Combination Therapy of *Lactobacillus plantarum* Supernatant and 5-Fluorouracil Increases Chemosensitivity in Colorectal Cancer Cells

JaeJin An¹ and Eun-Mi Ha²*

¹Medical Convergence Textile Center, Gyeongbuk Technopark, Gyeongsangbuk-do 38412, Republic of Korea

²Department of Pharmacology, College of Pharmacy, Catholic University of Daegu 38430, Republic of Korea

---

**Introduction**

Colorectal cancer (CRC) has the highest mortality among cancer types [39], and is the third most common malignant tumor [47]. 5-Fluorouracil (5-FU) is the most common chemotherapy drug used for CRC [61]. However, resistance to 5-FU occurs in every treatment circumstance, which leads to malignant cancers [48]. Most deaths of patients with colon cancer are due to the metastasis of chemotherapy-resistant cells [93]; therefore, combating such metastasis remains an important challenge in cancer diagnosis and treatment. Colorectal cancer is typically driven by cancer stem cells (CSCs) such as epithelial cancer [11, 26]. CSCs have self-renewal capacity, and invasion and metastasis are possible [11, 25, 28, 51]. Since CSCs are resistant to a number of conventional treatments, failure to treat colorectal CSCs leads to the recurrence of malignant tumors. Most of the regimens target the non-CSCs of tumors and fail to eliminate CSCs [27, 51]. This means that chemotherapy-resistant CSCs cause tumors that are incurable by chemotherapy, and this explains the difficulties in achieving a full recovery and the high chance of cancers recurring. Therefore, a treatment strategy specifically targeting colon CSCs is required for the effective treatment of colorectal cancer and for reducing the risk of recurrence and metastasis.

5-FU is a cytotoxic agent and the most representative and standardized chemotherapeutic regimen for colorectal cancer [3, 61]. With this regime, more than 50% of patients...
respond to treatment in the initial stage of the treatment process, but only a small proportion (4–15%) respond to the second-line therapy [48]. Because 5-FU induces cancer recurrence, the initiation of metastatic cancer cells and differentiation into multipotent CSCs occur owing to serious drug resistance [48, 77]. This phenomenon shows the limits of treatment, and the side effects to its cytotoxicity are serious. Therefore, the validation of nontoxic agents that can effectively treat cancer is currently required [48]; new chemotherapeutic agents should specifically target drug-resistant cells and metastatic CSCs.

The pathogenesis of CRC is highly correlated with the deregulation of the Wnt/β-catenin signaling pathway. The major effector of canonical Wnt signaling, β-catenin, has a variety of cellular functions [21, 94]. The degradation of β-catenin in the cytoplasm is regulated by the β-catenin destruction complex containing the tumor suppressor adenomatous polyposis coli, Axis inhibitor (Axin), and glycogen synthase kinase 3 [43, 55]. When there is no Wnt signal, the β-catenin level in the cytoplasm is regulated to be low by the destruction complex. However, the activating signal of Wnt inhibits the function of the destruction complex of β-catenin, and then β-catenin enters the nucleus [34]. The nuclear translocation of β-catenin leads to a variety of functions by binding to the transcription factor T-cell factor/lymphoid enhancer factor of the gene that is associated with cell proliferation [21, 46]. In particular, the accumulation of nuclear β-catenin induces various tumorigenesis, including colon cancer [8, 24, 62, 65, 68, 73, 88]. In addition, Wnt/β-catenin signaling is believed to promote the growth of CSCs and induce tumorigenesis in a wide range of solid tumors [24, 73]. Therefore, a number of cancer treatment studies have sought to develop drugs that inhibit the Wnt/β-catenin signaling [19, 24, 43, 89]. Axin is a key component of the β-catenin destruction complex that promotes the degradation of β-catenin [57, 80]. The level of Axin is regulated by tankyrase, which induces the ubiquitination and degradation of Axin [19, 43, 90].

