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The Antibacterial Properties of Filtrates from Chinese Cabbage Kimchi*

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Abstract

Lactobacillus plantarum and *Leuconostoc mesenteroides* are crucial functional starters and predominant isolates in a wide range of fermented foods, particularly kimchi, whose constituents exhibit bioactive properties. We previously developed a methodology using anion exchange resins to purify peptidyl compounds from *Lb. plantarum* LBP-K10. Antibacterial cultures of *Lb. plantarum* LBP-K10 were obtained from the respective cultures' supernatants and filtrates. However, conclusive evidence of the efficacy of kimchi filtrates in eradicating pathogenic bacteria is lacking. We aimed to simulate the potential effects of antibacterial filtrates that contained antibacterial compounds which were derived from cultures of *Lb. plantarum* LBP-K10. We acquired the kimchi filtrates using a combination of centrifugation and filtration methodologies, without the requirement for inoculation. The filtered liquid from Chinese cabbage kimchi, inoculated with *Lb. plantarum* LBP-K10 as a starter culture, and the non-inoculated liquid from Chinese cabbage kimchi (referred to as CCK and CCK_{Ref}, respectively) were were examined. CCK demonstrated greater inhibitory activity and a more significant bactericidal effect against the bacterial indicator strains. The minimum inhibitory concentration demonstrated comparable outcomes in tests against both Gram-positive and Gram-negative bacteria. This research offers a groundbreaking examination that displays the effectiveness of profiling peptidyl compounds within kimchi filtrates for curing bacterial infections.

Keywords: Korean traditional kimchi, *Lactobacillus plantarum* LBP-K10, Chinese cabbage kimchi, Kimchi filtrates, Antibacterial activity

Major Classifications: Food industry, Food biochemistry, Probiotics, Food Science (Food Nutrition, Healthy Food)

1. Introduction

Antimicrobial culture filtrates (CFs) are obtained by fermenting lactic acid bacteria (LAB) in traditional Korean kimchi, and they consist of low-molecular compounds. These compounds are anticipated to consist of hydrogen peroxide, carbon dioxide, organic acids, diacetyl, acetaldehyde, as well as both proteinaceous and nonproteinaceous bacteriocins, all of which are predicted to exhibit antimicrobial properties (Archer & Halami, 2015; Ji et al., 2015; Özogul & Hamed, 2017). The significant and verifiable impact of controlled microflora on both starter and non-starter kimchi can be demonstrated through the analysis of variations in secondary byproducts. Furthermore, the elevation of polar compounds due to alterations in metabolites within food products contributes

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to inadequate retention behaviors of analytes in chromatography (Jeong et al.,2013; Jung et al.,2014; Jung et al., 2012). Various filtrates derived from kimchi possess the potential to generate significant medicinal compounds owing to their distinctive orbital and target-specific chirality, as well as their diverse structural characteristics and potential utilization of cyclotides and related peptide scaffolds (Bellezza et al., 2014; Borthwick, 2012; Young, 2016). Further studies have substantiated previous research findings regarding starter and non-starter kimchi. The Leuconostoc mesenteroides starter consistently maintains an 88% dominance throughout the fermentation process (Eom et al., 2008), surpassing *Lactobacillus plantarum* and other microflora during the initial fermentation stage of kimchi (Jung et al., 2012). Precautionary measures should be implemented to mitigate the potential invasion of other types of kimchi microflora, including *Leuconostoc* spp., *Lactobacillus* spp., and *Weissella* spp. These microflorae have been found to adversely affect the fermentation process, microbial community, and metabolite production (Chang & Chang, 2010; Lee et al., 2008). Additionally, these studies provide evidence suggesting the involvement of *Ln. mesenteroides* is an effective candidate for utilization as an antimicrobial isolate (Jung et al., 2014; Jung et al., 2011; Jung et al., 2012).

Previously, our research team has provided evidence that filtrates derived from the culture of *Lb. plantarum* LBP-K10 has demonstrated efficacy in treating a wide range of pathogenic microorganisms, including multi-drug resistant strains, as well as human and plant pathogenic fungi, and the influenza A virus (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013). The antimicrobial effect was ascribed to a total of 15 proline-based cyclic dipeptides, a non-proline-based cyclo(Phe-Ala), and a non-peptidyl DL-3-phenyllactic acid. Additionally, the antimicrobial fractions found in kimchi filtrates from *Ln. mesenteroides* LBP-K06 and *Lb. plantarum* LBP-K10 were primarily identified ..

