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The Antifungal Efficacy of Extracts Derived from Kimchi Filtrates*

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Abstract

Secondary metabolites in the culture filtrates of lactic acid bacteria offer varied chiral moieties, making them a valuable resource for drug design scaffolding. Our previous methodology included using a combination of anion exchange resins, Amberlite IRA-67 and Purolite A420S, to purify significant quantities of *Lactobacillus plantarum* LBP-K10 peptidyl compounds. However, current experimental evidence regarding the impact of native culture extracts and/or filtrates on pathogenic fungi *in vivo/in vitro* is insufficient. This study analyzed the antifungal properties of two different probiotic cultures: the CH_2Cl_2-extracted filtrate of Chinese cabbage kimchi (CH_2Cl_2-extracted CCK_{WLB} and CH_2Cl_2-extracted CCK_{WOLB}) and the non-extracted filtrate of Chinese cabbage kimchi (non-extracted CCK_{WLB} and non-extracted CCK_{WOLB}). The samples were divided into two groups: one group was inoculated with probiotics while the other group remained non-inoculated. Filtrates from both experimental groups were utilized for antifungal assays. The treatments employing CCK_{WLB}, with an initial inoculation of *Lb. plantarum* LBP-K10 as a starter, demonstrated significant antifungal activity under various experimental conditions. Our study offers new perspectives on the antifungal properties of CH_2Cl_2-extracted kimchi filtrates, which are naturally produced by lactobacilli. The efficacy of antifungal compounds is supported by substantial evidence demonstrating their efficient uptake by cells and the antifungal properties exerted by metabolites.

Keywords: Korean traditional kimchi, Kimchi filtrates, Antifungal activity, Methylene chloride extraction

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

1. Introduction

The antimicrobial and antagonistic activities are commonly observed in the culture supernatants and culture filtrates (CFs) of lactic acid bacteria (LAB), as well as in various types of naturally fermented foods made from raw materials obtained from animals and plants (Archer & Halami, 2015; Ji et al., 2015). Several studies have underscored the significance of postbiotic metabolites and proteinaceous derivatives derived from probiotic lactic acid bacteria in assessing the potential antimicrobial properties of kimchi filtrates (Kim et al., 2009). For instance, six different strains of *Lactobacillus* produce postbiotic metabolites that display specific cytotoxic effects on malignant cancer cells (Chuah et al., 2019). This effect is accomplished through strain- and cancer cell-specific mechanisms that inhibit cell proliferation and induce apoptosis. This study discovered that protein-rich postbiotic metabolites produced by *Lactobacillus plantarum* I-UL4 using different media compositions, exhibit cytotoxic properties against MCF-7 breast cancer cells. The cytotoxic effects are mediated through strain- and cancer cell-specific

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antiproliferative and apoptotic mechanisms. The conclusions presented in this study are based exclusively on findings derived from cell culture experiments (Tan et al., 2015). Thus, *Lb. plantarum*, a bacterium frequently present in the gastrointestinal tract of humans, presents notable benefits as a prevailing strain for ensuring safe utilization in various food manufacturing and preservation procedures. Furthermore, it exhibits promising potential as a probiotic agent for anti-cancer applications (Kwofie et al., 2020).

In a previous study, we provided evidence that filtrates derived from *Lb. plantarum* LBP-K10 has been found to exhibit inhibitory effects on the proliferation of various pathogenic microorganisms, including multi-drug resistant strains, 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 attributed to 15 cyclic dipeptides derived from proline, one cyclo(Phe-Ala) not based on proline, and a non-peptidyl DL-3-phenyllactic acid. The antimicrobial fractions found in the filtrates of kimchi from *Ln. mesenteroides* LBP-K06 and *Lb. plantarum* LBP-K10 were predominantly found in the latter portion of the HPLC chromatogram, specifically between 15 to 32 minutes. These compounds were identified as N9 (F9) to N15 (F17) (Liu et al., 2017).

