The apoptotıc effects of SCFAs from *Lactobacillus reuteri* on (HT-29) human colon cancer cells

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Abstract

Colon cancer is one of the leading causes of death around the World. In colon or other types of cancer, chemical methods can be considered as a therapeutic strategy. However, probiotics can also be used as biotherapeutics to reduce recurrence and side effects in patients with colon cancer.

Short Chain Fatty Acids (SCFAs) the fermentation products of probiotic *L. reuteri* bacteria found in the gastrointestinal tract. There are several SCFAs including acetic, propionic, butyric and lactic acids that have been shown to have apoptosis inducing properties in human colon cancer cells. In this study, the anti-proliferative and pro-apoptotic activities of probiotics were explained and the effects of SCFAs produced by *L. reuteri* on mitochondrial apoptosis were observed with changes in ROS and LPO production levels on human colon cancer cells (HT-29).

Results suggested that SCFAs from *L. reuteri* increased the mortality rates of HT-29 cells, increased ROS and LPO production. In addition to these effects, downregulation of Bcl-2, elevation of cytochrome c and overproduction of caspase-3 protein in SCFAs-treated HT-29 cells were observed. It was concluded that SCFAs from *L. reuteri* have cytotoxic effects in HT-29 colon cancer cells.

Anahtar Kelimeler: Colon cancer, Probiotics, *L. reuteri*, HT-29, SCFAs, ROS, LPO, Apoptosis pathway

Lactobacillus reuteri'den elde edilen KZYA'nin insan kolon kanser hücrelerinde (HT-29) apoptotik etkileri

Özet

Kolon kanseri, dünyadaki önde gelen ölüm nedenlerinden biridir. Kolon veya diğer kanser tiplerinde, kimyasal yöntemler terapötik bir strateji olarak düşünülebilir. Bununla birlikte, probiyotikler, kolon kanserli hastalarda hastalığın tekrarı ve yan etkilerini azaltmak için biyoterapötikler olarak kullanılabilir.

Kısa Zincirli Yağ Asitleri (KZYA), bağırsakta bulunan probiyotik *L. reuteri* bakterilerinin fermentasyon ürünleridir. İnsan kolon kanseri hücrelerinde apoptoz indükleyici özelliklere sahip olduğu gösterilen asetik, propiyonik, bütirik ve laktik asitleri içeren birçok KZYA vardır. Bu çalışmada, probiyotiklerin anti-proliferatif ve pro-apoptotik aktiviteleri açıklanmış ve *L. reuteri*'nin ürettiği KZYA'nin, insan kolon kanseri hücrelerinde (HT-29) mitokondriyal apoptoz yolağına etkileri ve ROS ile LPO üretim düzeylerindeki değişimler gözlemlenmiştir.

Sonuçlara göre, *L. reuteri*'den elde edilen KZYA, HT-29 hücrelerinde mortalite oranını, ROS ve LPO üretimini anlamlı şekilde artırmıştır. Bu etkilere ilaveten KZYA ile tedavi edilen HT-29 hücrelerinde Bcl-2 seviyesinin azalması, sitokromun c ve kaspaz-3 miktarlarının yükselmesi gözlenmiştir. Tüm sonuçlar ışığında *L. reuteri* kaynaklı KZYA'nin kolon kanseri hücrelerinde sitotoksik etkileri olduğu sonucuna varılmıştır.

Keywords: Kolon kanseri, Probiyotikler, *L. reuteri*, HT-29, KZYA, ROS, LPO, Apoptoz yolağı

Abbrevations: Apaf-1: Apoptotic Proteaz Activate Factor-1, BSA: Bovine serum albumin, DCFH-D: 2,7'-Diclorofluorecein diacetate, DMEM: Dulbecco's Modified Eagle Medium, FBS: Fetal Bovine Serum, HT-29: Human Colon Cancer Cell Line, SCFAs: Short Chain Fatty Acids, LPO: Lipid peroxidation, MDA: Malondialdehite, ml: Mililiter, mM: milimolar, PAGE: Polyacrilamid Gel Elektrophoresis, PBS: Phosphate Buffered Saline, PIC: Proteaz Inhibitor Cocteyl, PMSF: Phenilmetansulfonylflorit, PVDF: Polyvinildenflorit, ROS: Reactive Oxygen Species, SDS: Sodyumdodesilsulfat, CM: Conditioned Medium, DM: Designed Medium, TBA: 2-tiobarbituric acid, TBARS: Tiobarbutiric acid reactive species, TBE: Tris boric acid EDTA, TBS: Tris buffered saline, TCA: Tricloro acetic acid, μl: microliter, μM: micromolar

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1. Introduction

Colon cancer is seen in the colon, the last part of the digestive system. Most colon cancer cases begin as small, non-cancerous cell masses called adenomatous polyps, and over time some of these polyps can turn into colon cancers [1]. Many colon cancer studies show that this cancer occurs via action of a series of mechanisms. These mechanisms have been suggested as mutations, oncogen activities and epigenetic alteration mechanisms (methylation, acetylation) in tumor suppressor genes (APC, p53) [2,3].