To evaluate the potential antimicrobial properties of *Lb. plantarum* LBP-K10 CFs containing antimicrobial compounds, the antibacterial activities of kimchi filtrates were compared. The present study focused on investigating the antibacterial properties of extracts from Chinese cabbage kimchi and LAB cultures. Based on the data obtained from the metabolites of *Ln. mesenteroides* kimchi metabolites (R. Liu et al., 2017), this study investigates the antimicrobial effects of kimchi filtrates and their mixtures, which are derived from the fermentation of kimchi with *Lb. plantarum*. This study represents a significant milestone as it is the first instance in which these findings have been documented.

2. Literature Review

2.1. The Combined Mixtures-kimchi Filtrates and Bacterial CFs: Synergistic Effects through the Formation of Conformational Enantiomeric Pairs or Diastereomers of Cyclic Dipeptides

In previous research, it was observed that different fractions obtained from bacterial cell-free extracts contained identical cyclic dipeptides, as confirmed by the electron ionization (EI) and chemical ionization (CI) data observed through a gas chromatography–mass spectrometry (GC-MS). However, these molecules exhibited clear peaks in the HPLC chromatograms. Despite displaying distinct peaks in the HPLC chromatograms differences in retention behaviors (Δt_R) may be due to the formation of conformational enantiomeric pairs or diastereomers. These pairs or diastereomers are formed by intramolecular hydrogen bonds between the carboxy and carboxyamide in the potential cyclic dipeptidyl moiety. The molecules suggest that differences in retention times may be due to the formation of conformational enantiomeric pairs or diastereomers. These pairs or diastereomers are formed by intramolecular hydrogen bonds between specific functional groups. This phenomenon is likely the result of the heterocomplexation of a chiral dipeptide, leading to the quantitative enrichment of enantiomers (Ishikawa et al., 2010; Shan et al., 2008). The folded structures of the five diastereomeric pairs, including [c-(L-X-L-Y) and c-(L-X-D-Y)], are derived from an aspartyl-tripeptide and a diketopiperazine. Additionally, five specific chiral derivatizing reagents, including amino acids such as L-Leu and D-Phe, are also utilized. Chiral auxiliaries consisting of L-valine, L-methionine, and L-alanine were proposed to influence retention behavior and facilitate the separation of diastereomers when using a reverse phase-HPLC column (Bhushan & Dixit, 2012; Brückner et al., 2012; Funasak et al., 1993). In a previous study, a mobile phase consisting of 10% methanol was utilized to separate cyclo(L-alanine-D-alanine) from the enantiomeric pairs cyclo(L-alanine-L-alanine) and cyclo(D-alanine-D-alanine) (Perzborn et al., 2013). Other studies suggested that the energy barrier for interconversion could be sufficiently high to enable the effective chromatographic separation of enantiomeric pairs or diastereomers, especially in peptides containing proline (Lesma et al., 2012; Swadesh, 2000). The earlier studies indicated that the isolated compounds F1/F6/F11, F2/F9, F3/F4/F12/F13, F5/F7, and F16/F17 in this research may be enantiomeric pairs. Our empirical findings suggest that it is possible to separate racemic diastereomers through CH_2Cl_2 extraction. Cyclic dipeptide peaks were identified through enantiomeric measurement. We classified these fractions into seven proline-based diketopiperazines with an EI-MS m/z value of

154, one non-proline-based cyclic dipeptide, and non-peptidyl molecules (Kwak et al., 2018).

2.2. The Synergistic Bioactivity of Combined Cyclic Dipeptides in Kimchi Filtrates and Bacterial CFs

In detail, we previously demonstrated that the individual cyclic dipeptides were bio-effectors, but the pooled cyclic dipeptides also exerted potent bioactive effects against various pathogens. It was also reported that the combination of cyclic dipeptides with or without antibiotics could exert synergistic antimicrobial effects (Deepa et al., 2015; Kumar et al., 2014; Kwak et al., 2018). However, although the MIC and antagonistic effects were observed for each cyclic dipeptides purified from *Lb. plantarum* LBP-K10 CFs in this study, we did not evaluate the combined effects of the cyclic dipeptides owing to the limited quantities of cyclic dipeptide collected using the semi-preparative HPLC system. Hence, we investigated this hypothesis by preparing a considerable amount of the complete cyclic dipeptide set (referred to as K10-CCDP) from the LAB CFs. For examples, we reported that the active concentrations of each cyclic dipeptide in the different K10-CCDP samples were noticeably lower than that previously required to demonstrate the synergistic antimicrobial activity of combined cyclic dipeptides, as reported in several previous studies. Finally, our data provide a potential framework for future research studies or commercial applications such as in agricultural pesticides, natural preservatives, or animal feed additives. Furthermore, our study offers a potential strategy that is capable of assessing the antimicrobial effects of cyclic dipeptides and their possible applications.

