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Increased Anticancer Activity of Organic Kimchi with Starters Demonstrated in HT-29 Cancer Cells

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Featured Application: Colorectal cancer (CRC) is the third most common cancer type worldwide; and the second most common cause of cancer death. CRC occurs due to unhealthy dietary habits and lifestyle. This study confirms that organic anticancer functional kimchi provides antioxidant and anticancer effects and can therefore be used as a preventive in the development of colon cancer. Accordingly, this study contributes to the health of general consumers by changing the recipe of kimchi in the market, as well as developing functional and fermented foods with anticancer and antioxidant effects.

Abstract: This study aims to investigate the effects of kimchi made with organic ingredients and lactic acid bacteria (LAB) as starters (*Leuconostoc mesenteroides* + *Lactiplantibacillus plantarum*) on HT-29 human colon carcinoma cells. Four types of kimchi (standard kimchi (SK), commercial kimchi (CK), anticancer kimchi (AK), and organic anticancer kimchi (OAK)) were evaluated. The results show that, among the different types of kimchi studied, OAK presents high DPPH free-radical scavenging activity and total phenol and flavonoid contents, and the MTT assay shows that the growth inhibition rate against HT-29 cancer cells is the highest. In addition, the quantitative reverse transcription polymerase chain reaction (RT-qPCR) results show that, compared to SK and CK, AK and OAK can effectively down-regulate the mRNA expression of anti-apoptotic gene Bcl-2 and up-regulate the mRNA expression of the cell cycle arrest genes p21 and p53; pro-apoptotic genes Bim, Bak, and Bad; and genes for caspases 3 9. Subsequently, a Western blot test confirmed that the expression of Bcl-2 decreased and the expressions of p53, Bax, and caspases 3 and 9 increased in OAK. The abovementioned results indicate that the anticancer kimchi prepared with organic ingredients and starters of lactic acid bacteria effectively present the best antioxidant activity and inhibit the proliferation of HT-29 cancer cells by promoting apoptosis and cell cycle arrest.

Keywords: kimchi; lactic acid bacteria; organic ingredients; HT-29 cancer cells; apoptosis; cell cycle arrest

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1. Introduction

Kimchi is a traditional Korean fermented food characterized by both the physiological activity of the raw materials and the probiotic function of lactic acid bacteria (LAB), the microorganisms used for fermenting it. Kimchi is a food made of vegetables, such as cabbage, radish, and green onions, with other seasonings, and is reported to have functional health properties, such as antioxidative, immune-enhancement, anti-obesity, and anti-diabetes

effects [1]. These properties are due to the presence of physiologically active substances, such as vitamins, beta-carotene, various minerals, dietary fiber, and phytochemicals, in the ingredients of kimchi [2]. Depending on whether the ingredients are added or whether they are organic or non-organic, the taste, flavor, and textural and functional properties, such as anticancer and anti-inflammatory effects, may change [3]. In particular, in the study of Yu et al. [4], when only organic cabbage was used, the proliferation of HT-29 cells was significantly inhibited through the activation of apoptosis, and the addition of sub-ingredients confirmed that the apoptosis effect improved.

Indeed, kimchi has been the subject of interest for many researchers because it contains various bioactive ingredients [5,6]. Since kimchi is a fermented food, the role of LAB is also extremely important. In fact, kimchi is known as a LAB-based functional and probiotic food. There are several types of LAB in kimchi, including *Leuconostoc*, *Lactobacillus*, and *Weissella* [1].

The natural fermentation of the unsterilized ingredients leads to the growth of different LAB, which can cause variations in the taste and quality of kimchi, making it difficult to produce on an industrial scale while maintaining a consistent quality. Therefore, the use of starter cultures has been considered as an alternative for the industrial production of standardized kimchi, and the demand for starter cultures has increased in recent years. The main LAB in kimchi are *Leuconostoc mesenteroides* (LM) and *Lactiplantibacillus plantarum* (LP). In the case of LM, it was shown to be the main microorganism involved in the optimal ripening of kimchi at low temperatures, and LP has been observed to be the dominant species in the late stage of fermentation [7]. These two LAB are not only produced during fermentation, but are also used as starter cultures before fermentation. Earlier studies by Lee et al. [8] and Bong et al. [9] revealed that starters (LM + LP) could enhance the anticancer and anti-obesity effects of kimchi. In addition, postbiotic metabolites produced from LP caused selective antiproliferative effects and apoptosis on cancer cells while preserving normal cells, and cell proliferation inhibitory effects and total phenol content on HT-29 colorectal cancer cells in soymilk fermented with LAB increased [10,11]. Dead nanometric *Lactiplantibacillus plantarum* (nF1), produced by the heat treatment of LP, is less than 1 μm in size and is absorbed faster through the Peyer's patches of M cells in the intestine, and it has immune-enhancing and anti-obesity effects [12]. However, the functional study of kimchi co-produced with LP, LM, and nF1 has not been comprehensively conducted to date. Hence, we aim to study the anticancer effects of kimchi co-produced with LP, LM, and nF1.

Colorectal cancer accounts for approximately 10% of all cancers and cancer-related deaths diagnosed worldwide each year, making it the second most common cancer in women and the third most common cancer in men [13]. Cancer cells are characterized by uncontrolled proliferation, angiogenesis, and inflammation, combined with the inhibition of apoptosis, cellular differentiation, and DNA repair, all of which are associated with the initiation and progression of cancer [14]. The HT-29 cell line is a human colon adenocarcinoma cell line that provides a desirable experimental system for studying the effective factors in epithelial cell differentiation. These morphological characteristics of HT-29 cells make them a suitable model for studying various cell signaling pathways, such as apoptosis and cell cycle arrest, as well as therapeutic agents or approaches [15]. Apoptosis is well known as one of the major pathways of tumor cell death [16]. A class of proteins closely related to the apoptosis pathway is the family of caspases, which includes initiator caspases (caspase 9) and effector caspases (caspase 3). Cell cycle arrest occurs to halt abnormal cell proliferation and to repair intracellular DNA damage [17]. Colorectal cancer is sporadic and only about 5% of cases have a genetic cause. Smoking, the use of anti-inflammatory drugs, unhealthy dietary habits, and the presence of health conditions, such as metabolic syndrome, are known to be the most important extrinsic risk factors for colorectal cancer [18]. Therefore, functional foods with anticancer bioactivity can lower the risk. With this aim, we chose to study the anticancer properties of kimchi, a popular food product.

The purpose of this study is to use the HT-29 human colon cancer cell line to detect the effect of organic anticancer kimchi (OAK) on the growth rate inhibition of cancer cells and the expression of mRNA and the proteins of genes associated with cell cycle arrest and apoptosis. Kimchi used in this study was prepared using organic ingredients, with starter cultures LP + LM and nF1 with enhanced anticancer activity, and improved taste and quality similar to or better than commercial products.