Accumulating evidences show that the incidence of colon cancer cases is estimated to be around 65 years of age and older [4]. In addition, irregular lifestyle and eating habits such as over-processed meat consumption, alcohol consumption, high fat or low-fiber diets are factors that increase the risk of colon cancer [5]. The increase in colon cancer has been observed to have a significant effect on dietary habits. Therefore, creating food supplements that reduce the risk of cancer is particularly important for optimal nutritional design [6].

Recent studies have suggested that chemotherapy can be considered a therapeutic and preventive strategy in colon cancer and other types of cancer [7]. However, probiotics can also be used as biotherapeutics to reduce recurrence and side effects in colon cancer patients [8,9,10]. Probiotics are non-pathogenic microorganisms and have positive effects on the patient's health or physiology [5,6]. Secondary metabolites, such as organic acids and peptides, produced in the intestine by feeding probiotics can be observed to be effective in cellular proliferation and differentiation, apoptosis and reducing colon cancer risk [5,11].

Short Chain Fatty Acids (SCFAs) are fermentation products of bacteria found in the gastrointestinal tract. These bacteria use carbohydrates as their main energy source and produce SCFAs [12]. The amount of production of SCFAs depends on different factors such as fermentation site, diet, time spent on the gastrointestinal tract and the composition of existing bacteria [12]. Acetic, propionic, butyric and lactic acids are SCFAs which are produced as a result of metabolic activity. They have been shown to have apoptosis induction and anti-inflammatory properties in human colon cancer cells [13]. They are also preferred source of energy for host cells in the gastrointestinal tract and are involved in many cellular processes such as repair of intestinal mucosal lining, stimulation of the autonomic nervous system, and production of hormones associated with the gastrointestinal tract [14].

Genetics of cancer can be defined as a combination of genetic factors and environmental factors such as radiation, chemical carcinogens and diet. Therefore, the role of nutrition in the development of cancer is strongly supported by epidemiological studies in cancers of the digestive system [15]. In some studies, anti-proliferative and pro-apoptotic effects of *Lactobacillus* and *Bifidobacterium* strains on various cancer cell lines have been demonstrated [16,17]. Studies have also shown that probiotic bacterial strains inhibit liver, bladder, and breast tumors in animal models with potential probiotic activities [17,18,19]. For example, *Lactobacillus reuteri* represents a probiotic species with potent anti-inflammatory and anti-proliferative effects that are naturally common in humans and animals [20,21].

Kahouli et al. [7] shows that *Lactobacillus fermentum* has a higher anti-proliferative effect against colon cancer cells compared to some other probiotic bacterial species (*L. acidophilus* and *L. rhamnosus*) whose anti-cancer effects are characterized by previous studies. It is also interesting to note that cancer cells are inhibited but normal cells are not inhibited by *L. fermantum* [7]. These effects are strongly related to the ability of *L. fermentum* to produce some

SCFAs. This bacterium has also been shown to produce antioxidant and anti-cancer compounds as an alternative biotherapeutic agent in the treatment of colon cancer [7].

Kahouli et al. [22] attempted to test whether *L. reuteri* bacteria could produce SCFAs to prevent colon cancer cells from multiplying. In this study, the potential production of SCFAs by probiotic *L. reuteri* was evaluated *in vitro* and compared with its anti-proliferative effect at the same time. Their findings demonstrated that, *L. reuteri* was an important effect in inhibiting colon cancer cell growth in connection with the production of SCFAs. Thus, it is suggested that *L. reuteri* has the ability to produce anti-carcinogenic compounds and that it has potential biotherapeutic effect in colon cancer [22].

Some probiotic bacteria produce SCFAs, especially propionate and acetate, by fermentation, which can inhibit human colon cancer cells through apoptosis [23]. In this process, bacterial culture supernatant or pure SCFAs increase reactive oxygen species (ROS) and lipid peroxidation (LPO). Then they decrease mitochondrial trans-membrane potential and accordingly change anti-apoptotic Bcl-2 activity, increase caspase-3 activity and induce degradation of the chromatin stability of the core [23,24,25,26,27]. In addition to this, butyrate, one of SCFAs, has been shown in previous studies to induce apoptosis in colon cancer cells but not in normal cells [28,29].

The aim of the present study was to determine the effect of SCFAs from *L. reuteri* and sythentically-added on the changes in human colon cancer cells (HT-29) by the evaluating cell viability, apoptosis and oxidative stress. Since the influence of SCFAs produced by *L. reuteri* on various aspects of colon cancer has not been investigated, we further purposed to study possible molecular mechanisms of SCFAs anti-cancer effects.

2. Material and Method

2.1. Cells

HT-29 (ATCC® HTB-38™) cell line was obtained from Uludag University. It is kept in the Central Laboratory of Bingöl University. Lactobacillus reuteri (ATCC®-23272) is obtained from ATCC and stored in the Laboratory of Molecular Biology and Genetics Department of Bingöl University.

2.2. Cell Culture

HT-29 cells were cultured in DMEM growth medium containing 10% FBS and 1% antibiotic at 5% CO2, 37 °C for 2 or 3 days. Cells cultured for experiments were used when they reach a density of approximately 70-80%. Lactobacillus reuteri bacterial cells were grown in deMan, Rogosa and Sharpe (MRS) liquid growth medium at 37 °C by shaking the shaker at 140 RPM per day.