To investigate the antimicrobial properties of *Lb. plantarum* LBP-K10 CFs, which contain antimicrobial substances, we conducted a comparative analysis on the antifungal abilities of Chinese cabbage kimchi filtrates. We conducted a comparative study to assess the antifungal activity of kimchi filtrates extracted using methylene chloride (CH_2Cl_2) in comparison to unextracted filtrates. Our study comprehensively examines the antifungal properties of kimchi filtrates and extracts derived from *Lb. plantarum*-fermented kimchi through analysis of *Ln. mesenteroides* kimchi metabolites (Liu et al., 2017). This publication represents a significant milestone as it is the first time that these findings have been made available to the public.

2. Materials and Methods

2.1. Strains and Culture Conditions

The bacterial and fungal strains are listed in Table 1. The bacterial strain was cultured on a regular basis using a modified de Man, Rogosa, and Sharpe (mMRS, MRS minus beef extract) medium, with or without 1.0% agar (De Man, Rogosa, & Sharpe, 1960). To investigate the bioactivities of kimchi filtrates, we conducted a culture of *Lb. plantarum* LBP-K10 in mMRS liquid broth at a temperature of 28℃ for either 72. For this study, kimchi filtrates were acquired by passing fermented foods through a 0.22-μm cellulose acetate membrane.

The *Ganoderma boninense* isolate (GMR3), a plant pathogen, and the *Candida albicans*, a human pathogen (Fonzi & Irwin, 1993), were cultivated and maintained in potato dextrose agar (PDA) and minimally defined synthetic (SD) medium, respectively. The medium was composed of 2% glucose, 0.5% ammonium sulfate [$(NH_4)_2SO_4$], and 0.17% yeast nitrogen base without amino acids and ammonium sulfate, respectively. The evaluation of the antifungal activity of the samples was conducted using a method that has been previously described (Kwak et al., 2014).

Table 1: Bacterial and viral strains utilized in this study comprise Gram-positive and Gram-negative multidrug-resistant bacteria, bacterial indicator strains, and the influenza A/H3N2 virus

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] Pathogenic fungi		
C. albicans		
SC5314	Wild type isolate	This study (Fonzi & Irwin, 1993)
G. boninense		
	GMR3, wild type isolate	This study (Kwak et al., 2014)

[1] Fungal pathogens.

2.2. Preparation of Fermented Foods and Their Filtrates

The filtrates obtained from Chinese cabbage were acquired via controlled and spontaneous fermentation processes, with and without inoculation by *Lb. plantarum* LBP-K10, which was used as the starter strain. These procedures are in accordance with previous proposals (Cheigh & Park, 1994; Jung et al., 2014; Jung et al., 2011; Jung et al., 2012), with some modifications. The Chinese cabbage underwent spontaneous fermentation and was acquired at the later stage of kimchi fermentation. In the context of controlled fermentation for the production of kimchi using Chinese cabbage, a supplementary fermentation process lasting 72 hours at 25℃ was carried out after the initial stage, continuing until the middle stage of kimchi fermentation, as previously described (Cheigh & Park, 1994). In contrast to LAB-inoculated Chinese cabbage kimchi (CCK_{WLB}), a distinct series of experiments was conducted involving the natural fermentation of Chinese cabbage kimchi (CCK_{WOLB}) without the use of any starter culture until it reached the intermediate stage of kimchi fermentation. The kimchi filtrates utilized in this study were derived from fermented products. The process involved

freeze drying, powdering, and filtration using an 80-mesh (180 micron) sieve to obtain the filtrates. The fermentation filtrates underwent processing in order to obtain a powdered form. The powder was subsequently dissolved in sterilized distilled water and filtered using a 0.22 μm-cellulose acetate membrane.

2.3. CH_2Cl_2 Extraction

The liquid-liquid extraction (LLE) process was performed by extracting the filtrates of kimchi using 10-fold volumes of CH_2Cl_2. The CH_2Cl_2 extracts were subsequently dissolved appropriate volumes of triple-distilled water and then filtered using a 0.22 μm cellulose acetate membrane. To prevent the accumulation of impurities, the filtered samples underwent a lyophilization process. This was followed by extraction using CH_2Cl_2 and subsequent filtration, in order to facilitate the antimicrobial activity assays.