2. Materials and Methods

2.1. Kimchi Preparation

The common and organic ingredients used to make kimchi were supplied by Damchaewon Co. (Taean, Chungcheongnam-do, Korea), and production was also conducted by the same company. Organic ingredients were grown without the use of chemical fertilizers and pesticides and were certified by the Ministry of Agriculture, Food and Rural Affairs of the Republic of Korea. The organic ingredients used in OAK were cabbage, red pepper powder, garlic, ginger, radish, green onion, pear, and leaf mustard. The starter strains *Lactiplantibacillus plantarum* PNU (KCCM 11352P) and *Leuconostoc mesenteroides* PNU (KCCM 11353P) were isolated from kimchi in our laboratory. These two starters were supplied as live bacteria from Mediogen Co. Ltd., which can manufacture food probiotic starters, and the starters were mixed in 0.9% physiological saline at a concentration of 1×10^6 CFU per 1 g of baechu cabbage. Dead nano-sized *Lactiplantibacillus plantarum* nF1 was provided by Immunobiotech Co., Ltd. (Seoul, Korea). Commercial kimchi (CK) was prepared using organic kimchi from Damchaewon Co. The cabbage was cut in 1/2 and pickled with sea salt for 24 h, then the water was discarded, and it was mixed with the other ingredients to prepare the different kimchi samples (Table 1). The standard kimchi (SK), CK, anticancer kimchi (AK), and organic anticancer kimchi (OAK) samples produced were fermented for 4 weeks at 5 °C. As the 3rd week was the optimal maturity period, the kimchi samples from that period were used for the experiment.

Table 1. Kimchi recipes of standard, commercial, anticancer, and organic anticancer kimchi.

Ingredients of Kimchi	SK(g)	CK(g)	AK(g)	OAK(g)
Baechu cabbage	5000	5000	5000	5000
Red pepper powder	175	280	175	175
Garlic	140	60	140	140
Ginger	30	50	30	30
Radish	550	760	550	550
Sugar	50	60	50	50
Anchovy juice	110	180	110	110
Green onion	100	190	100	100
Pear	-	-	140	140
Mustard leaf	-	80	375	375
Onion	-	190	-	-
Radish juice	-	380	-	-
Glutinous rice paste	-	30	-	-
Mushroom and sea tangle juice	-	-	125	125
Mistletoe extract	-	-	2.5	2.5
Lactiplantibacillus plantarum PNU (CFU/g)	-	-	1×10^6	1×10^6
Leuconostoc mesenteroids PNU (CFU/g)	-	-	1×10^6	1×10^6
Dead nano-sized *Lactiplantibacillus plantarum* nF1 (g/kg)	-	-	10	10

2.2. Preparation of Kimchi Extracts

The kimchi samples at week 3 were freeze-dried in a freeze dryer (FD5512, Ilshin BioBase Co., Gyeonggi-do, Korea) and ground into powder in a mill. Subsequently, the powder was then extracted twice for 24 h with 20 times its weight of methanol and filtered. Then, the extract was concentrated using a rotary vacuum evaporator (EYELA, Rikakikai

Co., Tokyo, Japan), and the concentrated extract was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and used in the subsequent experiments [8].

2.3. Measurements of pH Value, Acidity, and Salinity of Kimchi

Kimchi juice was collected weekly, and pH and acidity levels were measured using a pH meter (M220, Corning Co., Lowell, MA, USA). The acidity of the sample was diluted 20-fold according to the standard test method of the Association of Official Agricultural Chemists (AOAC) and titrated by adding 0.1 N NaOH until the pH reached 8.4. The amount of 0.1 N NaOH used was measured. To measure salinity, 300 uL of kimchi juice was placed into the salt meter (PAL-ES2, ATAGO Co., Tokyo, Japan) and the measurement was obtained [5]. Acidity was measured as follows:

$$\text{Acidity } (\%) = \frac{\text{mL of 0.1N NaOH} \times \text{normality of NaOH} \times 0.09}{\text{weight of sample (g)}} \times 100$$

2.4. Sensory Evaluation of Kimchi

This sensory evaluation was approved by the research review board of CHA University, Gyeonggi, Seongnam, South Korea. A total of 10 trained sensory panelists evaluated the samples, and the evaluation was conducted through quantitative descriptive analysis (QDA). There were total of 11 evaluation items, and for all the items, extremely disliked or weak was evaluated using the number 1, and extremely good or strong was evaluated using the number 9. The evaluation parameters were appearance, sour smell, green smell, moldy smell, salty flavor, bitter flavor, sour flavor, moldy flavor, refreshing flavor, hardness, and overall acceptability [19].

2.5. Assessment of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Inhibition Rate of Kimchi

The methanol-extracted kimchi samples were dissolved in DMSO and stored at 4 °C until needed. The 96-well plate was filled with 100 μL of SK, CK, AK, OAK, methanol, and 150 μM DPPH solution, and a dark reaction was conducted for 30 min. To confirm the DPPH inhibition rate, the absorbance was measured at 517 nm and calculated with the following formula [20]:

$$\text{DPPH inhibition rate } (\%) = [1 - \frac{(\text{SD} - \text{SM})}{(\text{MD} - \text{MM})}] \times 100$$

SD: sample 100 μL + DPPH 100 μL; SM: sample 100 μL + methanol 100 μL; MD: methanol 100 μL + DPPH 100 μL; and MM: methanol 100 μL + methanol 100 μL.

2.6. Assessment of Total Phenol (TP) Content

The content of total phenolic (TP) compound content was measured using the gallic acid equivalence method. A total of 75 μL of Folin–Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO, USA) was mixed with 25 μL of the methanol extract of kimchi of a certain concentration (250 mg/mL) and reacted at room temperature for 5 min. Then, 200 μL of 7.5% Na_2CO_3 solution and 700 μL of distilled water (D.W.) were added, and the light was blocked for 40 min at room temperature. The absorbance was measured at 765 nm with a Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA, USA). The standard curve was drawn using gallic acid as the reference (standard concentration was 0.03125–1 mg/mL), and the standard curve was used to calculate the TP content of the kimchi [21].

2.7. Assessment of the Total Flavonoid (TF) Content

A total of 1 mL of diethylene glycol was added to 100 μL of the kimchi extract and left at room temperature for 5 min. Then, 100 μL of 1N NaOH was added, the mixture was heated to 37 °C for 60 min, and the absorbance was measured at 420 nm. The flavonoid content was calculated from the standard calibration curve obtained using Quercetin (Sigma-Aldrich

Co., St. Louis, MO, USA) as the reference (standard concentration is 0–1280 μg/mL) to draw the standard curve [22].

2.8. HT-29 Cell Culture

An HT-29 human colon carcinoma cell line was purchased from the Cell Line Bank in Seoul, Korea. The cells were incubated at 37 °C in a 5% CO_2 incubator with Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene Inc., Daegu, Korea) containing a 1% penicillin–streptomycin solution (Gibco BRL., Rockville, MD, USA) and 10% inactivated fetal bovine serum (FBS) (Welgene Inc., Daegu, Korea). The cultured cancer cells were refed 2–3 times a week, and after washing with phosphate-buffered saline (PBS), 0.05% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) were detached and centrifuged. After centrifugation, the accumulated cancer cells and medium were mixed well using a pipette, and 10 mL of each solution was injected into a 75T cell culture flask, subcultured every 2–3 days, and used for the experiment.