2.3. Preparation of Conditioned Medium from *L. Reuteri*

The experiments were modified from Kahouli et al. [22]. Bacteria to be used in the experiments were cultured 3 times and collected between 14-16 hours which is the late growth phase of passage 3. Bacteria in the amount of 1×10^8 cfu/ml were determined by spectrophotometer (1 OD= 1×10^9 cfu/ml) by centrifugation at 1000xg for 15 min at 4 °C and then the cells were washed twice with cold PBS. The centrifuged and washed cells were seeded directly into antibiotic-free and FBS-free DMEM at 1×10^8 cfu/ml and incubated at 37 °C with shaking at 140 rpm for 4 hours. After the incubation, the DMEM medium was centrifuged

twice at 1000xg, 15 min, 4 °C, and the DMEM medium was filtered with sterile filters having a diameter of 0.2 μM-por. All sterilization procedures were completed and 10% FBS, 1% antibiotic were added to this conditioned medium. It is then stored at -80 °C for other uses by adjusting to pH 7 with 0.1 M NaOH and HCl and dividing in appropriate quantities.

2.4. Determination of SCFAs with HPLC/UV-Vis Detector

The standards for chromatographic separation of organic acids lactic acid, acetic acid, propionic acid and butyric acid were prepared from the main stocks of 1000 ppm as; 500, 250, 100, 50, 20, 10 and 5 ppm. Chromatographic separations were then performed using SIL-20A HT autosamplers, CTO-10AS column oven and SPD-20A UV-VIS detector using HPLC (LC-20AT, Shimadzu, Japan) system. For the chromatographic separation, Quiros et al. [30] method was modified and the isocrotic system was set to mobile phase 1 mM perchloric acid, column flow rate 1 ml/min, injection volume 5 μl, column temperature 60 °C and detection wavelength 210 nm. Analysis was performed against standard graphs of 5, 10, 20, 50, 100, 250 and 500 ppm using ODS-3, 25 mm x 4.6 x 3 μm (GL Sciences) column. Quantity determinations of SCFAs in the CM were calculated by comparing the specific detection time of the samples with the mass of the standards. The quantities determined at this stage were prepared by synthetically adding to the normal medium for Designed Medium (DM).

2.5. Cell Viabilty Assay

HT-29 cells were cultured at 10-15000 cells per well in an 8-well plate and cell viability assay were initiated using a real-time instrument capable of icelligence (Acea Biosciences, USA). Then, CM and DM were added at specific concentrations and incubated in a 5% CO_2 incubator at 37 °C for 24 hours and the results were recorded. Effective doses were determined by determining appropriate inhibitory doses for CM and DM.

2.6. Experimental Grouping

Control group; HT-29 cells were treated only with DMEM medium for 24 hours. Conditioned medium (CM) group; HT-29 cells were treated with effective dose of conditioned DMEM medium (1:1 ratio) for 24 hours. Designed medium (DM) group; HT-29 cells were treated with effective dose of designed DMEM medium (1:1 ratio) for 24 hours.

2.7. ROS Assay

The ROS analysis was carried out using 2',7'-dichlorodihydrofluorroresin diacetate (DCFH-DA). HT-29 cells were treated as described in the experimental group, then the cells were collected by centrifugation. Then, 2 μM DCFH-DA for $1×10^6$ cells was added to each sample and incubated at 37 °C for 1 hour in the dark. Fluorescence intensity measurements at 485 excitation and 525 emission values were then performed using a spectrofluorometer [31]. The results of Relative Fluorescent Units (RFU) were calculated as percentage.

2.8. LPO Assay

LPO is based on the measurement of the amount of MDA (malondialdehyde) in the cell. Cells were taken after treatment as indicated in the experimental group and reacted with 250 μl of trichloroacetic acid (70% w/v) and 1 mL of

thiobarbituric acid (0.8% w/v) and kept in a 95 °C boiling water bath for 30 min. Then, it was suddenly placed on ice for 5 min and then centrifuged at 10000 RPM and then absorbance measurements of 532 nm were taken using an ELISA reader device to calculate the MDA levels of each sample [32]. The MDA levels of the samples were calculated using the standard curve and the measurement results were expressed in nmol/ml.

2.9. QRT-PCR Assay

The HT-29 cells were grown to $3-4x10^6$ in 25 cm^2 flasks and then treated as indicated in the experimental group. Cells were washed with cold PBS and isolated by centrifugation at 2500 RPM for 3 min. Total RNA isolation was then performed using a total RNA isolation kit. After the purity of the isolated RNA was measured by the nanodrop method (260/280 = 1.8-2.1); mRNAs in total RNA were transformed into cDNA by cDNA synthesis kit. QRT-PCR experiments were performed with primers suitable for Bax, Bcl-2, cytochrome c, caspase-3, caspase-9 and housekeeping gene beta actin, whose expression is expected to change in the apoptotic process (Table 1). The results are plotted according to the Ct (Cycle threshold) method using the $2^{-\Delta\Delta Ct}$ formula [33].