2.4. Antifungal Activity Assay

The antifungal activity against the plant pathogen *G. boninense* and the human pathogen *C. albicans* was assessed using the method described (Kwak et al., 2014). To evaluate the inhibitory effects of purified cyclic dipeptides on *Ganoderma*, six-well PDA plates (3.0 mL) with mycelium and 8.0-mm perforations were utilized. A lyophilized fraction (1.5–25 mg), suspended in sterile TDW and then applied to the PDA plates in a six-well format. The plates were then incubated at 28℃ for seven days. The antifungal activity against *Candida* was evaluated by inoculating 1×10^4 cells of wild-type *C. albicans* SC5314 into six-well plates containing 3 mL of minimally defined SD agar medium. The plates were subsequently incubated at 28℃ for 3 days to observe the results.

2.4. Statistical Analysis

Results are reported as means ± standard deviation (SD). The statistical significance of the differences was analyzed using Student's *t*-test on Microsoft Office Excel (2013). A *p*-value less than .05 (*) was considered to be statistically significant in all comparisons.

3. Results and Discussion

3.1. The Antifungal Activity of CCK_{WLB} and CCK_{WOLB}

To investigate the production of bioactive antifungal compounds by *Lb. plantarum* LBP-K10 during kimchi fermentation, we conducted antifungal assay to determine the antifungal activities of two kimchi filtrates, including CCK_{WLB} and CCK_{WOLB} (Table 2). Based on previous research (Cheigh & Park, 1994), achieving a specific fermented state was vital for evaluating antifungal efficacy of spontaneously fermenting CCK_{WLB} and CCK_{WOLB} inoculated and non-inoculated with *Lb. plantarum* LBP-K10 and fermented to the late stage. To control the fermentation state, we implemented control over the duration of fermentation in both the CCK_{WLB} and CCK_{WOLB} fermentation processes. In the present study, the CCK_{WOLB} was subjected to spontaneous fermentation as a reference experiment, in addition to the bacterial culture filtrates. As described in the Materials and methods section, the antifungal assays against *G. boninense* and *C. albicans* were performed (Bivi et al., 2010; Chong et al., 2009) with minor modifications. Each *Ganoderma* mycelium was cultivated on Potato Dextrose Agar (PDA) plates with punctures measuring 8.0 mm in diameter as part of this experimental procedure.

Table 2: Comparison of antifungal activity in kimchi filtrates from Chinese cabbage with and without inoculation of *Lb. plantarum* LBP-K10 as a starter culture

Fungal strains	* Active concentration of complex (mg complex in 3 mL agar assay)		
	Culture filtrate without CH_2Cl_2 extraction (reference experiment)	[1] CCK_{WOLB} (kimchi without starter as a reference experiment)	[2] CCK_{WLB} (kimchi with starter)
Plant pathogen	(% inhibition)	(% inhibition)	(% inhibition)
Ganoderma boninense	12.5 ± 3.75 (100)	37.5 ± 10.0 (100)	25.0 ± 3.50 (100)
Human pathogen			
Candida albicans	10.50 ± 1.25 (100)	22.0 ± 1.26 (100)	14.50 ± 3.50 (100)

* Data represent means ± SD of three independent experiments expressed as mg/L of each sample.
[1] CCK_{WOLB} and [2] CCK_{WLB} samples used in this study were extracted identically to the culture filtrate of *Lb. plantarum* LBP-K10.