2.9. MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium Bromide) Assay

The MTT assay is commonly used to measure cellular metabolic activity as an indicator of the proliferation and death rates of cells. Cultured HT-29 cells were seeded in a 96-well plate at a concentration of 5.0×10^4 cells/mL. After incubation for 24 h, the kimchi extract samples were used to treat the cells at concentrations of 0.5, 1, 1.5, 2, and 2.5 mg/mL. After 48 h of incubation, the MTT solution (500 μg/mL) was dissolved in the medium and 100 μL was added to each well. Further incubation for 4 h was performed under the same culture conditions. After dissolving the formed formazan crystals in DMSO, the absorbance was measured at 540 nm using a Wallac Victor3 1420 Multilabel Counter [23].

2.10. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The cultured cells of the HT-29 human colon cancer cell line were counted using a cell counter (Luna automated cell counter; Logos Biosystems, Anyang, Korea), and cells at 1.0×10^5 cells/mL per well were dispensed into a 6-well plate and cultured for 24 h. Subsequently, the HT-29 cancer cells were treated for 48 h with the addition of a medium containing SK 2, CK 2, AK 2, and OAK 2 mg/mL. After the removal of the medium, RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA, USA) and dissolved in 0.1% diethyl pyrocarbonate (DEPC, Bioneer Co., Daejeon, Korea). The total dissolved RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), and the quantified RNA was synthesized into cDNA using Superscript II reverse transcriptase (Invitrogen). The synthesized cDNA was analyzed for gene expression using a thermal cycler BioRad CFX-96 real-time system (Bio-Rad, Hercules, CA, USA) [24]. The genes Bcl-2, p21, p53, Bim, Bak, Bad, caspases 3 9, and GAPDH were used, and their primer sequences are shown in Table 2.

2.11. Western Blot

Cell proteins were reacted with 1 mL radioimmunoprecipitation assay (RIPA, Invitrogen, USA) buffer, and the proteins were separated by centrifugation at 13,000 rpm for 15 min at 4 °C. The separated protein was quantified by the Bradford assay method. The extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred into a polyvinylidene fluoride (PVDF, Bio-Rad, Hercules, CA, USA) membrane, and non-specific proteins were blocked with 5% skimmed milk containing phosphate-buffered saline with Tween 20 (PBST). After blocking, the PVDF membrane was washed three times with PBST and once with PBS, and the primary antibody was reacted overnight at 4 °C. The PVDF membrane was then washed three times with PBST and once with PBS, and the secondary antibody was reacted for 2 h at room temperature. For Bcl-2, p53, Bax, caspases 3 9, and α-tublin, Santa Cruz (Dallas, TX, USA) primary antibodies were used, and the bands of the proteins were detected using an Amersham Imager 680 (GE Healthcare Life Sciences, Chicago, IL, USA) [25].

Table 2. Primer sequences of RT-qPCR assay.

Gene Name	Primer Sequence
Bcl-2	F: 5′-AAGATTGATGGGATCGTTGC-3′ R: 5′-GCGGAACACTTGATTCTGGT-3′
p21	F: 5′-ATGTCAGAACCGGCTGGGG-3′ R: 5′-GCCGGGGCCCCGTGGGA-3′
p53	F: 5′-ATGGAGGAGCCGCAGTCAGA-3′ R: 5′-TGCAGGGGCCGCCGGTGTAG-3′
Bim	F: 5′-AGATCCCCGCTTTTCATCTT -3′ R: 5′-TCTTGGGCGATCCATATCTC -3′
Bak	F: 5′-TCTGGCCCTACACGTCTACC -3′ R: 5′-AGTGATGCAGCATGAAGTCG -3′
Bad	F: 5′-CAATGACCCCTTCATTGACC -3′ R: 5′-GACAAGCTTCCCGTTCTCAG -3′
Caspase 3	F: 5′-TTTTTCAGAGGGGATCGTTG-3′ R: 5′-CGGCCTCCACTGGTATTTTA-3′
Caspase 9	F: 5′-CTAGTTTGCCCACACCCAGT-3′ R: 5′-CTGCTCAAAGATGTCGTCCA-3′
GAPDH	F: 5′-AGGTCGGTGTGAACGGATTTG-3′ R: 5′-GGGGTCGTTGATGGCAACA-3′

2.12. Statistical Analysis

The data were analyzed using Graph Pad Prism 9.4.1 (GraphPad, San Diego, CA, USA), and the data of all experiments were expressed as mean ± standard deviation. Two-way and one-way ANOVAs (analysis of variances) were used to confirm the significance between each group using Duncan's multiple-range test. The results were considered significant when $p < 0.05$ or less, and all experimental data were analyzed using the SPSS v18 statistical software package (SPSS Inc., Westlands, Hong Kong).

3. Result

3.1. Analysis of Physicochemical Characteristics of Kimchi Samples

When the kimchi was fermented, pH, acidity, salinity, and sensory evaluations were used as indicators to check the quality. Acidity, pH value, and salinity were evaluated during the fermentation period of a total of 4 weeks, and acidity was found to be at the most optimal ripening stage when the fermentation was conducted at 5 °C for 2~3 weeks [26]. As shown in Table 3, when fermented under these conditions, the acidity levels of SK, CK, AK, and OAK were 0.83 ± 0.04, 0.87 ± 0.01, 0.84 ± 0.01, and 0.85 ± 0.00%, respectively. This increased from 0.34 ± 0.01, 0.49 ± 0.01, 0.42 ± 0.01, and 0.43 ± 0.02%, respectively, at week 0. The pH values of 4.11 ± 0.02, 4.11 ± 0.00, 4.16 ± 0.02, and 4.19 ± 0.03, respectively, were measured at week 3, which tended to decrease from 5.43 ± 0.04, 5.48 ± 0.04, 5.38 ± 0.06, and 5.49 ± 0.05, respectively, at week 0. Salinity levels were 1.83 ± 0.03, 1.70 ± 0.05, 1.73 ± 0.02, 1.66 ± 0.03 at week 0 for SK, CK, AK, and OAK, respectively. In the third week of fermentation, the pH values measured were 1.40 ± 0.03, 1.26 ± 0.03, 1.49 ± 0.02, and 1.47 ± 0.04, respectively. A sensory evaluation was conducted in the 3rd week, when the ripeness of the kimchi was the best, and a total of 11 parameters were evaluated using a 9-point scale method (Figure 1). In the assessment of overall acceptability, SK scored 4.9, CK scored 5.7, and AK and OAK both scored 5.5. In the assessment of appearance, AK (6.1), SK and CK (6.4), and OAK (6.5) received high scores; however, there was no statistical significance to the variation in scores ($p > 0.05$). In the measurement of sour smell and flavor, SK scored 5.9 and 6.3 points, and CK scored 6.4 and 5.9 points, respectively, which were higher scores than AK and OAK, which scored 5.1 and 4.4 points and 4.6 and 5.4 points, respectively ($p < 0.05$). Other than these evaluation parameters, there was no variation in

statistical significance among the kimchi samples with respect to green appearance, moldy smell, salty flavor, bitter flavor, moldy flavor, refreshing flavor, and hardness ($p > 0.05$).