2.3. The Efficacy of CCK and LAB Cultures with Highly Cyclic Dipeptidyl Compounds

Based on a previous study (Liu et al., 2017), it has been proposed that antibacterial compounds can be produced through the utilization of bacterial filtrates derived from the supernatant and freeze-dried powder of different varieties of kimchi. The observed result can be attributed to the distinctive properties of each kimchi filtrate and the consistent implementation of a 10-fold CH_2Cl_2 extraction method, which demonstrates remarkable selectivity in the isolation of antibacterial compounds. The antibacterial compounds have undergone thorough purification to facilitate their analysis and confirmation as a singular compound, eliminating any potential presence of additional peptidyl or non-peptidyl compounds. This purification process involved the use of the 10-fold CH_2Cl_2 extraction method. This observation is substantiated by the increase in bioactive dipeptidyl molecules identified in the filtrates when employing the *Ln. mesenteroides* LBP-K06 strain is utilized as a starter culture, and its population gradually increases throughout the fermentation process. Based on the findings presented in the preceding report, modifications have been made to the starter strain by replacing *Ln. mesenteroides* LBP-K06 with *Lb. plantarum* LBP-K10. This can be attributed to the fact that this specific strain demonstrated the highest potency as an antibacterial probiotic strain in our previous research endeavors (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013).

The modified liquid-liquid extraction technique was utilized to isolate antibacterial cyclic dipeptidyl compounds from various kimchi filtrates (Liu et al., 2017). The kimchi fractions containing cyclic dipeptides were subjected to sequential re-chromatography using a semi-preparative Hypersil ODS C18 reverse-phase column. The isocratic composition of the mobile phase includes various combinations of methanol (3%, 5%, 10%, 15%, and 20%), acetonitrile (3%, 5%, and 10%), and HPLC-grade water (67-94%). Despite conducting multiple experiments using recursive CH_2Cl_2 extraction and fraction separations to capture pure components, quantifying impurity content through peak-area normalization has proven to be a recurring challenge. Our attempts to verify the proportion of relative retention peak area for the cyclo(Phe-Pro) fraction in all types of filtrates were unsuccessful, especially when compared to the isolated bacterial CFs. Chinese cabbage kimchi shows significant fractionation compared to other types of kimchi and bacterial isolates. The unique chromatographic profile of Chinese cabbage kimchi can be attributed to its specific fermentation properties, setting it apart from other substances. The EI and CI mass spectra of non-starter kimchi do not contain molecular ions. Various molecular ions do not exhibit distinct fragment ions, as observed in high-resolution mass measurements. Overlapping peaks in radish kimchi can cause the peaks to merge and affect each other. Various isocratic mobile-phase compositions are used at different pH levels to identify primary peaks and make accurate parametric estimates, enhancing peak separation. Despite attempts to isolate individual peaks from complex chromatograms, no compounds have been successfully isolated from any type of kimchi, even after controlling experimental parameters such as elution volume at peak maximum and peak height. The purification process yielded pure cyclic dipeptides that were suitable for identification and analysis using EI/CI gas chromatography-mass spectrometry as a single compound, with no other peptidyl or non-peptidyl compounds present. The purification model, combined with CH_2Cl_2 extraction, produced consistent fractions with patterns that facilitate antibacterial activity assays for cyclic dipeptides.

This experimental method utilized HPLC separation and a repeated 10-volume CH_2Cl_2 extraction, showing strong selectivity for isolating cyclic dipeptides (Kwak et al., 2018;

Kwak et al., 2014; Kwak et al., 2013). Additionally, the method required several modifications to the mobile phase. The process yielded pure cyclic dipeptides, which were analyzed and identified as individual compounds using EI/CI GC-MS. No other peptidyl or non-peptidyl compounds were detected. The cyclic dipeptide purification model, combined with CH_2Cl_2 extraction, allowed for the isolation of fractions with a consistent pattern of cyclic dipeptide fractionation in kimchi filtrates. The effectiveness of bioactive compounds in the solvent extraction process was assessed by analyzing cyclic dipeptides in powdered form using the EI/CI GC-MS technique every six months.