Table 2 presents the results of the antifungal assays conducted on two pathogenic fungi. The mycelia of *G. boninense* were inoculated onto six-well PDA plates (3.0 mL) with 8.0-mm punctures for seed inoculation. The lyophilized fraction samples weighing 1.5–25 mL were reconstituted in sterilized distilled water and subsequently applied onto six-well PDA plates. The plates were subsequently incubated at 28℃ for a period of 7 days. To determine the anti-*Candida* activity, the samples were applied onto minimal SD plates. *C. albicans* SC5314 (wild-type isolate) cells were inoculated into six-well plates

containing dextrose minimal SD agar medium (3.0 mL). The antifungal activity of the individual filtrates from kimchi was observed to varying degrees, as indicated in Table 2. Remarkably, the antifungal effectiveness of the fermented kimchi filtrate CCK_{WLB}, which was inoculated with *Lb. plantarum* LBP-K10, was observed to be significantly higher when compared to the different kimchi filtrate CCK_{WOLB}. However, the culture supernatant of LAB exhibited the most potent antifungal activity in these experiments.

3.2. Antifungal Activity of CCK_{WLB} and CCK_{WOLB} Using the CH_2Cl_2 Extraction Method

The growth of *G. boninense* and *C. albicans* was also suppressed upon administration of both types of CCKs via 10-fold CH_2Cl_2 extraction method (Table 3). The anti-*Ganoderma* and anti-*Candida* activities of CCK_{WLB} were observed to exhibit significantly greater efficacy in comparison to the kimchi filtrate CCK_{WOLB}. The concentrations of the filtrates, when tested against fungal pathogens, were found to be considerably lower than the concentration of 20 mg/mL of cyclo(L-Ile-L-Pro), as determined by the minimum inhibitory concentration (MIC) values (Lind et al., 2007). These concentrations exhibited similarity to the concentrations previously documented for cyclo(L-Phe-L-Pro) against *Fusarium sporotrichioides* and *Aspergillus fumigatus* (Ström et al., 2002). The antifungal activity of CCK_{WLB} and CCK_{WOLB} was found to be more potent in comparison to the synergistic activity of lactic acid, cyclo(L-Leu–L-Pro), and cyclo(L-Phe–L-Pro) derived from *Lb. casei* AST18 (Li et al., 2012). Additionally, the cyclo-(L-Pro-Gly), cyclo(L-Tyr-L-Tyr), cyclo-(L-Phe-Gly), and cyclo(4-hydroxy-L-Pro-L-Trp) compounds demonstrated inhibitory effects on *Aspergillus* species. Based on a previous study conducted (Liu et al., 2017), it has been suggested that the production of antifungal compounds can be accomplished by employing bacterial filtrates obtained from the supernatant and freeze-dried powder of different varieties of kimchi. The observed outcome can be attributed to the unique characteristics of each kimchi filtrate and the consistent application of a 10-fold CH_2Cl_2 extraction method, which exhibits exceptional selectivity in the isolation of antimicrobial compounds. The antifungal compounds have been sufficiently purified for the purpose of analysis and confirmation as distinct compounds, thereby excluding any additional peptidyl or non-peptidyl compounds. The purification process was achieved using the 10-fold CH_2Cl_2 extraction method. Gas chromatography-mass spectrometry (GC-MS) is employed for the analysis of compounds using both electron ionization (EI) and chemical ionization (CI) mass spectrometry techniques. This observation is supported by the finding of an elevated presence of cyclic dipeptides in the filtrates when utilizing the *Lb. plantarum* LBP-K10 strain serves as a starter culture, exhibiting demonstrating a growth population during fermentation process.

Table 3: Comparison of antifungal activity in kimchi filtrates extracted with CH_2Cl_2 from Chinese cabbage with and without inoculation of *Lb. plantarum* LBP-K10 as a starter culture

Fungal strains	* Active concentration of complex (mg complex in 3 mL agar assay)		
	Culture filtrate with CH_2Cl_2 extraction (reference experiment)	[1] CCK_{WOLB} (kimchi without starter as a reference experiment)	[2] CCK_{WLB} (kimchi with starter)
Plant pathogen	(% inhibition)	(% inhibition)	(% inhibition)
Ganoderma boninense	7.50 ± 1.00 (100)	22.5 ± 10.0 (100)	17.0 ± 5.00 (100)
Human pathogen			
Candida albicans	4.00 ± 1.75 (100)	12.0 ± 3.50 (100)	8.50 ± 2.50 (100)

* Data represent means ± SD of three independent experiments expressed as mg/L of each sample.
[1] CCK_{WOLB} and [2] CCK_{WLB} used in this study were extracted through CH_2Cl_2, which is identical to the culture filtrate of *Lb. plantarum* LBP-K10.