Table 3. Physicochemical characteristics analysis (pH, acidity, and salinity values) of kimchi samples. SK: standard kimchi; CK: commercial kimchi; AK: anticancer kimchi; OAK: organic anticancer kimchi; means with the different letters (a–d) are significantly different ($p < 0.05$) by Duncan's multiple range tests.

Weeks	pH Value				Acidity				Salinity			
	SK	CK	AK	OAK	SK	CK	AK	OAK	SK	CK	AK	OAK
0	5.43 ± 0.04 ^{ab}	5.48 ± 0.04 ^{a}	5.38 ± 0.06 ^{b}	5.49 ± 0.05 ^{a}	0.34 ± 0.01 ^{c}	0.49 ± 0.01 ^{a}	0.42 ± 0.01 ^{b}	0.43 ± 0.02 ^{b}	1.83 ± 0.03 ^{a}	1.70 ± 0.05 ^{bc}	1.73 ± 0.02 ^{bc}	1.66 ± 0.03 ^{c}
1	4.58 ± 0.04 ^{b}	4.55 ± 0.03 ^{b}	5.01 ± 0.05 ^{a}	5.01 ± 0.01 ^{a}	0.66 ± 0.02 ^{a}	0.55 ± 0.01 ^{b}	0.55 ± 0.01 ^{b}	0.57 ± 0.01 ^{b}	1.57 ± 0.03 ^{a}	1.34 ± 0.01 ^{c}	1.57 ± 0.02 ^{a}	1.48 ± 0.03 ^{b}
2	4.26 ± 0.02 ^{b}	4.29 ± 0.04 ^{b}	4.39 ± 0.02 ^{a}	4.41 ± 0.02 ^{a}	0.79 ± 0.01 ^{a}	0.66 ± 0.01 ^{c}	0.67 ± 0.01 ^{c}	0.69 ± 0.01 ^{b}	1.44 ± 0.01 ^{a}	1.30 ± 0.01 ^{c}	1.39 ± 0.01 ^{b}	1.39 ± 0.02 ^{b}
3	4.11 ± 0.00 ^{c}	4.11 ± 0.02 ^{c}	4.16 ± 0.02 ^{b}	4.19 ± 0.03 ^{a}	0.87 ± 0.01 ^{a}	0.83 ± 0.04 ^{b}	0.84 ± 0.01 ^{ab}	0.85 ± 0.00 ^{ab}	1.40 ± 0.03 ^{b}	1.26 ± 0.03 ^{c}	1.49 ± 0.02 ^{a}	1.47 ± 0.04 ^{a}
4	4.00 ± 0.02 ^{a}	3.95 ± 0.01 ^{c}	3.95 ± 0.03 ^{bc}	3.99 ± 0.01 ^{ab}	1.15 ± 0.01 ^{a}	1.03 ± 0.01 ^{ab}	1.10 ± 0.15 ^{a}	0.90 ± 0.00 ^{b}	1.41 ± 0.04 ^{b}	1.20 ± 0.02 ^{d}	1.35 ± 0.03 ^{c}	1.50 ± 0.03 ^{a}

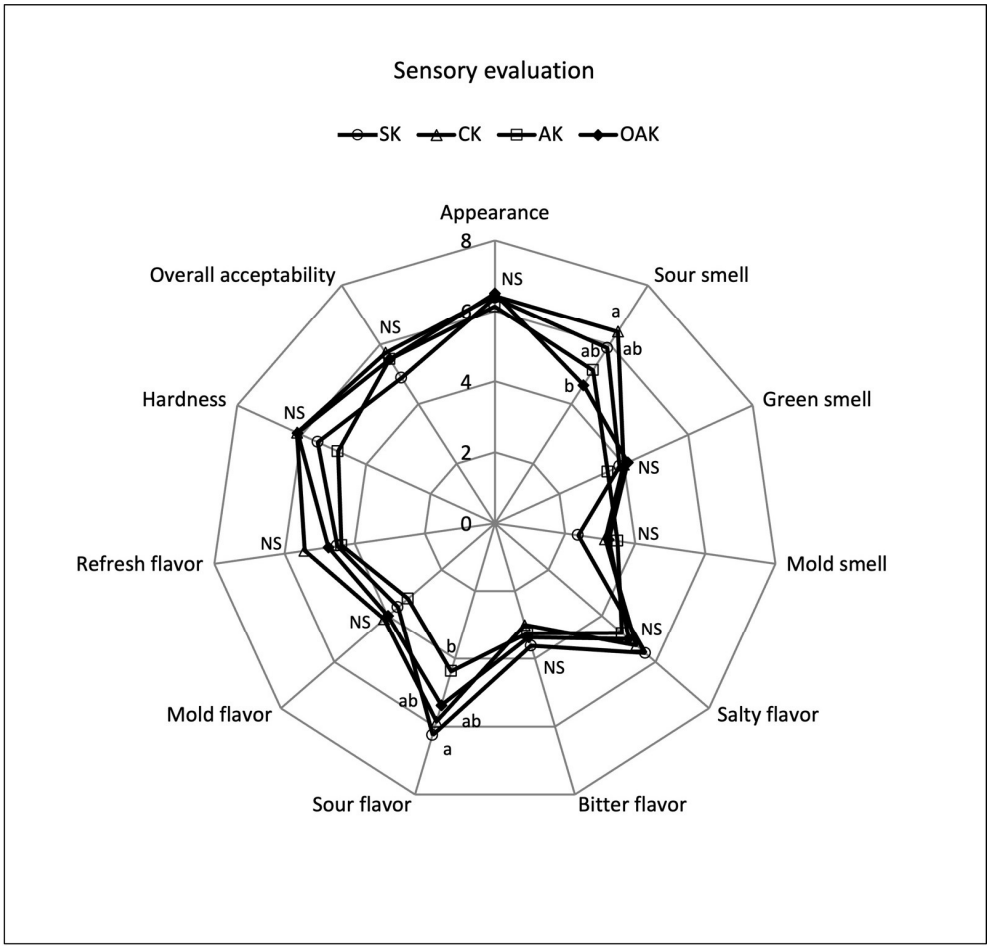

Figure 1. Sensory evaluation of kimchi fermentation at 3~5 °C at three weeks. SK: standard kimchi; CK: commercial kimchi; AK: anticancer kimchi; OAK: organic anticancer kimchi; means with the different letters (a–b) are significantly different ($p < 0.05$) by Duncan's multiple range tests. NS: no significant difference.