2.4. Concentration and Efficacy of Antibacterial Compounds in Filtrates Related to Filter Pore Size

The primary purpose of filters is to eliminate microorganisms from liquids. Filtration efficiency depends on the filter material utilized but usually eliminates a broad spectrum of active metabolites and chemicals irrespective of the size of filter pores. In previous studies, we observed no differences when using nitrocellulose membranes with varying filter pore sizes. The mechanisms of retention during membrane filtration are interestingly demonstrated in the scanning electron micrographs (Todd & Kerr, 1972). The tracketched filter and cellulose membrane filter differ in their screen filter and depth filter functionalities, respectively. A previous study depicted scanning electron micrographs revealing the 0.2 μm-rated membrane's pores to be bigger than the trapped Brevundimonas diminuta cells (Osumi et al., 1996). Typically, bacteria resided in the top 30 μm of the membrane. Previous studies have acknowledged the significant influence of adsorptive effects in filtering plasma proteins and influenza vaccines through membrane filters of sizes 0.22 and 0.45 μm, respectively. However, track-etched filters have less adsorptive properties as they are narrow and entirely sieve-like, without twists and turns. Adsorptive sequestration is not an inherent trait of filters, and rather points to their ability to trap organisms of specific sizes (Lukaszewicz & Meltzer, 1979; Lukaszewicz et al., 1978). Depth-type filters with a wide range of pore sizes are thought to capture organisms primarily through adsorption. Another investigation conducted a comparative study on bacterial retention utilizing pore sizes with screen-type polycarbonate and cellulose-ester membranes (Bobbitt & Betts, 1992). The former demonstrated a significant size threshold that prevented further cell passage, making it more selective in eliminating bacteria based on their size. Sieve retention plays a crucial role in achieving sterile filtration; however, it should not be considered as the sole factor. Membrane filters have advanced beyond their primary function as sieves and provide several benefits compared to conventional depth filters. Their thinness and uniform pore size make them extensively preferred in filtration technology.

3. Materials and Methods

3.1. Bacterial Strains and Culture Condition

The bacterial and fungal strains are listed in Table 1. The strains of lactic acid bacteria (LAB) were cultivated on a regular basis using a modified de Man, Rogosa, and Sharpe (mMRS) medium, with or without 1.0% agar, excluding beef extract (De Man et al., 1960). To examine the bioactivities of kimchi filtrates, *Lb. plantarum* LBP-K10 was cultured in mMRS liquid broth at 28℃ for 72 h. In order to conduct this research, kimchi filtrates were acquired from fermented food samples utilizing a cellulose acetate membrane with a pore size of 0.22 μm.

The antibacterial properties of the filtrates were subsequently assessed using gram-positive and gram-negative bacterial indicators, as well as multidrug-resistant bacteria, in accordance with established protocols (Liu et al., 2017). The bacterial specimens were provided by the Korea National Institute of Health (KNIH).

Table 1: Bacterial strains utilized in this study comprise one LAB strain and Gram-positive and Gram-negative bacterial indicator strains

Strain	Types or strains	Source or reference
LAB strains		
Lactobacillus plantarum LBP-K10	Original isolate from fermented Chinese cabbage	This study (Kwak et al., 2013)
[1] Bacterial indicator strains		
Gram-positive bacteria		
	Bacillus subtilis	This study, KNIH (Kwak et al., 2018)
	Staphylococcus aureus	This study, KNIH (Kwak et al., 2018)
	Streptococcus pneumoniae	This study, KNIH (Kwak et al., 2018)
Gram-negative bacteria		
	Salmonella Typhimurium	This study, KNIH (Kwak et al., 2018)

Escherichia coli	This study, KNIH (Kwak et al., 2018)
Shigella dysenterii	This study, KNIH (Kwak et al., 2018)

[1] Gram-positive and Gram-negative bacteria in this study were provided by the Korea National Institute of Health (KNIH).

3.2. Preparation of Fermented Foods and Their Filtrates

The filtrates derived from Chinese cabbage were obtained via controlled and spontaneous fermentation procedures. Inoculation with *Lb. plantarum* LBP-K10 was used as the starter strain, following the methods proposed previously (Cheigh & Park, 1994; Jung et al., 2014; Jung et al., 2011; Jung et al., 2012), with certain modifications. The Chinese cabbage underwent a process of natural fermentation and was obtained during the later phase of kimchi fermentation. In the context of controlled fermentation for the production of kimchi using Chinese cabbage, a supplementary fermentation process was conducted at a temperature of 25 ℃ for a duration of 72 hours subsequent to the initial stage of fermentation, until the kimchi attained the intermediate stage of fermentation. The Chinese cabbage kimchi (CCK) underwent a natural fermentation process, supplemented with a starter culture, until it reached the advanced stage of kimchi fermentation, as described in a previous study (Cheigh & Park, 1994). In contrast to *Lb. plantarum* LBP-K10 inoculation on Chinese cabbage kimchi (CCK), a separate series of experiments was conducted involving the natural fermentation of Chinese cabbage kimchi (CCK_{Ref}) without the use of any starter culture until it reached the intermediate stage of kimchi fermentation. Kimchi filtrates were obtained from fermented products using a sequential procedure that included freeze drying, powdering, and filtration with a #80-mesh (180 micron) sieve. The filtrates derived from the fermentation process were subsequently dissolved in sterilized distilled water. The solution was filtered using a 0.22 μm-cellulose acetate membrane.