3.3. The Effectiveness of the CH_2Cl_2 Extraction Technique Utilizing Kimchi Filtrates

Previously, the modified LLE technique was employed to isolate antifungal cyclic dipeptidyl compounds from various kimchi filtrates (Liu et al., 2017). The fractions of kimchi containing cyclic dipeptides were subjected to sequential re-chromatography on a semi-preparative Hypersil ODS C18 reverse-phase column. The isocratic composition of the mobile phase consists of various combinations utilizing 3%, 5%, 10%, 15%, and 20% methanol, 3%, 5%, and 10% acetonitrile, alongside 67-94% HPLC-grade water. Despite conducting multiple experiments capturing pure components via recursive CH_2Cl_2 extraction and further fraction separations, we encounter regular challenges in quantifying impurity content via peak-area normalization. Our efforts to confirm the relative retention peak area proportion for the cyclo(Phe-Pro) fraction across all types of filtrates were unsuccessful, particularly when compared to the isolated bacterial CFs. Despite this, Chinese cabbage kimchi displays a marked fractionated profile compared to other kimchi types and bacterial isolates. The unique fermentation properties of kimchi species may explain the varied chromatographic profile of Chinese cabbage kimchi, distinct from other

substances. In addition, the electron ionization (EI) and chemical ionization (CI) mass spectra of non-starter kimchi are deficient in molecular ions. Several molecular ions do not clearly exhibit fragment ions, as observed through high-resolution mass measurements. Multiple peaks found in radish kimchi can coalesce, resulting in overlaps that can impact each other. To identify primary peaks and make precise parametric estimates, diverse isocratic mobile-phase compositions are employed at varying pH levels, ensuring better peak separation. Despite efforts to isolate individual peaks from complex chromatograms, no compounds have been isolated in any type of kimchi, even after controlling experimental parameters such as elution volume at peak maximum and peak height. This purification process generated pure cyclic dipeptides suitable for identification and analysis via EI/CI gas chromatography–mass spectrometry as a singular compound, without the presence of any other peptidyl or non-peptidyl compounds. The purification model for cyclic dipeptides, paired with CH_2Cl_2 extraction, produced uniform fractions with cyclic dipeptide fractionation patterns that enable antifungal activity assays.

This experimental approach, which employed HPLC separation combined with a repeated 10-volume CH_2Cl_2 extraction, exhibited a distinctive feature of strong selectivity for the isolation of cyclic dipeptides (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013). Additionally, it incorporated multiple alterations of the mobile phase as mentioned. This procedure yielded cyclic dipeptides of high purity, which were subsequently analyzed and identified using EI/CI GC-MS as individual compounds, without the presence of any other peptidyl or non-peptidyl compounds. The cyclic dipeptide purification model, in conjunction with CH_2Cl_2 extraction, resulted in the isolation of fractions that exhibited a consistent cyclic dipeptide fractionation pattern in kimchi filtrates. To ensure the preservation of the bioactive compounds' efficacy during the solvent extraction process, the cyclic dipeptides were evaluated in powdered form using the EI/CI GC-MS technique every six months. For over three years at room temperature, the antifungal activities of the powdered samples remained unchanged after utilizing HPLC fractionation in combination with repeated CH_2Cl_2 extraction.