3.2. Antioxidant Capacities of Kimchi Samples

The evaluation of the antioxidant capacities of the kimchi samples was performed using DPPH free-radical scavenging activity, and the total phenol and flavonoid contents were ascertained and are shown in Figure 2. When the extracted kimchi sample was treated with 2 mg/mL, the free-radical scavenging activity increased in the order of SK, CK, AK, and OAK. The total phenolic content of OAK was highest among the kimchi samples. OAK showed a significant difference at 0.5 ($p < 0.05$) and 1 ($p < 0.01$) mg/mL versus the corresponding concentrations of the other kimchi samples, and the phenol content increased in the order of SK, CK, AK, and OAK at 2 mg/mL. With regard to the total flavonoid content, there was no difference between the kimchi samples up to 0.5 mg/mL; however, at 1 mg/mL, the flavonoid contents of AK and OAK increased compared to SK, and at 2 mg/mL, OAK showed the highest flavonoid content.

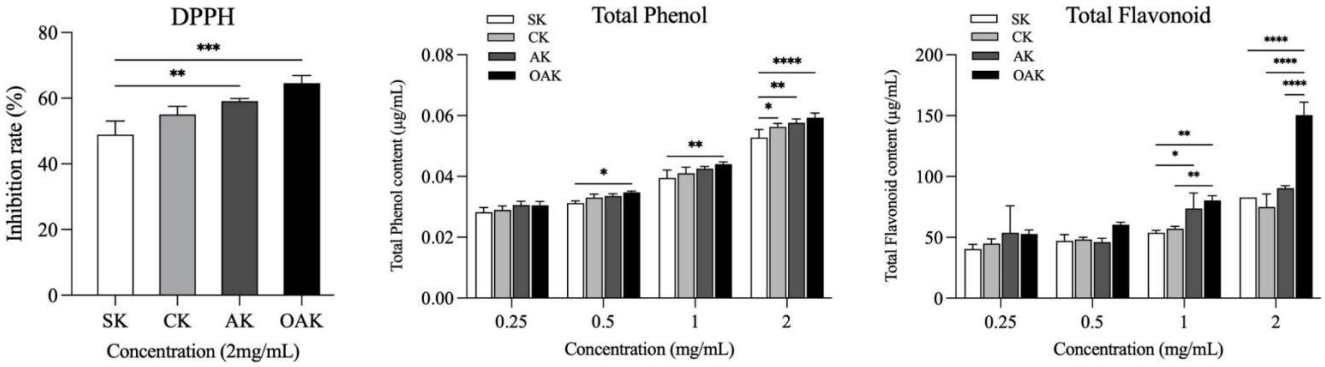

Figure 2. DPPH free-radical scavenging, total phenol, and total flavonoid contents of kimchi at different concentrations. SK: standard kimchi; CK: commercial kimchi; AK: anticancer kimchi; OAK: organic anticancer kimchi. *, **, ***, **** symbols mean significantly different ($p < 0.05$), ($p < 0.01$), ($p < 0.001$), ($p < 0.0001$), respectively, by one- and two-way ANOVA tests.

3.3. Inhibition Rate of HT-29 Human Colon Carcinoma Cells by Kimchi

The HT-29 cells were treated with kimchi extract in increasing concentrations from 0.5 to 2.5 mg/mL in units of 0.5 mg/mL (Figure 3). As the concentration increased, the inhibition rate of HT-29 cancer cell growth also increased. At all concentrations, OAK showed the highest rate of inhibition of HT-29 cell growth, followed by AK. A concentration of 2 mg/mL was chosen for the follow-up experiment, during which SK showed an inhibition rate of 18.99 ± 0.55%, CK 22.37 ± 2.09%, AK 25.63 ± 5.65%, and OAK 35.49 ± 2.17%.

3.4. mRNA Expression of Genes Associated with Cell Cycle Arrest

SK, CK, AK, and OAK significantly increased the mRNA expression levels of p21 and p53 genes compared to the control group (Figure 4). AK and OAK increased the mRNA expression levels of p21 1.70 and 2.33 times, respectively, compared to the control group. AK increased the expression levels of p53 1.59 times and OAK 2.65 times. Among all the groups, the highest increase in mRNA expression levels of p21 and p53 was observed with OAK. Thus, it was confirmed that OAK increases the expression of genes related to cell cycle arrest.

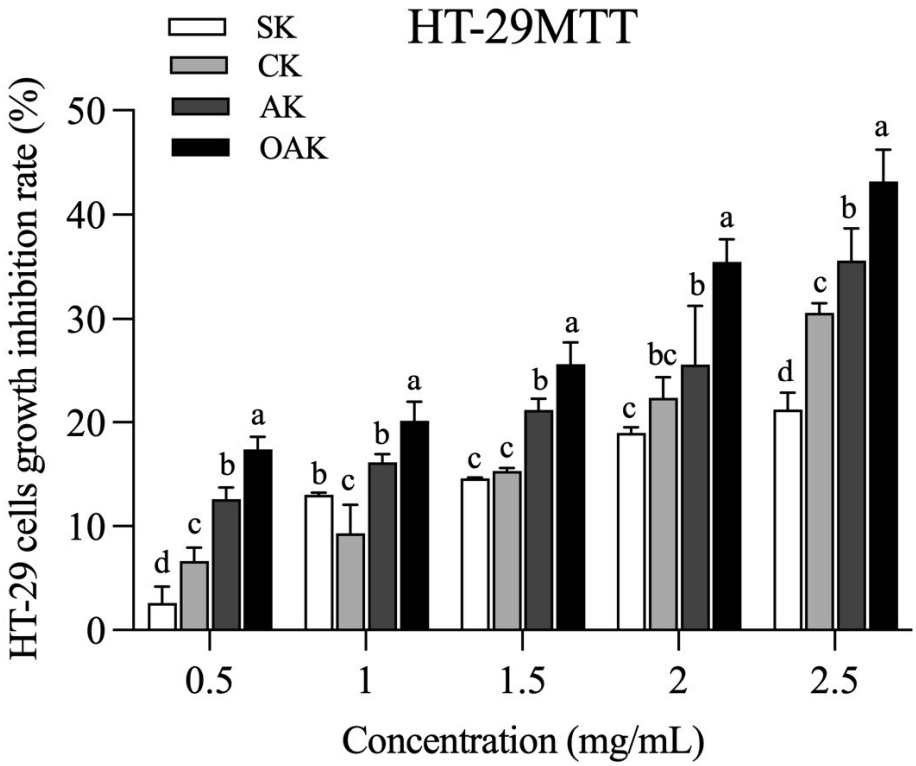

Figure 3. Inhibition rate of HT-29 colon cancer cells according to the kimchi type. SK: standard kimchi; CK: commercial kimchi; AK: anticancer kimchi; OAK: organic anticancer kimchi. Means with different letters (a–d) are significantly different ($p < 0.05$) by Duncan's multiple range tests.