3.3. Antibacterial Assay

The determination of the minimum inhibitory concentration (MIC) was conducted using a broth microdilution assay following the guidelines set forth by the National Committee for Clinical Laboratory Standards (Wikler, 2006). Prepared samples were assessed utilizing Gram-positive and Gram-negative bacterial indicators, in addition to multidrug-resistant bacteria, as previously describe (Kwak et al., 2018).

Samples were subjected to a two-fold dilution, resulting in concentrations ranging from 400 to 3.125 μg/mL. The MIC was then determined after incubating the samples for 17 hours at 37 ℃. The minimum bactericidal concentration (MBC) of the compound was determined by measuring the lowest concentration that resulted in a reduction of >99.9% in the initial inoculum growth. This measurement was conducted in three independent experiments. The analysis of purified compounds involved the utilization of bacterial indicators and multidrug-resistant bacteria at a concentration of 5 ×105 CFU/mL (Wiegand et al., 2008). In the conducted reference experiments, the antibacterial activity of LBP-K10-CF was measured at 24-hour intervals following seed inoculation in order to ascertain its MIC.

3.4. Statistical Analysis

Results are presented as means ± standard deviation (SD). The statistical significance of the observed differences was assessed by conducting a Student's *t*-test using Microsoft Office Excel (2013). A *p*-value less than .05 (*) was considered to indicate statistical significance in all comparisons.

4. Results and Discussion

4.1. The CCK and LAB Cultures with Noticeable Antibacterial Characteristics

Approximately 400 strains were previously isolated from three traditional Korean kimchi preparations. Among these isolates, 200 types of LAB with antimicrobial activities were identified. Finally, 30 strains with potent antimicrobial activity were selected (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013). Among these strains, *Lb. plantarum* LBP-K10 CF showed the greatest activity against the bacterial indicators used. These findings align with previous studies screening Cyclic dipeptides using *Lb. plantarum* strains derived from various kimchi cultivars, which demonstrated greater antimicrobial efficacy than other isolates (Li et al., 2012; Lim & Im, 2009; Yang et al., 2011). *Lb. plantarum* WCFS1 was found to possess the ability to encode pyruvate-dissipating potential and is believed to function as a fermenter with exceptional nutritional and bioactive characteristics (Liu et al., 2015; Mayo et al., 2008). The in vitro evaluation of the probiotic and functional potential of Lactobacillus strains indicated antagonistic activity against several types of pathogens. Furthermore, these strains exhibited other physiological functions, such as reducing cholesterol and nitrate levels, scavenging free radicals, stimulating the immune response, and producing an abundance of exopolysaccharides (Ren et al., 2014). Based on the previously conducted comparative studies with other LABs, including other Lactobacillus strains, we conducted experiments to determine the distinct effectiveness of using *Lb. plantarum* LBP-K10 and compared it to non-inoculated kimchi filtrates.

Table 2: Relative antibacterial activity of each kimchi filtrate without CH_2Cl_2 extraction

Strain	Culture filtrate without CH_2Cl_2 extraction (reference experiment)	CCK_{Ref} (kimchi without starter as a reference experiment)	CCK (kimchi with starter)
[1, a,*] Antagonism test	+++	+++	+++
[1, b,*] Antagonism test	+++	++	+++
[1, c,*] Antagonism test	+++	++	+++
[1, d,*] Antagonism test	+++	+++	+++
[1, e,*] Antagonism test	+++	++	+++
[1, f,*] Antagonism test	+++	++	+++

[1] Symbol: +, <15 mm; ++, <22 mm; +++, >22 mm (Indicator strains: a B. subtilis, b S. aureus, c S. pneumoniae, d S. Typhimurium, e E. coli, f S. dysenterii)
[*] All experiments represent the mean of three separate trials ± standard error.