3.4. The Bio-Effector Potential of Kimchi Filtrate as a Potent Antifungal Agent

In particular, we previously suggest the extent of production of antifungal *cis*-cyclo(L-Val-L-Pro)) and antiviral *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013) by growing *Lb. plantarum* LBP-K10 with various previously identified types of isolates (i.e., *Lb. sakei* LBP-S01, *Lb. plantarum*/*pentos* LBP-S02, *Lactococcus lactis* LBP-S03, *Lc. lactis* LBP-S06, *Ln. mesenteroides* LBP-K06, *Weissella cibaria* LBP-K15, and *W. confusa* LBP-K16). Additionally, the cyclic peptides cyclo(Phe-Pro) and cyclo(Ile-Pro) derived from Propionibacterium CF have been found to exhibit antifungal activity against *A. fumigatus* and *Rhodotorula mucilaginosa* (Lind et al., 2007). These observations are also in agreement with our previous results showing increased amount of filtrates exhibiting antimicrobial properties (Kwak et al., 2018; Kwak et al., 2014; Kwak et al., 2013). These filtrates may contain cyclo(Tyr-Pro), *cis*-cyclo(L-Val-L-Pro), cyclo(Ser-Pro), cyclo(Leu-Pro), *cis*-cyclo(L-Leu-L-Pro), and *cis*-cyclo(L-Phe-L-Pro).

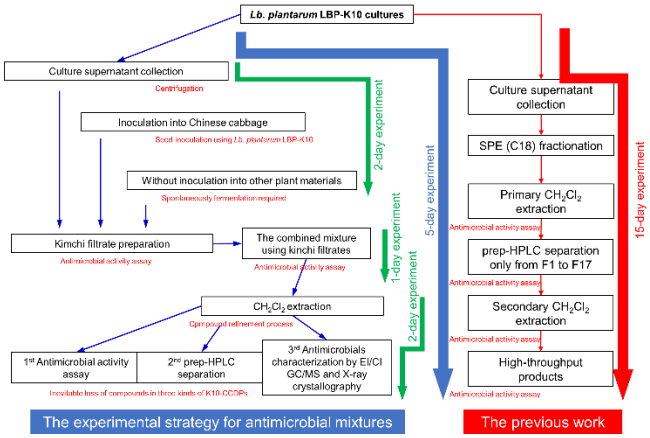

Figure 1. Direct comparison between the previous purification technique and the newly demonstrated probiotic mixtures, composed of kimchi filtrates, indicates a significant improvement in efficiency regarding both yield and time when using LAB cultures in kimchi. Compared to the previous technique combining CH_2Cl_2-HPLC, the presented data shows a more efficient approach to purifying a considerable amount of combined antimicrobial compounds.

Hence, this study has provided evidence indicating that the individual filtrates and extracts of kimchi possess bio-effector properties (Fig. 1). Furthermore, the combined antifungal compounds found in kimchi filtrates have demonstrated significant bioactive effects against various pathogens. It has been reported in several studies that the simultaneous administration of antifungal peptides, either alone or in combination with antibiotics, can result in synergistic antimicrobial effects (Deepa et al., 2015; Kumar et al., 2014; Kwak et al., 2018; Kwak et al., 2013). The data presented in this study provide a potential framework for future research investigations or commercial applications, including the development of agricultural pesticides, natural preservatives, or animal feed additives. Additionally, our study introduces a potential methodology for evaluating the antimicrobial properties of kimchi filtrates and exploring their potential practical uses.