The colon consists of complex microorganisms and their diverse community [37, 58]. Gut flora are essential for maintaining intestinal function and homeostasis [6, 50, 53]. However, although many recent studies have presented evidence that shows a correlation between gut microbiota and CRC [1, 13, 14, 41], the mechanism for the effect of gut flora on the development of colorectal cancer is very complex and remains unclear. The symbiotic relationship between the host and their gut microbiota is essential for maintaining the balance of gut flora in the intestine. An abnormal intestinal environment results in changes to the balance (imbalance), called dysbiosis, in accordance with the change in the composition of microorganisms. Dysbiosis negatively affects the host; it is associated with the overgrowth of opportunistic pathogens [16, 91], and inhibits the growth of beneficial commensal bacteria [9]. The imbalance of gut flora eventually leads to intestinal inflammation [40, 87] and colorectal cancer [20, 30, 69, 81, 95]. Since many virulence-associated pathogens have been discovered around tumor tissues in particular, these results suggest that the gut microbiome plays an important role in the development and progression of CRC. Thus far, many studies have found correlations between intestinal microorganisms and CRC; there are reports that Lactobacillus strains inhibit cancer cell proliferation and prevent malignant transformation in colorectal cancer cell lines in vitro [2, 54, 56, 86]. Clinical trials that seek to elucidate the potential role of probiotics in regard to CRC suppression are very rare; the most frequently used probiotics for these clinical trials are Lactobacillus species [42, 59, 74, 79].

This study intends to determine an effective treatment strategy for 5-FU-resistant colorectal cancer cells. We tested whether applying the metabolites secreted by intestinal flora to the therapeutic regime was effective in the inhibition of viability and removal of chemoresistant CRCs. Recently, we reported that the growth rate of Lactobacillus plantarum (which is one of the gut flora that is first settled in the human gut) increases and its ability to secrete antimicrobial substances is improved in specific response to pathogen infection [38]. Here, with interest in the potential role of Lactobacillus species, we confirmed the influence of chemoresistant colorectal cancer and cancer stem-like cell lines.

Altogether, this paper demonstrates that Lactobacillus plantarum (LP) supernatant (SN) inhibits specific markers of the 5-FU-resistant colorectal cancer cells that consist of the CSCs population, cell survival, and colonsphere formation while inducing apoptosis. This effect of LP SN had synergy with 5-FU in combination therapy. Moreover, we determined that 5-FU-resistant CRCs induce an increase in the mRNA of tankyrase and the downregulation of Axin. In a series of processes, β-catenin is accumulated in the nucleus; we observe that this phenomenon is reversed by LP SN treatment.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from...
GIBCO BRL (Bethesda, MD, USA), 5-FU and other reagents were purchased from Sigma (St. Louis, MO, USA). Anti-CD44, anti-CD133, and anti-β-actin were purchased from Cell Signaling (Beverley, MA, USA), and anti-β-catenin and anti-histone were purchased from Abcam Biotechnology (Cambridge, MA, USA).

**Culture Condition of Lactobacillus plantarum and Isolation of Culture Supernatant**

**LP SN:** The strains used in this study, LP strain, were obtained from the bank of antibiotic-resistant bacteria (CCARM 0067). These were statically cultured at 37°C for 24 h in Difco Lactobacilli MRS broth (Becton Dickinson, USA). The LP with OD_{600} = 8 that were cultured in the MRS broth were inoculated at 5% (v/v) into 1L of M9 minimal broth and then statically cultured at 37°C for 36–48 h. After removing the pellets via centrifugal separation of 10^9 LP at 10,000 × g for 10 min, the supernatant was taken. In this supernatant, the pH of the M9 broth was adjusted to 6.8–7 using Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 2.5 mM NaVO_{3}, 25 μg/ml aprotinin, 2 μg/ml leupeptin, 25 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail. Then, after reacting them at 4°C for 20 min, they were centrifuged at 14,000 × g for 15 min, and the supernatant was then taken. Nuclear extracts were obtained by resuspending the insoluble pellets (which were obtained after being incubated with cytosolic lysis buffer (10 mM, HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, pH 7.6) and centrifuged) with nuclear lysis buffer (20 mM HEPES, 20% glycerol, 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT). Then 50 μg of the protein was electrophoresed by 10% SDS-PAGE and transferred onto immobilon-P nylon membranes (Millipore, Bedford, MA, USA). This was then hybridized with the antibody of the corresponding protein. After washing this with membrane and reacting this with the secondary antibody with the tagged radish peroxidase for 1 h, it was analyzed using a chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). This experiment was conducted at least three times, and the analysis of the relative intensity of protein bands as a result of western blotting was performed through the Image J program (The National Institute of Health).