To examine the synthesis of active antibacterial compounds by *Lb. plantarum* LBP-K10 during the fermentation process of kimchi, we performed disc diffusion assays (Table 2) to evaluate the antibacterial properties of CCK and to compare them with those of LAB cultures. We hypothesized that the attainment of a specific fermented state is crucial for the manifestation of antibacterial properties in spontaneously fermenting CCK inoculated with *Lb. plantarum* LBP-K10 and fermented until reaching the advanced stage, as previously suggested (Cheigh & Park, 1994). To regulate the fermentation process of CCK and other types of kimchi, including CCK that has not been inoculated with *Lb. plantarum* LBP-K10, CCK_{Ref}, it was imperative to regulate the duration of fermentation. Additionally, experiments were conducted to compare the spontaneous fermentation of CCK_{Ref} using bacterial culture filtrates as references. This action was undertaken with the objective of retaining authority over the fermentation process.

The filtrates obtained from kimchi displayed diverse levels of antibacterial activity, as evidenced by the results of the disc diffusion assay. The kimchi filtrate, which was subjected to fermentation and inoculated with *Lb. plantarum* LBP-K10, also known as CCK, exhibited the highest level of antibacterial activity, surpassing other filtrates such as CCK_{Ref} (Table 2). The exceptional antibacterial efficacy against bacterial indicators, encompassing Gram-positive bacteria such as B. subtilis and S. aureus, as well as Gram-negative bacteria like S. typhimurium and E. coli, can be ascribed to the inherent capability of *Lb. plantarum* LBP-K10 to produce antibacterial compounds either in the culture medium or in starter kimchi. Nevertheless, the production of these agents in non-starter fermented materials is limited.

4.2. The MIC of Kimchi Filtrates in Relation to Their Antibacterial Activity

Subsequently, an experimental investigation was conducted to validate the antibacterial properties of the kimchi filtrates by determining the determination of MIC (Table 3). The experiment was carried out under identical conditions as specified in Table 2. Notably, the kimchi filtrate exhibited significant antibacterial effects. However, the antibacterial activity demonstrated by CCK_{Ref} was relatively lower compared to that of CCK. The findings indicate that the *Lb. plantarum* LBP-K10 strain as a starter culture, along with the incorporation of spontaneously fermenting CCK with *Lb. plantarum* LBP-K10, contribute to the variations observed in the content of antibacterial compounds. The findings depicted in Table 3 illustrate the influence of antibacterial metabolite biosynthesis on the consumption of amino acids throughout the process of kimchi fermentation. The aforementioned process plays a critical role in the primary metabolic processes of both starter and non-starter ripening flora. This observation aligns with previous studies that have documented a progressive decline in amino acid concentrations during the intermediate stages of fermentation (Jeong et al., 2013).

This highlights the importance of kimchi metabolites as crucial constituents of the diet, and their potential usefulness in predicting, estimating, or evaluating antimicrobial-producing behaviors in conjunction with kimchi taste and flavor profiles. Alterations in the composition of metabolites, particularly organic acids such as lactic and acetic acids, as well as flavoring compounds like mannitol and amino acids, have been recognized as potential indicators of antimicrobial activity during the fermentation process of kimchi. The variation in metabolite composition has been observed to be dependent on the type of kimchi material (Ha et al., 1989). Therefore, we propose the utilization of *Lb. plantarum* starter culture, as opposed to *Ln. mesenteroides* starter culture, in the manufacturing process of Chinese cabbage kimchi enriched with antimicrobial compounds. This recommendation is made in order to fulfill the requirements related to antibacterial compounds. Our study provides empirical evidence of the production of antibacterial compounds in a carefully controlled fermentation process of cabbage. This synthesis is achieved by precisely manipulating variables such as the dominance of the starter culture, modification of the fermentation time-course, and adjustment of temperature.

However, the primary drawback of the MIC and MBC tests is the lack of information regarding time-dependent parameters, such as the rate of killing, subMIC effects (Powers et al., 1984), and the post-antibiotic effect, known as PAE (Pillai et al., 2005). Consequently, research efforts have shifted towards studying bacterial growth responses in the presence of dynamically changing antibiotic concentrations, using the in vitro kinetic models for this purpose. Multiple drug-microorganism interactions have been examined with various dosing schedules. Therefore, in vitro time-kill kinetic studies can simulate the effect of specific antibiotics, including Penicillin-binding proteins (PK), to study their pharmacodynamics against pathogens. It is noteworthy that measuring the static value of antibiotic concentration after incubation of bacteria remains the primary limitation of determining the MIC (Sy & Derendorf, 2014). There is no information about the changing concentrations of antibiotics and their effect on bacterial growth. Antibiotic concentrations in host organisms are subject to continuous change, as previously noted. Consequently, in vitro time-kill kinetic studies provide a more precise representation of what happens in host (Michael et al., 2014). These studies in humans allow for the analysis of various factors such as altered clearance, infection site, protein binding, starting inoculum size, and dosing regimens, among others. Several designs have been created for this purpose. The pharmacokinetic/pharmacodynamic (PK/PD) modeling can estimate specific parameters that characterize the pharmacodynamics of antibiotics (Schmidt et al., 2008), while in vitro time-kill studies are primarily used in drug development due to their high complexity (Velkov et al., 2013).