Table 1. The primer sequences of the apoptosis related genes used in the QRT-PCR (R: Reverse; F: Forward)

Gene	5'-3' Primer Sequences
Bax (F)	TGGAGCTGCAGAGGATGATTG
Bax (R)	CGGGGATTGATCAGACACGTAA
Bcl-2 (F)	TTTAATTGTATTTAGTTATGGCCT
Bcl-2 (R)	CAATAAACAATTCTGTTGACG
Cytchrome C (F)	AACAAAGGCATCATCTGGGGAG
Cytchrome C (R)	CACAGGTGAATCTTGCTTGGT
Caspase-9 (F)	ATTGTGAACATCTTCAATGG
Caspase -9 (R)	AGTAGGACACAAAGATGTCA
Caspase -3 (F)	TAGTTGCAATTGAATTAAATTAGGA
Caspase -3 (R)	TAGAATACACAGTCTTAAGTGG
Beta Actin (F)	AAAGCGGCCTTGGAGTGTGT
Beta Actin (R)	CATGGCTGGGGTGTTGAAGG

2.10. Western Blotting Assay

HT-29 cells were grown to $3-4x10^6$ in 25 cm^2 flasks, treated as described in the experimental group, then washed with cold PBS and centrifuged to prepare for protein isolation. Cells were homogenized with the ratio of 1:5 (w/v) cold protein isolation kit (Abcam, UK). In order to prevent proteins from being degraded by protease activity, both protease inhibitor cocktail (PIC) and PMSF were used during homogenization and all treatments were carried out on ice. The homogenates were centrifuged at 14000 RPM for 20 min at +4 °C and the supernatants were taken up in microcentrifuge tubes. The amount of protein in each sample was determined using the Bradford method. All samples were then stored at -80 °C until western blot experiments were performed.

The protein lysates obtained from the cells were run on a gel of 12% concentration by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Subsequently, the separated proteins were transferred to PVDF membrane and blocked with 5% BSA for 1 hour. Then, the membrane

were incubated for 3 hours via appropriate primer antibodies (Table 3.4) and washed with TBS-T (Tris Buffer Saline-Tween20 0.1%) as 5 times 5 min. After washing, the membrane was incubated with primer-appropriate secondary (Table 2) for 1.5 hours and washed with 5 times 5 min in TBS-T. The membrane was then incubated with ECL buffer (Abcam, UK) for approximately 3-4 min and the protein bands were fixed to the X-ray films with the developer. The relative level of protein bands were calculated the software program (Image Lab, Bio-Rad). The method used for the expression levels of each target protein; The target genes were normalized with the housekeeping gene beta actin and the percent change relative to the control.

Table 2. Usage characteristics of antibodies used in Western Blotting experiments

Antibodies	Antibody Usage Features
Bax (23 kDa)	Dilution: 1:500; Santa Cruz, sc-20067; Mouse monoclonal
Bcl-2 (26 kDa)	Dilution: 1:500; Santa Cruz, sc-7382; Mouse monoclonal
Cytochrome c (15 kDa)	Dilution: 1:500; Santa Cruz, sc-13156; Mouse monoclonal
Procaspase-9 (46 kDa)	Dilution: 1:500; Santa Cruz, sc-81663; Mouse monoclonal
Beta Actin (43 kDa)	Dilution: 1:500; Santa Cruz, sc-47778; Mouse monoclonal
Secondary	Dilution: 1:1000; Santa Cruz, sc-2005; Anti-Mouse HRP linked

2.11. Statistical Analysis

All results have been repeated at least three times. Statistical analysis was performed with GraphPad Prism 5.01 software and comparable data sets were evaluated by the one-way ANOVA Newman-Keuls Post-Hoc Test; $p < 0.05$ was considered significant.

3. Results

3.1. Amounts of SCFAs in CM

The presence and amounts of some short chain fatty acids in the conditioned DMEM medium were determined by HPLC/UV-Vis Detector. Standard graphics and equations were prepared for each fatty acid by standard graphs of 5, 10, 20, 50, 100, 250 and 500 ppm. Table 3 shows the amounts and retention times of lactic acid (2.289 μL / mL), acetic acid (0.937 μL / mL), propionic acid (0.62 μL / mL) and butyric acid (0.112 μL / mL) in CM. The 4 hour incubation of *L. reuteri* bacteria in DMEM resulted in the highest lactic acid production, but at least the production of butyric acid (Table 3).

Table 3. Amounts of the constituents analysed in the conditioned DMEM medium

Constituents	μl/ml	Retention Time
Lactic acid	2.289	6.83
Acetic acid	0.937	6.60
Propionic acid	0.682	16.62
Butyric acid	0.112	10.21

3.2. Effects of SCFAs on Cell Viability

We investigated the effects of some SCFAs from the secondary metabolites from the *L. reuteri* on the viability of HT-29 colon cancer cells. As seen in Figure 1, the 1:1 ratio of CM compared with the cells in the control group significantly reduced the proliferation of HT-29 cells. Similar to this result, DM significantly decreased cell viability in HT-29 cells. In view of these results, it is observed that some SCFAs obtained from *L. reuteri* bacteria are effective in decreasing the amount of HT-29 colon cancer cells.
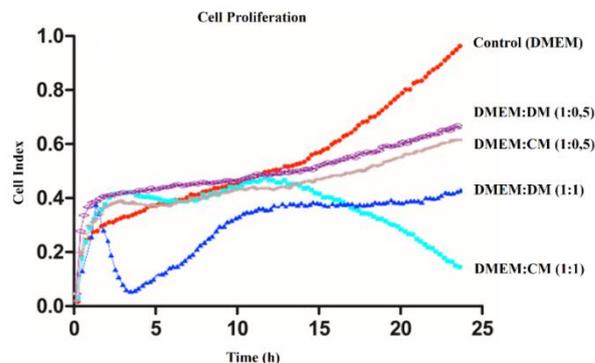
Figure 1. Effect of SCFAs on cell proliferation in HT-29 colon cancer cell. Control; HT-29 cells were treated only with DMEM medium for 24 hours. CM (Conditioned Medium); HT-29 cells were treated for 24 hours with the effective dose (1:1 or 1:0.5) of CM. DM (Designed Medium); HT-29 cells were treated for 24 hours with the effective dose (1:1 or 1:0.5) of DM

