (Figure 13 A, B, and C). In contrast, the diameters of clear zones with exposure to kimchi metabolites were higher than the control. Furthermore, in distance A, necrosignals reduced the diameter of clear zones formed by ampicillin, ceftezole, and meropenem to 1.5, 1.2, and 0.9 cm, respectively, much lower than when kimchi LAB metabolites were present (Figure 13 A, B, and C).

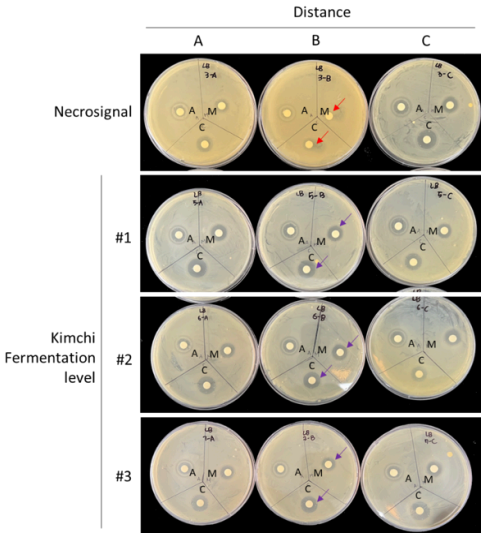


Figure 12. Qualitative effect of Kimchi LAB Metabolites on Inhibition of Necrosignal

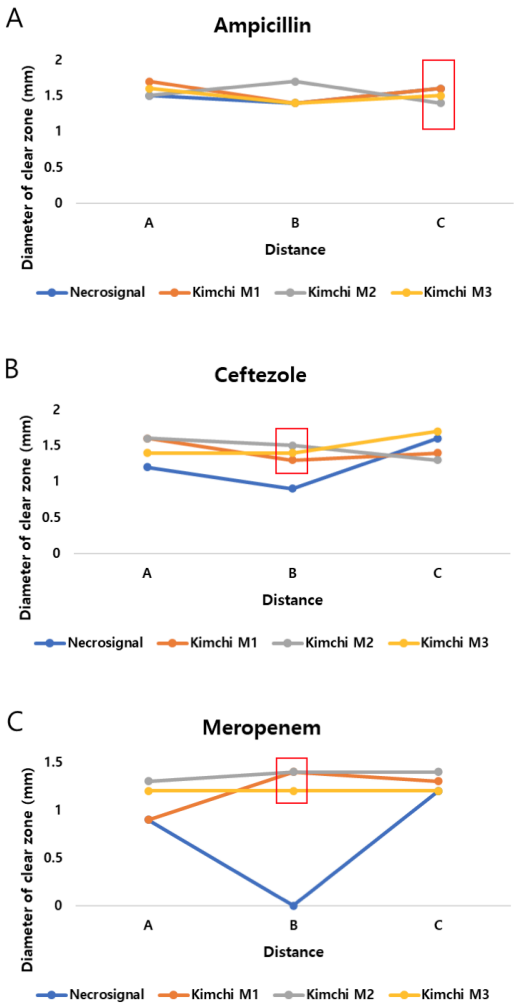
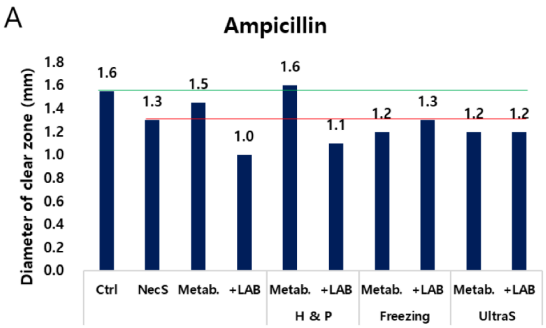


Figure 13. Diameters of clear zones formed by antibiotics depending on distance from border and inclusion of kimchi LAB metabolites

Finally, Kimchi LAB metabolites maintained necrosignal inhibitory activity even under high temperatures, deep freezing, and ultrasonic conditions. Clear zones formed by ceftezole and meropenem under necrosignaling conditions were smaller in diameter (Figure 14 B & C) than those under various treatment conditions.