**RNA Extraction and Quantitative Polymerase Chain Reaction (PCR) Analysis**

The mRNA expression levels of the CSC markers, Axin and tankyrase, in 5-FU chemoresistant colorectal cancer cells were analyzed through real-time PCR. Afterward, the total RNAs of parental and 5-FU-resistant colorectal cancer cells were extracted using TRIzol (Invitrogen, CA, USA). Then, PCR was conducted after synthesizing the cDNA using a SuperScript First-Strand Synthesis System (Invitrogen). cDNA was amplified and determined through a CFX96 real-time PCR system using a SYBR Premix Ex Taq kit (Takara Bio Inc., CA, USA). β-Actin was used as the internal control of the expression analysis, and the nucleotide sequence of the gene used in the experiment is summarized in Table 1.

**Measurement and Analysis of the Survival Rate of Cancer Cells**

The cytotoxicity of 5-FU was measured using a water-soluble tetrazolium salt (WST) assay. For this, 2 × 10³ cells of parental HT-29, chemoresistant HT-29, parental HCT-116, and chemoresistant HCT-116 cells were aliquoted into a 96-well plate and cultured for 24 h. Subsequently, each concentration of 5-FU was treated in each cell sample, and the cells were cultured at 37°C for 48 h. At this time, the control group was treated with 0.1% DMSO. After they were cultured for 72 h, 100 μl of WST assay reagent (Daellab, Seoul, Korea) was added to each well and was then reacted at 37°C for 90 min. Then, the absorbance was measured through a microplate reader (450 nm). Additionally, in the experimental group treated with LP SN, 5-FU was treated at the same time in the same manner as described above after pretreatment with LP SN for 24 h. Cell death was measured using the WST assay; the measurement value was calculated as the average value obtained from experiments repeated three times.

**Analysis of Tumorosphere/Colonosphere Formation and Size**

Chemoresistant HT-29 and HCT-116 cells were cultured in
The experiments were repeated three times.

Laboratories, USA). The average value was calculated by repeating the experiments three times, and the percentage (%) of apoptotic cells was measured.

Apoptosis analysis and measurement of caspase-3 activity.

The caspase activity was measured by a colorimetric assay kit provided by Biovision Research Products (Germany). This kit detected the chromophore p-nitroanilide cut from the labeled DEVD-pNA substrate and formed color.

5-FU chemoresistant cells were reacted with 5-FU and LP SN for 72 h, and the reacted cells were allowed to stand on ice for 10 min after being dissolved in a lysis buffer. Then, the supernatant was separated through centrifugation (10,000 × g) and the results were calculated as the average values obtained by repeating the experiments three times.

Statistical analysis.

All experiments were repeated three times or more, and all findings were shown as the mean ± standard deviation (SD). The statistical significance (p < 0.05) of the analyzed experimental data was verified from the experimental data of the control group and each treatment group using Student’s t test.

Results.

LP SN inhibits the expression of specific cancer stem cell markers in 5-FU-resistant HT-29 and HCT-116 cells.