3.3. Effects of SCFAs on ROS

To determine whether SCFAs would increase ROS production in HT-29 colon cancer cells, we determined the ROS density in the control and CM and DM-added cells. As seen in Figure 2, ROS production was significantly increased in CM-added cells compared to control (p <0.001), but DM-added cells also showed a significant increase compared to control. However, the increase in the amount of ROS was observed to be greater in CM-added cells than in DM-added cells (p <0.001).
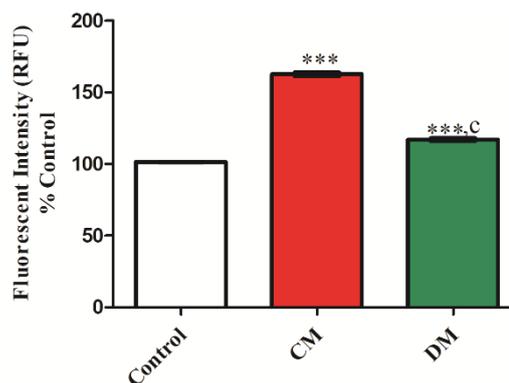
Figure 2. Effect of SCFAs on ROS production in HT-29 colon cancer cell. Cells were incubated as described in the experimental group. As seen in the graph, the ROS production was significantly increased (p <0.001) in the cells added to the CM compared to the control, and the DM added cells also showed a significant increase compared to the control (p <0.001). However, the increase in the amount of ROS was observed to be more frequent in CM added cells than in DM added cells (p <0.001). Data were calculated with mean ± SEM. (N: 6), ***p <0.001 Control vs CM, ᶜp <0.001 CM vs DM. CM (Conditioned Medium), DM (Designed Medium)

3.4. Effects of SCFAs on LPO

One of the widely used biological markers for LPO assessment is MDA. The MDA density in CM and DM treated cells was measured to determine whether SCFAs that significantly increased ROS production could increase MDA production in HT-29 colon cancer cells. As seen in Figure 3, MDA concentration was significantly increased in CM and DM-added cells compared to control (p <0.001). However, CM-induced MDA levels were significantly increased compared to DM-induced cells (p <0.001).
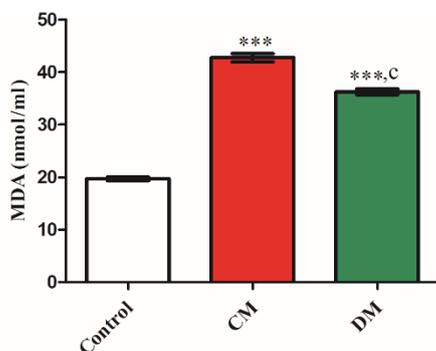
Figure 3. Effect of SCFAs on LPO accumulation in HT-29 colon cancer cell. Cells were incubated as described in the experimental group. As seen in the graph, MDA (LPO is summarized as MDA in the graph) was significantly increased (p <0.001) in the cells added to the CM compared to the control, and DM added cells also showed a significant increase compared to the control (p <0.001). However, the increase in the amount of MDA was observed to be greater in CM added cells than in DM added cells (p <0.001). Data were calculated with mean ± SEM. (N: 6), ***p <0.001 Control vs CM, ᶜp <0.001 CM vs DM. CM (Conditioned Medium), DM (Designed Medium)

3.5. Effects of SCFAs on Apoptosis Associated mRNA Expression Levels

We examined whether short chain fatty acids can cause any change in apoptosis-related Bcl-2 family. Bax, Bcl-2, cytochrome c, caspase-9 and caspase-3 gene expression levels were examined to determine if SCFAs could alter mRNA of these apoptotic enzymes levels in HT-29 cells. The expression of the apoptotic Bax gene levels significantly increased compared to the control in both CM and DM added cells (Figure 4.A). Expression levels of the anti-apoptotic Bcl-2 gene were significantly decreased in the samples added with both CM and DM compared to the control (Fig. 4.B). In addition, the Bax/Bcl-2 gene expression ratio was calculated assuming that the calculation of the Bax/Bcl-2 gene expression ratio is more important than the expression of the Bax gene alone or the expression of the Bcl-2 gene [34]. In the calculations, the ratio of Bax/Bcl-2 gene expression was markedly increased in HT-29 cells incubated with both CM and DM (Figure 4.C).