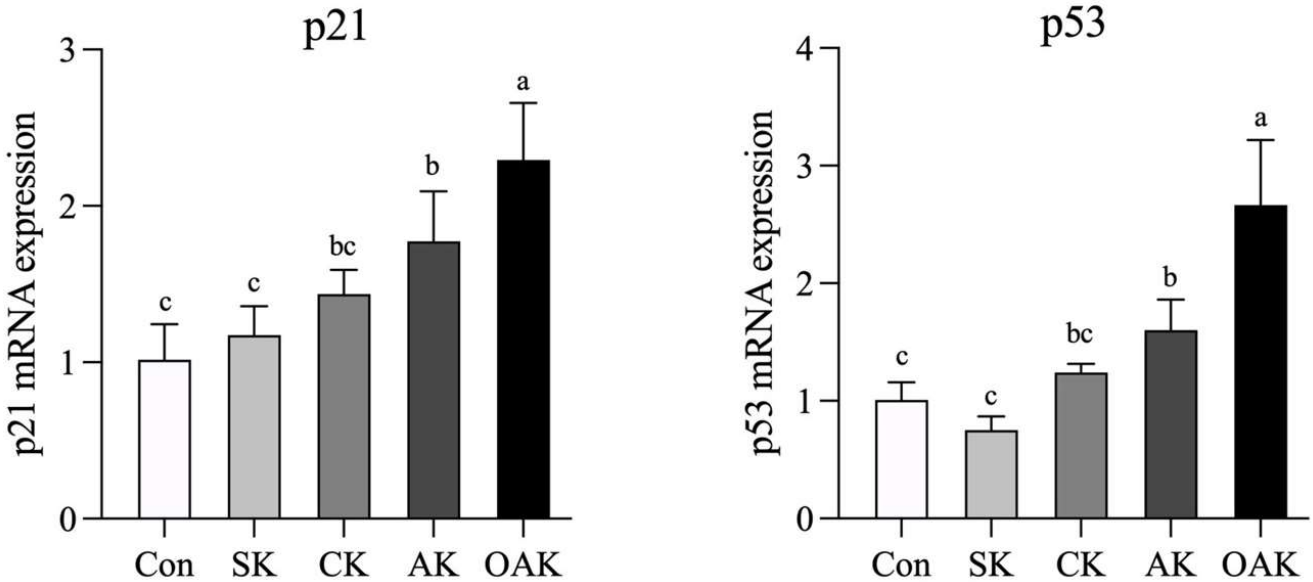

Figure 4. Effects of kimchi on mRNA expression levels of p21 and p53 in HT-29 cells. SK: standard kimchi, 2 mg/mL; CK: commercial kimchi, 2 mg/mL; AK: anticancer kimchi, 2 mg/mL; OAK: organic anticancer kimchi, 2 mg/mL. Fold ratio: gene expression/GAPDH×control numerical value (control fold ratio = 1). Means with different letters (a–d) above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

3.5. mRNA Expression of Genes Associated with Apoptosis

The mRNA expression levels of Bim, Bad, Bak, Bcl-2, and caspases 3 9 related to apoptosis are presented in Figure 5. AK and OAK increased the mRNA expression levels of Bim related to pro-apoptosis 5.07 times compared to the control group. AK increased the mRNA expression levels of Bad and Bak by 4.74 and 5.03 times, and OAK by 5.63 and 5.41 times, respectively. AK reduced the mRNA expression levels of Bcl-2, an anti-apoptosis-related gene by 0.34 times and OAK by 0.20 times. It was confirmed that OAK significantly increased the expression levels of pro-apoptosis genes and decreased the expression levels of anti-apoptosis-related genes. In addition, the mRNA expression levels of caspases 3 and 9 were increased 2.59 and 2.01 times, respectively, by OAK compared to the control group, showing the greatest increase. These results confirm that kimchi samples, especially OAK, promote the apoptosis of HT-29 cells by regulating the expression levels of apoptosis-related mRNA.

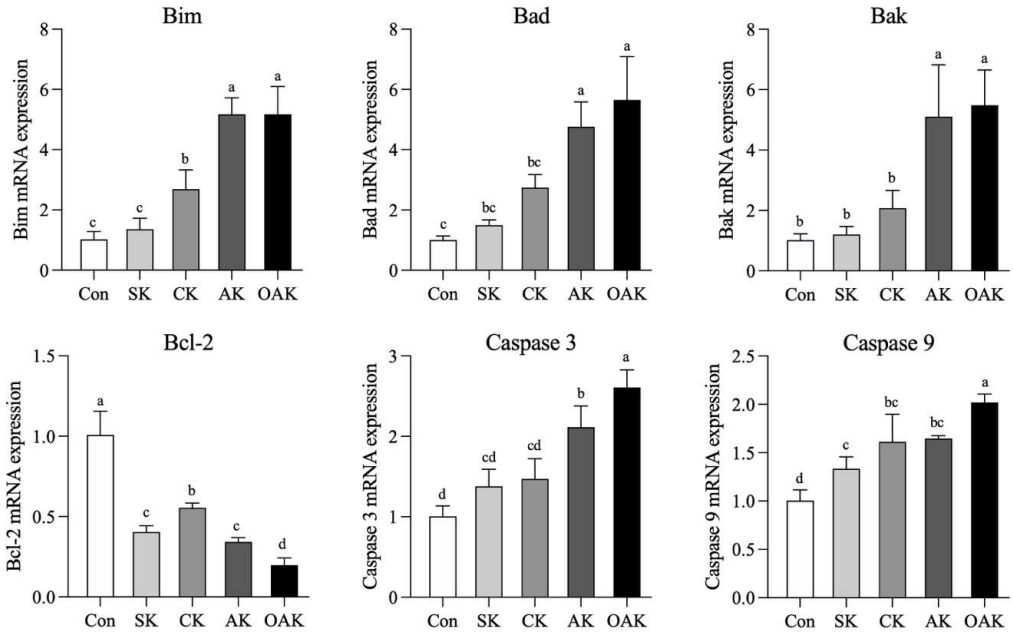

Figure 5. Effects of kimchi on the mRNA expression levels of Bim, Bad, Bak, Bcl-2, and caspases 3 and 9 in HT-29 cancer cells. SK: standard kimchi, 2 mg/mL; CK: commercial kimchi, 2 mg/mL; AK: anticancer kimchi, 2 mg/mL; OAK: organic anticancer kimchi, 2 mg/mL. Fold ratio: gene expression/GAPDH×control numerical value (control fold ratio = 1). Means with different letters (a–d) above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

3.6. Expression Levels of Proteins Associated with Cell Cycle Arrest and Apoptosis

Western blot analysis was performed to further confirm the mechanisms of cell cycle arrest and apoptosis of HT-29 cells using kimchi extracts (Figure 6). These extracts significantly reduced Bcl-2 protein expression and the lowest expression level was observed for OAK. Expressions of p53 and Bax proteins increased significantly in the order of SK, CK, AK, and OAK. OAK showed the highest expression levels of caspases 3 and 9, followed by AK. The Western blot results are almost consistent with the mRNA analysis, suggesting that OAK, an organic functional kimchi, inhibits the growth of HT-29 cancer cells through cell cycle arrest and apoptosis pathways.