Most colorectal cancers are thought to be derived from stem cells, and the specific CSC markers of colon tumors have been identified [26]. These CSC markers are the surface epitopes CD44, CD133, CD166, and aldehyde dehydrogenase 1 (ALDH1) [4, 29, 67, 92]. In particular, the expression of CSC markers was increased in chemotherapy-resistant cells with increased resistance to 5-FU, a chemotherapeutic agent, compared with those of the parental cells, and the resistant cells showed the characteristics of the CSC phenotype [27]. We constructed 5-FU-resistant cells by applying colorectal cancer cells, HCT-116 and HT-29, according to standard methods [71]. Fig. 1 shows that the expression of specific CSC markers was increased at the protein (Figs. 1A, 1B) and mRNA levels (Figs. 1C, 1D) in 5-FU-resistant cells. However, the expression of colon CSC markers was inhibited when treating LP SN in 5-FU-resistant colon cancer cells. In particular, after LP SN treatment in 5-FU-resistant HT-29 cells, CD44 and CD166 protein levels were decreased in a similar manner to those of the parental cells (Fig. 1A, 1B). Moreover, the LP SN treatment also decreased the mRNA expression of CD44, CD133, CD166, and ALDH1 in a similar manner to that of the parental cells, all of which were increased in 5-FU-resistant HT-29 and HCT-116 cells (Fig. 1C, 1D).

In addition, the increased mRNA expression of CSC markers in 5-FU-resistant CRCs did not show any reaction to the treatments of the SN of different kinds of Lactobacillus spp. (Lactobacillus brevis and Lactobacillus acidophilus) and potential pathogens (Escherichia coli and Salmonella Typhimurium) (Fig. S1). Thus, these results clearly show that LP SN treatment specifically reduces the expression of CSC markers in 5-FU-resistant colon cancer cells. In all of the experimental results above, treating the parental cells with LP SN did not affect the protein and mRNA expression of the CSC markers.

LP SN increases cell death by combination therapy with 5-FU in chemoresistant cells.

After 5-FU treatment, the viability of parental HT-29, chemoresistant HT-29, parental HCT-116, and chemoresistant HCT-116 cell lines were analyzed by cytotoxicity analysis (Fig. 2A). First, we treated different concentrations (0, 25, and 50 μM) of 5-FU in chemoresistant cells and analyzed their viability (Fig. 2A). We observed that 5-FU inhibited the proliferation of the parent cell lines HT-29 and HCT-116 in a dose-dependent manner. When comparing every experimental group treated with 25 and 50 μM of 5-FU with the non-treated group (0 μM), survival was inhibited by greater than or equal to 50%. However, greater than or equal to 80% of 5-FU-resistant HT-29 and HCT-116 survived even after 50 μM 5-FU treatment. Therefore, we confirmed that they had become resistant to 5-FU treatment as expected (Fig. 2A). Next, we determined whether LP SN affected the viability of 5-FU-survival colorectal cancer (Figs. 2B, 2C). This experiment was conducted on 5-FU-resistant cells.
Fig. 1. The culture supernatant of *Lactobacillus plantarum* inhibits the expression of cancer stem cell markers in 5-FU-resistant HT-29 and HCT-116 cells.

(A) The cancer stem cell markers were confirmed by western blot analysis. The expression levels of CD44 (80 kDa) and CD166 (100 kDa) proteins were significantly increased in 5-FU-resistant colorectal cells, HT-29, compared with that of the parental cells. However, in 5-FU-resistant HT-29, the increased expression levels of CD44 and CD166 were significantly decreased by LP SN treatment (10 μg for 72 h). The CD44 and CD166 protein expression of parental cells (lane 2) was not affected by the treatment with LP SN. The control (-) of each cell was treated with the same amount of concentrated M9 as that of LP SN. The protein expression levels were corrected to β-actin. (B) The expression levels of the relative proteins were analyzed using the ImageJ program on the basis of band intensity. (-) was only treated with M9 broth, and (+LP) was treated with 10 mg of LP SN. *p < 0.05, compared with the parental cells of each cell (n = 3). *p < 0.05, compared with the 5-FU-resistant cell lines treated with only M9 broth (n = 3). N.S.: not significant compared with the parental cells of each cell (n = 3). (C, D) The CSC markers were identified by real-time PCR analysis in HT-29 (C) and HCT-116 (D) cells. The CSC markers were CD44, CD133, CD166, and ALDH1, and β-actin was used as an internal reference for normalization. The data for the expression level of each gene were presented by setting the expression level in parental cells to 100% and comparing the relative expression levels of 5-FU-resistant cells and those treated with LP SN (10 μg for 72 h). The control group was treated with only M9 broth. Values are the mean ± SD. *p < 0.05 indicates the comparison of the value of parental cells (control), and *p < 0.05 compares the values of 5-FU-resistant cell lines (5-FU-resistant) treated with only M9 broth and chemoresistant cells treated with LP SN (5-FU-resistant + LP). LP SN treatment was not significant (N.S.) in parental cells compared with the control.
resistant cells and viability was analyzed after treatment with only LP SN or with a combination therapy of LP SN and 5-FU (50 μM). When treated with 2.5 or 5 μg of LP SN alone, the two cell types exhibited a tendency to slightly induce proliferation. When treated with 10, 15, and 20 μg of LP SN, we confirmed that proliferation was slightly inhibited (by about 10%) in a concentration-dependent manner (Fig. 2C). However, when treated with LP SN and 5-FU together, cell death increased compared with cells treated with only LP SN at the same concentration (Fig. 2C). Cell death was increased in a concentration-dependent manner, and particularly notable differences in cell death