In addition, in the apoptotic cytochrome c gene expression, SCFAs caused a significant increase compared to the control group (Figure 4.D). Furthermore, gene expression levels of apoptotic caspase-9 (Figure 4.E) and caspase-3 (Figure 4.F) enzymes were significantly increased in CM and DM treated samples compared to control.
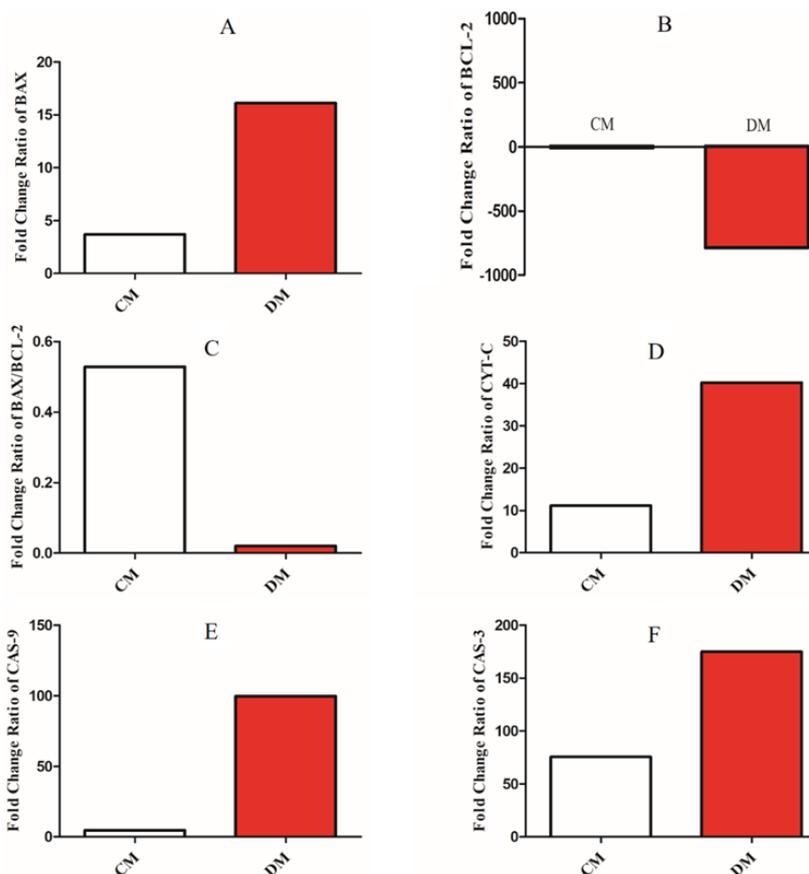
Figure 4. Effects of SCFAs on apoptosis-related gene expression level in HT-29 colon cancer cell. Cells were incubated as described in the experimental group. (A) Pro-apoptotic Bax, (B) anti-apoptotic Bcl-2, (C) Bax/Bcl-2 ratio, (D) cytochrome c, (E) caspase-9 and (F) caspase-3 gene expression rates were measured by QRT-PCR. CM (Conditioned Medium), DM (Designed Medium)

3.6. Effect of SCFAs on Expression Levels of Apoptosis-Related Proteins

We focused on the expression of apoptotic Bax and anti-apoptotic Bcl-2 proteins (Figure 5.A) to examine the molecular mechanisms of the apoptotic effects of SCFAs in detail. The expression of the Bax protein was not significantly altered in both CM and DM-added cells compared to the control (Figure 5.BI). The amount of Bcl-2 protein expression was significantly decreased in both the CM and DM cell groups compared to the control (Figure 5.BII). In addition, the calculation of the Bax/Bcl-2 protein expression ratio is more important than the expression of only the Bax protein or only the Bcl-2 protein [34], since the increase of ratio causes the release of cytochrome c and

hence the induction of apoptosis. In the computations performed, the Bax/Bcl-2 protein expression ratio in the HT-29 cells incubated with both CM and DM markedly increased (Figure 5.BIII). In addition, SCFAs in CM and DM caused a significant increase in apoptosis-inducing cytochrome c protein expression compared to the control group (Figure 5.BIV).