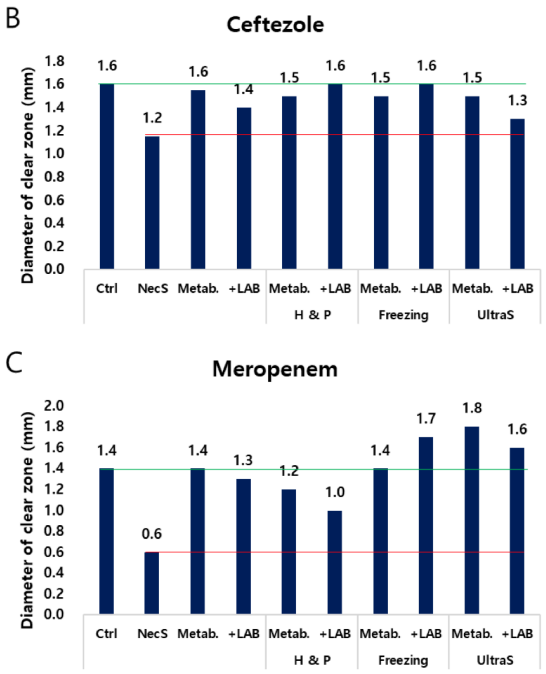


Figure 14. Diameter of clear zones formed by antibiotics depending on treatment of kimchi LAB metabolites in comparison to necrosignaling conditions (red lines) and control (green lines)

IV. Discussion

Our study found that necrosignals enhance the survival of bacteria in the presence of antibiotics, thus inducing the transformation from regular bacteria to superbugs. We discover that kimchi's LAB metabolites have necrosignaling-inhibiting effects. This could be due to that during kimchi LAB fermentation, the major metabolites produced are a heterogeneous mixture of lactic acid and ethanol, which works to break down structures of proteins in bacteria, along with bacteriocins and exopolysaccharides that are known to have antimicrobial effects [6].

While this experiment demonstrated that kimchi LAB metabolites inhibit necrosignal transmission, the effectiveness of these substances under environmental stressors (i.e. when the conditions deviate from typical kimchi fermentation conditions) was still uncertain. We must carefully assess the range of LAB metabolites' inhibitory activity because if kimchi is to be incorporated into future medications, it must withstand various processing and packaging treatments, subjected to heating and pressure, deep freezing, and ultrasonication. We tested some of these conditions as shown in Figure 14 and concluded that kimchi LAB metabolites overall maintain necrosignal inhibitory activity when subjected to high temperature, deep freezing, and ultrasonic conditions and are made up of very durable substances. However, we note that the clear zones made by ampicillin under necrosignaling conditions (Figure. 14 A), were greater in size than those under various treatment conditions, deviating from the pattern shown in Figure 14 B and C. This could be due to the fact that the bacteria were previously exposed to ampicillin in order to produce dying bacteria, making this result dispensable.

We also noted that the results of this study were different from what we expected. According to Inatsu et al, when Staphylococcus aureus, a gram-positive bacterium, was inoculated with kimchi for a prolonged time (i.e. fermentation occurred for a long time), the *S. aureus* level decreased rapidly to minimum detectable level [7]. In my study, however, bacterial growth was limited mostly effectively when exposed to moderately-fermented kimchi. One factor that could have accounted for this difference was the presence of dying bacteria in my study. While my study analyzed necrosignaling, which involves the transfer of chemical signals from dying to living bacteria, Inatsu et al only considered the effect of kimchi fermentation stages on bacterial proliferation.

V. Conclusion

The primary purpose of this study was to detect whether dying bacteria secrete necrosignals for living bacteria to become multi-resistant bacteria, and, if detected, to discover whether kimchi LAB metabolites can effectively suppress necrosignaling between dying and living bacteria. First, border crossing assays confirmed that necrosignals enhance survival of bacteria in the presence of antibiotics, thus inducing the transformation from regular bacteria to superbugs. In addition, it was found that LB agar plates treated with kimchi LAB metabolites had larger clear zones, proving that kimchi LAB metabolites suppress the transfer of necrosignals, especially those of moderate fermentation. Hence, through this research, it was discovered that kimchi LAB metabolites have repressing effects against the transfer of necrosignals and maintain this inhibitory activity even under extreme conditions, thus being a novel candidate for future treatment of multi-resistant superbugs. However, as this study was limited to revealing the overall effects of kimchi LAB metabolites, future research should be conducted to identify and confirm the exact chemical components of kimchi metabolites that enable it to prevent the transfer of necrosignals using chromatography, precipitation, or microscopic analysis. Future research can isolate the specific substances of kimchi LAB metabolites and offer new insights on how these substances can be used to develop effective treatment against superbugs formed through necrosignaling.

Acknowledgment

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