Fig. 2. In 5-FU survival colorectal cancer cell lines, cell death is increased by the co-treatment of LP SN with 5-FU.
The degree of cell survival was confirmed by analyzing cytotoxicity through WST assay. (A) Although the viability of parental cells HT-29 and HCT-116 was significantly reduced when treated with 25 or 50 μM 5-FU for 72 h, the 5-FU chemoresistant cell lines had a very high resistance to 5-FU-induced cell death compared with the parental cell lines. Control was presented as relative values (%) by setting the cells treated with 0.1% DMSO to 100%, and *p < 0.05 was represented through Student’s t test. (B) Parental cells HT-29 and HCT-116 exhibited high lethality by treatment with 50 μM of 5-FU and were not affected by additional co-treatment with LP SN. (C) Cell death was increased in 5-FU survival colorectal cells HT-29 and HCT-116 when co-treated with 5-FU (50 μM) and LP SN simultaneously. Cell survival was observed after treatment with LP SN and 5-FU for 72 h. LP SN treatment reversed the cell survival in response to 5-FU resistance in a concentration-dependent manner, and the control was presented as the relative value by setting the viability of chemoresistant cell lines treated with only 50 μM of 5-FU to be 100%. *p < 0.05 indicates a comparison of the value of the experimental group treated with only 5-FU.
were observed at 10, 15, and 20 μg concentrations of LP SN. In contrast to the results above, two parental cells originally showed high cell death in 50 μM of 5-FU and were not affected by the co-treatment of LP SN (Fig. 2B, blue line). Furthermore, in 5-FU-resistant CRCs, the SN from different species of Lactobacillus and pathogens that underwent co-treatment with 5-FU had no effect on cell death (Fig. S2). Therefore, this means that the LP SN combination therapy showed a synergetic effect on the decrease in viability of FU-resistant colorectal cancer cells.

**LP SN Increases Apoptosis by Hypersensitivity in Response to 5-FU in Chemotherapy-Resistant Colon Cancer Cells**

Whereas the 5-FU-resistant HT-29 treated with only M9 broth (control) showed 6.5% apoptosis, 5-FU-resistant HT-29 treated with 50 μM 5-FU showed 18.5% apoptosis. However, when treated in combination with 10 μg of LP SN and 5-FU, apoptosis was increased by more than 45% (a 2.2-fold increase compared with those treated with only 5-FU) (Fig. 3A, left graph). These results were similar in the chemoresistant HCT-116 cell line (Fig. 3A, right graph). This result was related to the activity induction of caspase-3, as a positive marker of apoptosis [12]. The caspase-3 activity was more increased (greater than or equal to 2.5-fold in both cells) in the 5-FU survival HT-29 and HCT-116 cells treated with 5-FU and LP SN compared with those treated with only 5-FU (Fig. 3B). In all of the results above, the two 5-FU-resistant cells were not affected by the single treatment of LP SN (Figs. 3A, 3B). Furthermore, the above phenomenon did not significantly affect the co-treatment with 5-FU in addition to the single treatment with the different bacterial SN (Fig. S3).