We also examined the expression of the apoptotic procaspase-9 enzyme. The level of protein expression of procaspase-9 showed a marked decrease when compared with control in CM and DM-treated cells (Figure 5.BV), meaning that the decreased level of procaspase-9 elevated in the active caspase-9 level. These results provide new evidence that SCFAs can play an apoptotic role in HT-29 colon cancer cells.
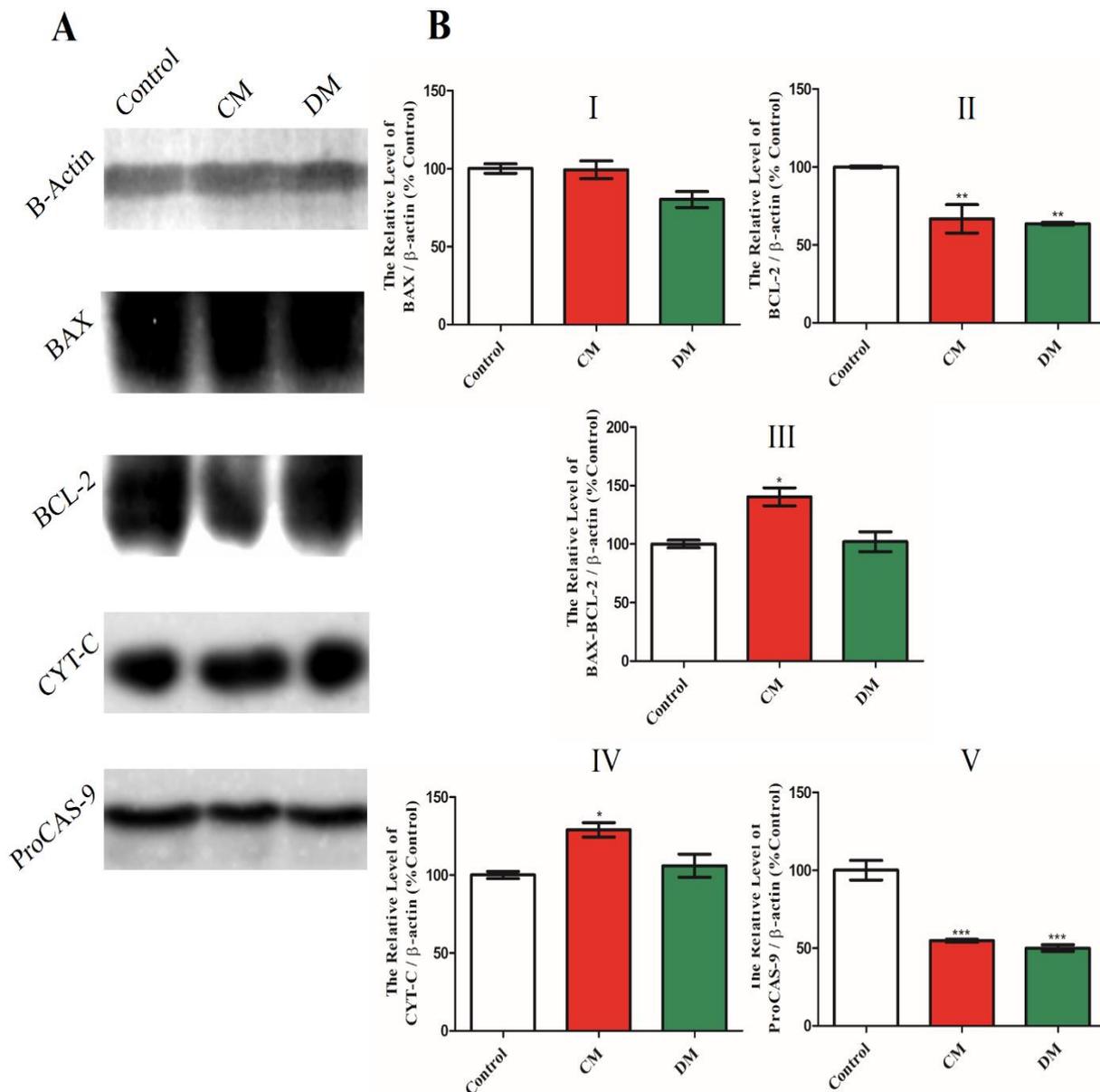
Figure 5. The effects of SCFAs on the expression levels of apoptosis related proteins in the HT-29 colon cancer cell. Cells were incubated as described in the experimental group. (A) Western blot analysis of Bax (23 kDa), Bcl-2 (26 kDa), cytochrome c (15 kDa) and procaspase-9 (46 kDa) protein levels expression. B-Actin (43 kDa) was used as loading control. (B) Incubation of HT-29 cells with CM increased the ratio of Bax/Bcl-2 and cytochrome c protein, thereby reducing the amount of procaspase-9. (I) Pro-apoptotic Bax, (II) anti-apoptotic Bcl-2, (III) Bax/Bcl-2 ratio, (IV) cytochrome c and (V) procaspase-9 protein expression ratios were measured using the Graphpad analysis program. Data are presented as mean ± SEM (n: 3). *p <0.05 Control vs CM, **p <0.01 Control vs CM, ***p <0.001 Control vs CM. CYT-C = cytochrome c, ProCASP-9 = procaspase-9). CM (Conditioned Medium), DM (Designed Medium)

4. Discussion

Cytotoxicity caused by SCFAs from different species of *Lactobacillus* has been implicated in the cancer treatment, however, the direct impacts of Synthetic SCFAs and SCFAs from L. reuteri on cancer cells and the mechanism by which it might induce apoptosis-related cytotoxicity have not been clarified yet. In the current study, we investigated the pro-oxidant activity of SCFAs from *Lactobacillus reuteri* in HT-29 colon cancer cells, which increased the ROS production and MDA as a marker of LPO.

Previous studies have represented the effectiveness of SCFAs that inhibits cancer cells proliferation [35], our findings which have also indicated anti-cancer effects of synthetic SCFAs and SCFAs from *L. reuteri* in HT-29 cells. Studies on cell viability show that the SCFAs regulate cell proliferation *in vitro* and *in vivo* in gastrointestinal tissue [35]. It has been noted that consumption of dose-depended SCFAs induces levels of genes which were related to cell proliferation and the expression levels of cyclin B1, cyclin D1, cyclin E1, CDK1, CDK2, CDK4 and CDK6 genes were increased in the medium-feeding group [35]. Based on this result, the SCFAs diet can regulate the expression of genes that control cell proliferation [35]. Inhibition of cancer cells was demonstrated in studies with human-derived probiotic bacteria such as *Propionibacterium pentosaceus*, *Lactobacillus salivarius*, which are thought to have an alternative biotherapeutic potential for colon cancer [36]. Proliferative inhibition mechanisms of colon cancer cells suggest that probiotic bacteria can directly attach to colon cancer cells, or produce some SCFAs, especially butyric and propionic acids, can trigger the anti-cancer feature by acting synergistically [36]. All of them could suggest that SCFAs are clearly safe to inhibit tumorigenesis progression.