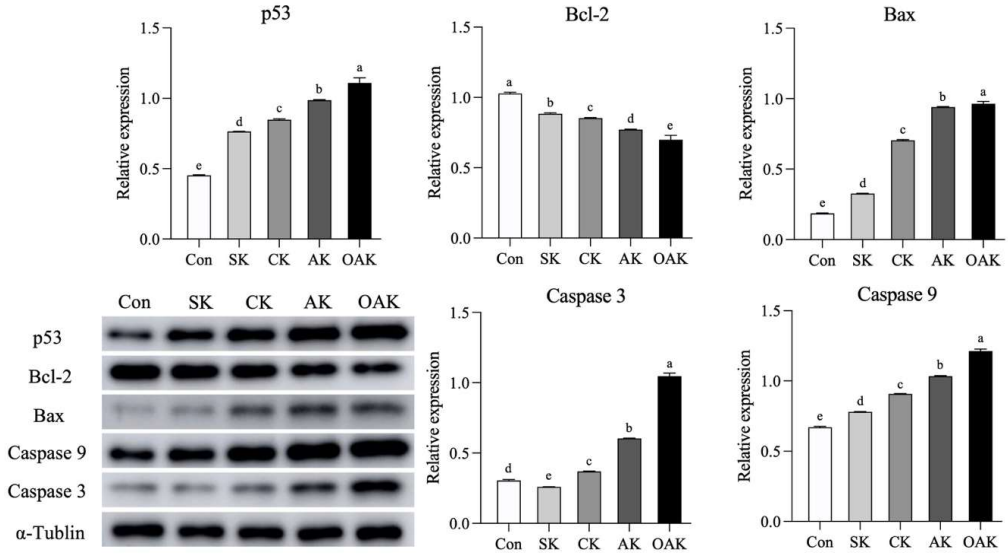

Figure 6. Effects of kimchi on the protein expression levels of p53, Bcl-2, Bax, and caspases 9 and 3 in HT-29 cancer cells. SK: standard kimchi, 2 mg/mL; CK: commercial kimchi, 2 mg/mL; AK: anticancer kimchi, 2 mg/mL; OAK: organic anticancer kimchi, 2 mg/mL. Means with different letters (a–e) above the bars are significantly different ($p < 0.05$) by Duncan's multiple range test.

4. Discussion

Kimchi is a fermented food that is prepared using LAB at 5 °C for 2 to 3 weeks to reach the optimal ripening stage. At this time, the pH range is 4.2~4.5 and the acidity level is 0.6~0.9% [27]. LAB produce organic acid through the fermentation process in food while lowering the pH level and increasing acidity to inhibit the growth of other bacteria, thus preserving the product's shelf life and quality [3]. The salinity level of kimchi distributed in Korea is 1.4~2.5%, and it is known that, as kimchi matures, the salinity reduces from the initial salinity level following a certain storage period due to the osmotic pressure inside and outside the cabbage [5]. According to the results of this study, the pH levels between kimchi samples in the initial week 0 ranged from 5.4 to 5.54, acidity ranged from 0.33 to 0.50%, and salinity ranged from 1.63 to 1.86%. As the fermentation progressed, CK tended to decrease in pH level more rapidly than AK and OAK. These results are similar to those observed by Choi et al. [28], in which the fermentation rate decreased when mistletoe extract was mixed in. In the sensory evaluation, CK showed the highest score for sour smell compared to the other samples, probably due to the production of a large amount of organic acid as the fermentation rapidly progressed.

Kimchi contains natural antioxidants, such as carotenoids, vitamins, flavonoids, and other phenolic compounds, because it basically composed of cabbage, radish, red pepper powder, garlic, and ginger [29]. In addition, the characteristics of kimchi change depending on the cultivation method of these ingredients. According to a study by Seong et al. [30], organic cabbage has higher levels of dietary fiber, ascorbic acid, chlorophyll, and carotenoids than conventional cabbage. This is because organic plants do not benefit from pesticides and must endure and survive pests and diseases on their own, thus increasing their phytochemical content [31]. In addition, Jung et al. [32] confirmed that the total phenol and flavonoid contents in organically grown young radish kimchi increased by 1.5 to 3 times compared to general young radish kimchi. Therefore, in this study, an antioxidant evaluation was conducted for the kimchi samples, and free-radical scavenging activity, total phenolic content, and total flavonoid content were measured. Oxygen free radicals are known to be involved in the development of systemic inflammatory response syndrome due to oxidative stress when free radicals exceed the antioxidant capacity of the body, and it has been confirmed that antioxidants that remove these free radicals can reduce inflammatory response [33]. In this study, OAK showed the highest free-radical scavenging effect,

followed by AK. In addition, OAK was found to have the highest total phenol and flavonoid contents at 1 and 2 mg/mL sample treatment concentrations, respectively, suggesting that the antioxidant effect was significantly increased with the use of organic ingredients.

According to Park and Hong [29], kimchi has been confirmed to present anticancer, anti-aging, antioxidant, and anti-obesity effects due to the complex action of its ingredients, fermentation products, and LAB. Furthermore, Kim et al. [34] and Cui et al. [35] confirmed that kimchi presents anticancer and anti-obesity effects through in vivo experiments. In the case of mistletoe extracts added to AK and OAK, it was confirmed that they had significant anticancer effects on HT-29 and A549 cells when mixed with kimchi [36]. In addition, it was also confirmed that kimchi mixed with mistletoe extract, pear, mustard leaf, mushroom, and sea tangle juice was confirmed to present higher anticancer activity than standardized kimchi on HT-29 cells [4,37]. In the case of LP and LM, which were used as starter cultures LAB isolated from kimchi, they presented all the basic properties of probiotics, such as acid resistance, bile resistance, intestinal adhesion, and heat stability [38]. LP has antimutagenic effects, phagocytosis, and inhibits the growth of pathogenic bacteria, and LM is mainly used as a starter for kimchi and imparts a refreshing taste with non-carcinogenic properties [39,40]. When these two were used as starters for kimchi, Lee et al. [8] confirmed that they had an anti-obesity effect, and Bong et al. [9] found that starter-added kimchi had highly effective anticancer effects on HT-29 and HCT-116 cells. nF1 is a heat-treated LP biogenic consisting of non-viable *Lactiplantibacillus plantarum* cells. In a recent study by Choi et al. [41], it was shown to have an immune-enhancing effect on immunosuppressed animal models, and in the study by Kim et al. [42], it was confirmed that nF1-added kimchi improved the symptoms of irritable bowel syndrome (IBS). As seen in Figure 3, AK and OAK display a higher HT-29 cancer cell death rate than SK, which appears to be due to the addition of sub-materials, starter strains, and nF1 included in AK and OAK, as mentioned above. In addition, OAK was manufactured with the same organic ingredients as CK; however, there were differences in the contents of ingredients and sub-ingredients. Specifically, mushroom and sea tangle juice, mistletoe extract, starters, and nF1 are believed to have increased the death rate of HT-29 cells in OAK.