**The Combination Therapy of LP SN and 5-FU Inhibits Activated Wnt/β-Catenin Signaling in Chemoresistant CRC Cells**

Wnt/β-catenin signaling is activated in colorectal cancer cells [24, 73]. We confirmed the Wnt/β-catenin pathway and the effect of LP SN in 5-FU-resistant CRCs (Fig. 4). In HT-29 and HCT-116, which were 5-FU-resistant cell lines, the expression of tankyrase 1 was increased compared with the parental cells (Fig. 4A). However, the treatment of LP SN recovered expression to a similar level to that in parental cells. In 5-FU-resistant colon cancer cells, the expression of Axin 2 was reduced in contrast to that of tankyrase, and the LP SN treatment increased the downregulated expression of Axin (Fig. 4B). In the parental cells HT-29 and HCT-116, LP SN treatment did not affect the expression of the two genes. Next, we analyzed the expression of β-catenin in HT-29 and HCT-116 parental and chemoresistant cells by western blot analysis to determine the effect of tankyrase 1 and Axin 2 expression on the stability of β-catenin, a downstream protein. Compared with the parental cells, β-catenin was stably accumulated in the nucleus in HT-29 and HCT-116 5-FU-resistant cell lines (Fig. 4C, lane 3). However, LP SN treatment in 5-FU-resistant CRC cells inhibited the accumulation of β-catenin in the nucleus (Fig. 4C, lane 4). The relative expression levels of these proteins were confirmed through the results in Fig. 4D. Through additional experiments that treated the other bacterial SN (Fig. S4), we confirmed again that the activated Wnt/β-catenin signaling in 5-FU-resistant CRCs was specifically inhibited by the combination therapy of LP SN and 5-FU.
LP SN Inhibits Colonosphere Formation in 5-FU-Resistant Colon Cancer Cells

Next, we tested whether LP SN inhibited the formation of the colonosphere, one of the most critical features of colon CSCs (Fig. 5). In the microscopic image, it was observed that the LP SN treatment inhibited the sphere-forming
potential in both 5-FU-resistant HT-29 and HCT-116 cells very effectively (Figs. 5A, 5B). Although 5-FU-resistant CRC lines HT-29 and HCT-116 cultured for 10 days formed well-defined spheroids, those treated with LP SN and 5-FU simultaneously (5-FU + LP) showed significantly smaller spheres/spheroids compared with the control, treated with only 5-FU (Figs. 5A–5C). The average sizes of the colonospheres that were formed by 5-FU-resistant HT-29 and HCT-116 cells treated with only 5-FU were 110 and 91 μm, respectively (Fig. 5C). However, the respective average sizes of the colonospheres of the 5-FU-resistant cells additionally treated with LP were 46.5 and 37 μm (Fig. 5C). In addition, the respective numbers of colonospheres formed by 5-FU-resistant HT-29 and HCT-116 were decreased by 59.6% and 64.9% (Fig. 5D). This suggests that the combination therapy of LP SN and 5-FU effectively removed them by specifically targeting 5-FU-resistant colon cancer and had a synergetic therapeutic effect on the 5-FU resistance.