3.5. The Low Cytotoxicity Potential of Kimchi Filtrate Containing Cyclic Dipeptides as a Promising Antifungal Agent

Despite the limited number of toxicity assessments conducted to ensure the safety of kimchi filtrates for commercial use, the antimicrobial activity of the cyclic peptides has been sufficiently supported by their structural characteristics. In addition to the aforementioned aspects of the debates surrounding bioactive structures, it is imperative to consider the factor of cytotoxicity associated with cyclic dipeptides. The antiviral activities of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) were evaluated for their cytotoxicity at different concentrations using 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Kwak et al., 2013). It was observed that both compounds exhibited minimal cytotoxicity. However, the viability of the host cells is marginally impacted by elevated concentrations, particularly those surpassing 10.0 mM, of the two cyclic peptides. The concentrations of cyclic dipeptides that exhibit efficacy against the influenza virus are markedly lower compared to the concentrations that induce cytotoxicity in host cells. As a consequence, the proliferation of normal cells remains unimpeded even at elevated levels of the cyclic peptides. Another study employing the MTT assay demonstrates the cytotoxic effects of cyclo(-Pro-Tyr) on HepG2 cells. The results of this study demonstrate that the compound cyclo(-Pro-Tyr) induces dose-dependent cytotoxic effects in HepG2 cells. However, no significant toxicity was observed in mouse Fibroblast McCoy cells at the concentrations that were tested (Karanam & Arumugam, 2020). A research investigation was conducted to assess the cell viability of three cyclic dipeptides, namely cyclo(L-Leu-D-Arg), cyclo(2-hydroxy-Pro-L-Leu), and cyclo-(L-Pro-L-Val). The findings of the study indicate that cyclo(L-Leu-D-Arg) demonstrates the highest level of cytotoxicity, with cyclo(2-hydroxy-Pro-L-Leu) exhibiting a closely comparable level. Cyclo(L-Leu-D-Arg) exhibits the most potent activity against MDAM-B231, a breast cancer cell line, with an IC_{50} value of 25 μM. Furthermore, the compound exhibits promising activity against the A549 lung cancer cell line, with an IC_{50} value of 50 μM. Cyclo(2-hydroxy-Pro-L-Leu) demonstrates inhibitory activity against MDAM-B231 with an IC_{50} value of 100 μM. However, it has been observed that these three diketopiperazines do not demonstrate any cytotoxic effects on normal human fibroblast cells (FS) even at concentrations as high as 50 μM. Conclusively, this study makes a significant contribution to the understanding of cyclic dipeptides derived from probiotic strains. These compounds have the potential to serve as valuable sources for the development of novel drugs in the pharmaceutical industry, specifically as potent antifungal agents.

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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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References

Archer, A. C., & Halami, P. M. (2015). Probiotic attributes of *Lactobacillus fermentum* isolated from human feces and dairy products. *Applied Microbiology and Biotechnology, 99*, 8113-8123.

Bivi, M. R., Farhana, M. S. N., Khairulmazmi, A., & Idris, A. (2010). Control of *Ganoderma boninense*: A causal agent of basal stem rot disease in oil palm with endophyte bacteria *in vitro*. *International Journal of Agriculture and Biology, 12*, 833–839.

Cheigh, H. S., & Park, K. Y. (1994). Biochemical, microbiological, and nutritional aspects of kimchi (Korean fermented vegetable products). *Crit Rev Food Sci Nutr, 34*, 175−203.

Chong, K. P., Rossall, S., & Atong, M. (2009). *In vitro* antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. *Journal of Agricultural Science, 1*, 15–20.

Chuah, L.-O., Foo, H. L., Loh, T. C., Mohammed Alitheen, N. B., Yeap, S. K., Abdul Mutalib, N. E., . . . Yusoff, K. (2019). Postbiotic metabolites produced by Lactobacillus plantarum strains exert selective cytotoxicity effects on cancer cells. *BMC complementary and alternative medicine, 19*, 1-12.

De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of Lactobacilli. *Journal of Applied Microbiology, 23*, 130–135.

Deepa, I., Kumar, S. N., Sreerag, R. S., Nath, V. S., & Mohandas,

C. (2015). Purification and synergistic antibacterial activity of arginine derived cyclic dipeptides, from *Achromobacter* sp. associated with a rhabditid entomopathogenic nematode against major clinically relevant biofilm forming wound bacteria. *Frontiers in Microbiology, 6*, 876.

Fonzi, W. A., & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics, 134*, 717–728.

Ji, K., Jang, N. Y., & Kim, Y. T. (2015). Isolation of Lactic Acid Bacteria Showing Antioxidative and Probiotic Activities from Kimchi and Infant Feces. *Journal of Microbiology and Biotechnology, 25*, 1568-1577.

Jung, J. Y., Lee, S. H., & Jeon, C. O. (2014). Kimchi microflora: history, current status, and perspectives for industrial kimchi production. *Applied Microbiology and Biotechnology, 98*, 2385–2393.