Uncontrolled cell proliferation and abnormal gene function and expression are the hallmarks of cancer, and tumor cells usually result from damage to genes that regulate cell cycle arrest [43]. To inhibit the growth of these cancer cells, cell cycle arrest and apoptosis are achieved by regulating the expression of transcription factors and suppressing growth signaling pathways [44]. The tumor suppressor gene p53 is involved in cell cycle arrest and apoptosis and is activated by signals, such as DNA damage, oncogene activation, and stress [45]. When p53 is activated, it plays an important role in determining the duration and outcome of cell cycle arrest at the G2 checkpoint, an important cell cycle checkpoint, and it can activate pro-apoptosis genes, such as Bax, or down-regulate anti-apoptosis genes, such as Bcl-2 [46]. p21, a downstream mediator of p53, induces G1 arrest in the cell cycle by binding to cyclin E/cyclin-dependent kinases (Cdk)2 and cyclin D/Cdk4 complexes. It also inhibits DNA replication by promoting the transcriptional silencing of transcription factor E2F1 targets important for DNA replication and cell cycle progression [47]. The p53 and p21 genes are involved in cell division throughout the G1, S, and G2/M phases of the cell cycle, and by measuring the expression of these genes, it is possible to determine how much a substance, such as an anticancer drug, inhibits cancer cell division [48]. Therefore, we investigated the effect of kimchi samples on the expression levels of p21 and p53. Both AK and OAK significantly increased the mRNA expression levels of p21 and p53 in HT-29 cells (Figure 4), and also increased the protein expression level of p53 (Figure 6). Between them, OAK was observed to increase the mRNA and protein expression levels the most. Previous studies have reported that kimchi inhibits the growth of cancer cells by up-regulating the expression of cell cycle arrest-related genes [49]. Similarly, in this study, the expression levels of p21 and p53 were increased the most by OAK, suggesting that functional recipes, starters, and organic ingredients induce the up-regulation of cell cycle arrest-related factors in HT-29 cells.

Apoptosis is a type of programmed cell death observed in multicellular organisms. This is summarized as cell death due to cell morphology and internal biochemical changes, which include cell rupture, cell membrane changes, nuclear fragmentation, chromatin condensation, and chromosome cleavage, and ends with the cells being phagocytosed and processed by other cells [50]. The first link between apoptosis and cancer began in the late 1980s, when it was understood that the Bcl-2 gene specifically blocks B-cell apoptosis in lymphomas [51]. The mitochondrial pathway of apoptosis is the major mechanism of physiological cell death, which is initiated by the pro-apoptotic members of the Bcl-2 family that cause mitochondrial outer-membrane permeabilization (MOMP). Pro-apoptosis, which induces the release of cytochrome c from mitochondria, and anti-apoptosis, which inhibits the release, play a role in apoptosis by interacting with each other [52]. Anti-apoptosis genes include Bcl-2 and Bcl-xL, which inhibit apoptosis and pro-apoptosis genes, such as Bax and Bak, which are downstream effectors that directly induce and promote apoptosis [53]. In addition, the pro-apoptotic BH3-only protein is an upstream member of the chain reaction, and when activated, suppresses anti-apoptosis and activates pro-apoptosis factors, such as Bax and Bak, to induce mitochondrial apoptosis [54]. Yu et al. [4] reported that kimchi supplemented with functional additives down-regulated Bcl-2 and up-regulated pro-apoptotic protein expression in HT-29 cells. Furthermore, Bong et al. [9] confirmed that kimchi with starters increased the mRNA expression of Bax and decreased the expression of Bcl-2, making it more effective in regulating apoptosis-related factors. In this study, the mRNA expression of anti-apoptotic Bcl-2 was decreased in HT-29 cells by treatment with OAK, an organic functional kimchi, and the mRNA expressions of pro-apoptotic Bak and BH3-only proteins Bim and Bad were up-regulated (Figure 5). In addition, the measurement of protein expression levels (Figure 6) confirmed that the expression of Bcl-2 decreased and the expression of Bax increased.

The caspase–cascade system plays an important role in the induction, transduction, and amplification of intracellular apoptotic signals. Caspases are classified according to their roles into initiator (caspases 2, 8, 9, and 10), executioner (caspases 3, 6, and 7), and inflammatory caspases (caspases 1, 4, and 5) [55]. Caspases 3, 7, and 9 have been reported to play a central role in the apoptotic process. When caspase 9 is activated, it cleaves and activates caspases 3/7 to further cleave other endogenous substrates and induce apoptosis [56]. Caspase 3 degrades intracellular structural and functional proteins and causes apoptosis [57]. In this study, the expression levels of caspases 3 and 9 were the highest in OAK, and OAK regulated the expression levels of caspase family members, resulting in high apoptotic activity in HT-29 cells. These results are similar to those of Kim et al. [37], in which the significant up-regulation of caspases 3 and 9 was observed in aged kimchi. These results show that kimchi supplemented with a functional recipe of nF1, starters, and organic ingredients can play an important anticancer role by regulating the expression of cell cycle arrest, apoptosis, and caspase-related genes. Considering the difference between kimchi samples prepared with different ingredients and lactic acid bacteria, it seems that the functional recipe of OAK prepared with the abovementioned ingredients improves the fermentation quality and antioxidant and anticancer effects of kimchi. The kimchi recipes prepared in this way were the best anticancer kimchi recipes confirmed after 20 years of research in this laboratory. Therefore, OAK seems to be an ideal kimchi for the development of premium organic anticancer kimchi. The kimchi prepared with the previous recipes has also been confirmed to have anti-obesity and anti-inflammatory effects through various in vitro and in vivo experiments; however, the kimchi made with this recipe has not yet been confirmed to display the various effects. Therefore, it is necessary to confirm the functionality of the kimchi made with this recipe and conduct a follow-up experiment.

5. Conclusions

These results confirm that OAK, an organic anticancer functional kimchi prepared in this laboratory, shows an increased antioxidant capacity (DPPH inhibition rate and total

phenol and flavonoid contents), while maintaining the physicochemical characteristics and taste of the existing kimchi. In addition, OAK significantly inhibited the growth of HT-29 human colon cancer cells and up-regulated the mRNA expression of cell cycle arrest-related genes p53 and p21 and apoptosis-promoting genes Bim, Bad, Bak, and caspases 3 and 9. At the same time, the mRNA expression of Bcl-2, an anti-apoptosis gene, was decreased. Similarly, the protein expression levels of Bak, p53, and caspases 3 and 9 increased, and the expression of Bcl-2 decreased. In conclusion, kimchi produced using organic ingredients, starters, and nF1 maintains the taste and quality of existing kimchi and maintains constant fermentation to produce kimchi with a uniform quality. In addition, there is a possibility of developing a functional food with high anticancer activity using organic ingredients and LAB probiotics components.

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