Discussion

CRCs are among the most common health issues in industrialized countries [18]. Approximately 75% of the causes of CRC are determined by environmental factors rather than genetic factors [66]. The introduction of the westernized lifestyle leads to increased risk of CRC onset, due to the increased consumption of meat and a high-calorie diet, decrease in dietary fiber intake, and elongated life expectancy [17]. Diet habits based on meat and fat consumption lead to the overgrowth of opportunistic pathogens. The increase in pathogens promotes the onset and progress of CRC through various processes [22] such as chronic inflammation [36, 45, 63], the creation of reactive oxygen species [52], cell cycle regulation [5], toxic metabolite production [23, 82], and the creation of pro-diet carcinogenic compounds [15, 35, 70, 75]. These environmental changes result in dysbiosis by inducing changes in the diversity and composition of the commensal bacteria that are ultimately beneficial. Therefore, manipulation of the gut microbiota is required to prevent the onset of CRC and treat CRC [53]. Recently, there have been many research studies examining animal models. The studies confirm that the introduction of probiotics prevents CRC and has a positive effect on treatment by transforming the dysbiosis that is close to CRCs [10, 31]. In particular, many results from clinical studies...
trials on *Lactobacillus* species have been reported, as *Lactobacillus* species inhibit harmful bacteria in CRC patients and regulate immune responses [32, 96]. There is a report of the inhibition of the growth of patients’ colorectal tumors through treatment over 2–4 years [44]. Therefore, researching the clinical applications of probiotics as treatment methods for CRC is required.

Metastatic colorectal cancer is very incurable and has a poor prognosis. In cases in which it is impossible to perform surgery on metastatic colorectal cancer, the survival period is short, approximately 2 years [39, 49]. Treatment with 5-FU such as FOLFOX (a combination of 5-FU and oxaliplatin) is a standard therapy for metastatic colorectal cancer [3, 76]. However, the development of 5-FU-resistant cells is a general phenomenon that induces the recurrence of tumors. In addition, since the application of these drugs is associated with additional cytotoxicity, there are several side effects [33, 60]. In many recently magnified studies, the composition of the gastrointestinal microbiota has been changed by chemotherapeutic agents; this result shows that chemotherapeutic agents cause intestinal mucositis [83-85]. The changes in the composition of gut microbiota may have a serious impact on hosts, because the gut microbiota are responsible for a number of key functions such as hosts’ immune regulation, protection against pathogen invasion, and nutrient metabolism [5, 31]. Although there has been insufficient research on these changes, it is suggested that the changes in the composition of gut flora are significantly correlated with chemotherapy [83]. The most common human commensal bacteria are *Lactobacillus* or *Bifidobacterium* genera; in particular, 5-FU induces a decrease of *Lactobacillus* species within the gastrointestinal tract [75], thereby ultimately making them more sensitive to chemotherapy damage and leading to successive pathogen infection and cytotoxicity [75]. Although the importance of probiotic-based therapy trials has come to prominence [72], chemotherapeutic drugs have limitations in their capacity to treat intestinal diseases, because these drugs induce dysbiosis and affect the composition of the live microbiota. This means that with a supplement of live probiotic, the prognosis varies according to the environment; therefore, the discovery of probiotic-derived secretory factors capable of stable treatment is required in the future rather than therapy with live probiotics. Probiotics-derived substances as non-toxic agents may present an alternative that can enhance the effects of treatments that seek to prevent colorectal cancer, or as adjuvants for use in combination with drugs.

In this study, we formed 5-FU-resistant cells from two colorectal cancer cell lines, HT-29 and HCT-116, and used them for experiments. Chemoresistant HT-29 and HCT-116 cells increased the expression of several of the biomarkers of CSCs, and their abilities to form colonospheres, which are known to be increased in CSCs populations, were observed. These characteristics of the 5-FU-surviving colorectal cancer stem-like cells were reduced by treatment with LP SN. Interestingly, caspase-3 activities were increased by the combination therapy of LP SN and 5-FU, and 5-FU-resistant CRCs were sensitive to apoptosis.

In the existing studies, probiotics administration inhibited [64] or increased [12] apoptosis in various chemotherapy-induced damage conditions. Although all of the above results showed the inhibition and activation of caspase-3, which is a positive marker of apoptosis, the mechanism remains unclear. The present authors believe that future research should examine the specific probiotic strains that affect the function and regulation of caspase related to chemotherapy-induced apoptosis. In this respect, our research presents the potential of *Lactobacillus plantarum* as a specific probiotic that can inhibit 5-FU-resistant CRCs. Besides this, it was observed in this study that the secretory substances of *Lactobacillus plantarum* both increased chemotherapeutic-induced apoptosis in neoplastic cells while also inducing apoptosis. Thus, the present study