Table 3: Antibacterial activity observed in the filtrates of Chinese cabbage kimchi that underwent fermentation with *Lb. plantarum* LBP-K10 as a starter culture in addition to being present in the non-inoculated filtrates of Chinese cabbage kimchi

Indicator strains	[1,2] MIC (mg/L)		
	Culture filtrate without CH_2Cl_2 extraction	CCK_{Ref} (kimchi without starter)	CCK (kimchi with starter)
[3] Gram-positive bacteria			
Bacillus subtilis	3.42±0.62	6.11±0.50	4.93±0.62
Staphylococcus aureus	3.84±0.25	6.95±0.78	5.08±0.44
Streptococcus pneumoniae	3.19±0.33	7.36±0.49	5.23±0.88
[4] Gram-negative bacteria			
Salmonella Typhimurium	2.95±0.27	6.09±0.92	4.40±0.27
Escherichia coli	2.84±0.19	6.73±0.86	5.02±0.35
Shigella dysenterii	3.35±0.41	7.22±0.79	4.84±0.51

1 The values represent the average of three independent experiments ± standard errors.
2 MIC: Minimum inhibitory concentration.
3 Gram-positive and 4 Gram-negative bacteria used in this study were supplied by the National Institute of Health (NIH) in Korea.

4.3. The Utilization of *Lb. plantarum* Starter in Kimchi Filtrate with Potent Antibacterial Activity

Our MIC data, as presented in Table 3, is consistent with the results of prior research, specifically studies involving proline-based cyclo(Leu-Pro) and cyclo(Phe-Pro) from Lactobacillus isolates, Streptomyces sp. KH-614, and Achromobacter xylosoxidans (Dal Bello et al., 2007; Rhee, 2002; Ström et al., 2002; Yan et al., 2004). This observation aligns with our previous findings, which indicated a higher occurrence of proline-based fractions that possess antimicrobial properties (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013). The fractions analyzed in this study consist of cis-cyclo(L-Leu-L-Pro) and cis-cyclo(L-Phe-L-Pro). Hence, it is plausible that kimchi filtrates, which contain specific cyclic dipeptides, exhibit potent inhibitory properties against bacteria, fungi, and viruses. These compounds have demonstrated noteworthy antibacterial activity against multidrug-resistant bacteria, with a specific focus on cis-cyclo(L-Leu-L-Pro). This finding aligns with the results of a previous study (Rhee, 2004) which examined the effects of cyclo(L-leucyl-L-prolyl) and cyclo(L-phenylalanyl-L-prolyl) on the growth of five strains of vancomycin-resistant enterococci (VRE) and pathogenic yeasts. Additionally, the study also investigated the effects of cyclo(Leu-Pro) derived from *Ln. mesenteroides* LBP-K06 has been examined for its efficacy against multidrug-resistant Gram-positive and Gram-negative bacteria, as reported (Liu et al., 2017).

The present study provides conclusive evidence of the synergistic effect of probiotic compounds on antimicrobial activity, as demonstrated through the use of the LAB approach. This is illustrated in Table 2 and 3. Our research findings indicate that the microbial community derived from starter cultures in the production of antimicrobial compounds in kimchi within a closed system. Through the implementation of an investigative study on the byproduct composition produced as a consequence of metabolite alterations during the process of fermentation, it has been noted that there exists a significant association between the type of kimchi material utilized and the resulting content. The underlying assumption in this particular context is that variations in the dietary composition and the ratio of plant or animal-derived raw materials, in conjunction with regulation facilitated by the application of specific starter cultures, can lead to diverse production of fermentation metabolites. Our hypothesis is supported by the results of a content analysis conducted in a previous study (Jung et al., 2012). The study unveiled notable variations in carbon sources found in kimchi filtrates, including glucose and fructose. The concentrations of carbon sources in raw materials remain consistent until the middle stage of fermentation, which can significantly influence the metabolic processes of LAB during the production of diverse fermentation products. Chinese cabbage demonstrates elevated concentrations of free sugars in comparison to other commonly utilized plant materials for the production of raw materials and fermented kimchi.