Jung, J. Y., Lee, S. H., Kim, J. M., Park, M. S., Bae, J. W., Hahn, Y., . . . Jeon, C. O. (2011). Metagenomic analysis of kimchi, a traditional Korean fermented food. *Applied and Environmental Microbiology, 77*, 2264-2274.

Jung, J. Y., Lee, S. H., Lee, H. J., Seo, H. Y., Park, W. S., & Jeon, C. O. (2012). Effects of *Leuconostoc mesenteroides* starter cultures on microbial communities and metabolites during kimchi fermentation. *International Journal of Food Microbiology, 153*, 378-387.

Karanam, G., & Arumugam, M. K. (2020). Reactive oxygen species generation and mitochondrial dysfunction for the initiation of apoptotic cell death in human hepatocellular carcinoma HepG2 cells by a cyclic dipeptide Cyclo (-Pro-Tyr). *Molecular Biology Reports, 47*(5), 3347-3359.

Kim, B. H., Lee, H. S., Jang, Y. A., Lee, J. Y., Cho, Y. J., & Kim, C.-i. (2009). Development of amino acid composition database for Korean foods. *Journal of Food Composition and Analysis, 22*, 44-52.

Kumar, S. N., Lankalapalli, R. S., & Kumar, B. S. (2014). *In vitro* antibacterial screening of six proline-based cyclic dipeptides in combination with β-lactam antibiotics against medically important bacteria. *Applied Biochemistry and Biotechnology, 173*, 116-128.

Kwak, M.-K., Liu, R., & Kang, S.-O. (2018). Antimicrobial activity of cyclic dipeptides produced by *Lactobacillus plantarum* LBP-K10 against multidrug-resistant bacteria, pathogenic fungi, and influenza A virus. *Food Control, 85*, 223-234.

Kwak, M.-K., Liu, R., Kim, M.-K., Moon, D., Kim, A. H., Song, S.-H., & Kang, S.-O. (2014). Cyclic dipeptides from lactic acid bacteria inhibit the proliferation of pathogenic fungi. *Journal of Microbiology, 52*, 64-70. doi:10.1007/s12275-014-3520-7

Kwak, M.-K., Liu, R., Kwon, J.-O., Kim, M.-K., Kim, A. H., & Kang, S.-O. (2013). Cyclic dipeptides from lactic acid bacteria inhibit proliferation of the influenza A virus. *Journal of Microbiology, 51*, 836-843. doi:10.1007/s12275-013-3521-y

Kwofie, M. K., Bukari, N., & Adeboye, O. (2020). Probiotics potential of yeast and lactic acid bacteria fermented foods and the impact of processing: a review of indigenous and continental food products. *Advances in Microbiology, 10*(09), 492.

Li, H., Liu, L., Zhang, S., Cui, W., & Lv, J. (2012). Identification of antifungal compounds produced by *Lactobacillus casei* AST18. *Current Microbiology, 65*, 156–161.

Lind, H., Sjögren, J., Gohil, S., Kenne, L., Schnürer, J., & Broberg, A. (2007). Antifungal compounds from cultures of dairy propionibacteria type strains. *FEMS Microbiology Letters, 271*, 310–315.

Liu, R., Kim, A., Kwak, M.-K., & Kang, S.-O. (2017). Proline-Based Cyclic Dipeptides from Korean Fermented Vegetable Kimchi and from *Leuconostoc mesenteroides* LBP-K06 Have Activities against Multidrug-Resistant Bacteria. *Frontiers in Microbiology, 8*, 761.

Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and Environmental Microbiology, 68*, 4322–4327.

Tan, H. K., Foo, H. L., Loh, T. C., Alitheen, N. B. M., & Rahim, R. A. (2015). Cytotoxic effect of proteinaceous postbiotic metabolites produced by Lactobacillus plantarum I-UL4 cultivated in different media composition on MCF-7 breast cancer cell. *Malaysian Journal of Microbiology*, 207-214.