### Table 1. List of real-time PCR primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5'-CCAAGACGACATGAAATCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCAATCATGCTGGAAGTGAC-3'</td>
</tr>
<tr>
<td>CD44</td>
<td>Forward</td>
<td>5'-AAGGTGGAGACAAACATC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCAATGAGACAAACTGCAAG-3'</td>
</tr>
<tr>
<td>CD133</td>
<td>Forward</td>
<td>5'-GGTGTCTTCTGACTGTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACCAACTGAGACCACCA-3'</td>
</tr>
<tr>
<td>CD166</td>
<td>Forward</td>
<td>5'-TACGAGGAATGCAACTCCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGCAGACATAGTTCCAGCA-3'</td>
</tr>
<tr>
<td>ALDH1</td>
<td>Forward</td>
<td>5'-GTGGTCAACCCAGCAGAGCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTGTAAGCCCATACACCAAGA-3'</td>
</tr>
<tr>
<td>Axin 2</td>
<td>Forward</td>
<td>5'-CTGGTCAACCCAGCAGAGCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ATCCTCCCAAACACCCGCTCA-3'</td>
</tr>
<tr>
<td>Tankyrase 1</td>
<td>Forward</td>
<td>5'-ATGCCCCCAAGGCGCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGTGAGATGCTGGTGAAGATCA-3'</td>
</tr>
</tbody>
</table>
presents a cell status-dependent specific treatment.

It is thought that current chemotherapy technology fails to remove CRCs/CSCs, and this is an important obstacle to treating colorectal cancer. CSCs have the potential for self-renewal and can be differentiated into heterogeneous populations within tumors. In particular, colon CSCs express specific surface and cytoplasmic markers, including CD133, CD144, CD166, and ALDH1 [4, 26, 29, 92]. We observed that CSC markers were consistently expressed in 5-FU-resistant colon cancer. The previous study reported that tumorigenicity was more specifically and limitedly generated in CSCs that expressed the markers CD44 and CD166 [26]. Interestingly, we determined that CD44 and CD166 expression was increased in all 5-FU-resistant HT-29 and HCT-116 cells compared with those in the corresponding parental cell lines, and the combined therapy of LP SN and 5-FU decreased these expressions levels.

Our current data demonstrate that chemical combination therapy with LP SN is highly effective at inhibiting carcinogenesis by reducing the formation of the colonospheres that are formed in chemoresistant colon cancer CSCs. This suggests that the chemical combination therapy of LP SN and 5-FU may be an effective treatment strategy for specifically targeting chemoresistant cancer cells. In addition, the combination therapy of LP SN and 5-FU is very effective at removing the cancer stem-like population among 5-FU-resistant colon cancer cells. Therefore, this study shows that chemical combination therapy with probiotics is very effective at inhibiting the population of primary tumor stem cells and colorectal cancer cells. This can be an excellent strategy for removing current CSCs by targeting them specifically, and can inhibit the recurrence of colon cancer.

Although the Wnt pathway is an important target for cancer treatment, the discovery of nontoxic agents related to Wnt inhibitors is still insufficient. The current research is limited to the anticancer mechanism that inhibits Wnt signaling by applying several limited small molecules. A recent study reported that a small molecule, WAV939, inhibited cell growth and weakened cell migration by suppressing tankyrase and stabilizing Axin [7]. Our study suggests that probiotic secretory substances can regulate cell proliferation in colorectal cancer by controlling tankyrase-Axin-β-catenin.

The application of gut flora offers the best means of reducing the toxicity of chemical drugs. The metabolites of these intestinal microorganisms do not lead to drug resistance and can be ingested simply in the long term by being added to food; furthermore, they will prevent the formation or recurrence of primary tumors.

References

cancer, when microbiota-host mutualism breaks. World J. Gastroenterol. 28: 908-922.


and Bifidobacterium lactis has minor effects on selected immune parameters in polypectomised and colon cancer patients. Br. J. Nutr. 97: 676-684.