Thus, the components in kimchi filtrates indicate that it is a significant source of vitamins, such as the vitamin B group, beta-carotene, ascorbic acid, and their relevant precursors (Jung et al., 2014). It is also a valuable source of dietary fiber, and consuming kimchi filtrates may lower lipid levels in individuals. Some kimchi filtrates contain bioactive proline-containing cyclic dipeptides and non proline-based cyclic dipeptidyl compounds (Kwak et al., 2018; Liu et al., 2017). Certain raw vegetables, including cabbage, perilla leaves, parsley, pepper leaves, red pepper, and garlic, are used in kimchi filtrates derived from plant materials. These vegetables undergo an in vitro test system to demonstrate their antimutagenic properties. In addition, animal experiments have indicated that kimchi extract has anti-carcinogenic effects (Thakur & Belwal, 2022). This outcome could be attributed to the reinforcement of the host's immune system or kimchi's ability to impact mutagen metabolism. Additionally, LAB produced from kimchi impeded the attachment of Helicobacter pylori to human gastric epithelial cell lines and impeded H. pylori growth in vitro (Jung et al., 2014). The utilization of research results and expected outcomes via industrial development would encompass: the development of alternative antibiotics for human medicine as a substitute for antibiotics, animal medicine as a replacement for antibiotics, functional foods, and food preservatives.

4.4. Future Prospects

Vulnerabilities in current technology include the limited use of lactic acid bacteria as antibacterial agents. The only domestic and foreign cases where lactic acid bacteria have been successfully isolated, purified, identified and commercialized as pure substances are found with NISIN. While NISIN holds symbolic value as the first product of its kind, the narrow spectrum of antibacterial activity renders it ineffective as a food preservative for meeting the actual goal of the product. In most cases, antibacterial substances from lactic acid bacteria are utilized in food products by capturing all components of the fermentation liquid. There has not yet been a case at home or abroad in which comprehensive research and development has been attempted to develop antibiotic substitutes, such as isolating and identifying the expression genome of antibiotic substances found in specific kimchi extracts with potentially high-value industrialization prospects due to their distinct utilization methods and applications. Additionally, identifying the structure of the expression substance, isolating and purifying it, and evaluating its efficacy of utilization have also not been attempted. To achieve the objective of industrializing antibiotic substitutes for human and animal use, it is crucial to isolate and identify the expression genome of lactic acid bacteria that produce the active ingredient of kimchi extract, utilize it efficiently, identify the mechanism for high concentration expression to maximize expression output, isolate and purify the product to obtain high-purity yield, and attempt extensive productization through substance and efficacy identification.

Future prospects are as follows. Products containing lactic acid bacteria and metabolites have been utilized in various forms such as food, probiotics, and medications due to their acknowledged efficacy and safety. Recently, licenses for antibacterial purified substances have been granted in Japan following Europe. Several industries have made attempts to industrialize these substances, thus highlighting their immense potential for commercialization. On the other hand, when it comes to antibiotic substances isolated from kimchi extract, the progress in genome isolation and analysis technology, material fermentation technology, and material structure analysis technology is facilitating both strategy and research and development. As the amount of antibiotics continues to rise, the need for alternative medical treatments is also increasing. This is due to the emergence of superbugs and the persistent occurrence of infectious diseases. Additionally, there is a growing awareness of the benefits of immune-boosting functional foods and the necessity for natural, non-harmful preservatives. In the future, it is anticipated that diverse industrialization methods using antibacterial substances derived from lactic acid bacteria or more specifically, kimchi extract will emerge. This is expected to generate alternative product markets or new markets for existing human and animal medicinal antibiotics significantly (Fig. 1).

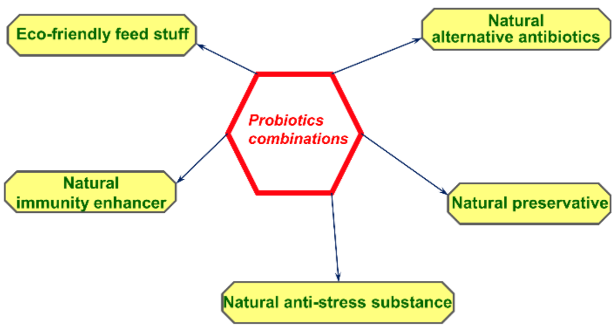

Figure 1. Probiotic applications - a strategic approach. The present study aims to investigate the efficacy of different kimchi filtrates in combating bacterial and fungal growth, with potential applications in various commercial sectors.

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Availability of Data and Materials

All supporting information including table of results and detailed methods is available upon request.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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