A number of studies demonstrated that the increase in ROS is usually observed in the progression of apoptosis, and ROS production may be a sign of apoptosis. It is known that many anticancer drugs and natural compounds increase the ROS level because they induce apoptosis in cancer cells [37,38]. ROS has high chemical activity and plays important roles in regulating apoptosis through cell proliferation [39]. However, antioxidants generally inhibit cell damage under normal conditions [40]. However, an excessive increase in the amount of ROS induced by different pro-oxidants leads to an oxidative stress, leading to apoptosis, overcoming the clearing activity of the cellular antioxidant defense system [39]. The increase in ROS level is a common biochemical characteristic observed in cancer cells. ROS can react with unsaturated fatty acids in cell membranes to induce LPO [37].

Present study clearly showed that treatment of HT-29 colon cancer cells with synthetic SCFAs and SCFAs from *L. reuteri* significantly decreased cell viability and triggered MDA and ROS productions. Previous study has also revealed that *Lactobacillus* is a potent inducer of endogenous ROS production and ROS-dependent cell proliferation in fruit juice and mouse intestines [41]. In addition, it suggests that ROS production from *Lactobacillus* and cell proliferation are linked the enzyme Nox1 (NADPH oxidase 1), which is functional in intestinal epithelial cells. In germ-free animal models ROS production has not occurred and the growth of suppressed epithelial cells is associated with this condition. This result suggests that bacterial activation of an enzyme producing ROS in enterocytes affects cell proliferation [41]. It might be suggested that increase of SCFAs-induced cell death is occurred by the raise of intracellular ROS productions. SCFAs produced by fermentations of probiotic bacteria

may kill human colon cancer cells as a result of apoptosis [23]. This is achieved by increasing ROS and LPO levels [37]. Therefore, SCFAs led to reducing mitochondrial trans-membrane potential, altering anti-apoptotic Bcl-2 activity, enhancing caspase-3 activity and impairing chromatin stability in the nucleus [23,24,25,26]. These findings together with our results strongly put forward that the anti-cancer ability of synthetic SCFAs and SCFAs from *L. reuteri* has main responsibility to increase ROS generation and oxidative stress for induction of apoptosis.

Herein, we assessed mechanisms by which synthetic SCFAs and SCFAs from *L. reuteri* can be effective to induce apoptosis in HT-29 cells. The mitochondrial (intrinsic) pathway is one of the most important mechanisms to promote apoptosis. In the intrinsic pathway of the apoptosis of the cells, increased permeability of the mitochondrial membrane structure is observed and as a result, the amount of cytochrome c in the cytoplasm can increase [42]. In the regulation of apoptosis by the intrinsic pathway, there are closely related genes and proteins. One of these is anti-apoptotic Bcl-2, which protects cells against apoptosis by inhibiting the cytochrome c increase in its cytoplasm, while another is Bax and stimulates apoptosis as a pro-apoptotic member [43]. The permeability of mitochondria to cytochrome c varies with the translocations of Bcl-2 and Bax proteins found in mitochondrial pores [34,44]. Cytochrome c reacted with Apaf-1 in the cytoplasm leads to the activation of procaspase-9 and activated caspase-9 also activates procaspase-3 [42,45].

In the present study, synthetic SCFAs and SCFAs from *L. reuteri* induced the decrease of Bcl-2 expression levels. Decline in expression level of Bcl-2 also led to an enhancement on Bax/Bcl-2 expression ratio that have crucial role to decide sensibility of cells to apoptosis [34]. Furthermore, we suggested that the increase of Bax/Bcl-2 ratio resulted in a change on the expression level of cytochrome c which was increased by SCFAs treatment. We put forwarded that treatment with SCFAs increased the expression level of caspase-3. The balance of the Bax/Bcl-2 ratio and the induction of caspase-3 are important for the cell to survive or die in intrinsic pathway [34].

We further found that the transcription level of caspase-9 and -3 were up-regulated by synthetic SCFAs and SCFAs from *L. reuteri* induction. Gui and Shen [35] demonstrated that SCFAs control cell apoptosis both *in vitro* and *in vivo*. Expression levels of apoptotic genes (caspase-3, caspase-8, caspase-9, p53 and Bax) and expression levels of Bax/Bcl-2 ratio in the medium-feeding group of SCFAs were significantly increased in regulating mRNA levels of genes involved in cell apoptosis. Therefore, the SCFAs diet consumed in the medium level induces apoptosis [35]. In another study, apoptosis induction is tested in apoptosis resistant colon cancer cell (Caco-2) by an anti-tumor agent in butyrate from SCFAs [46]. Butyrate reduced anti-apoptotic Bcl-XL protein expression while enhancing pro-apoptotic Bak protein expression. This resulted in the release of cytochrome c from the mitochondria to cytoplasm and the activation of procaspase-9, -3 and -1 respectively and the formation of active caspase cascades [46]. It was shown that the butyrate from SCFAs induces apoptosis in the Caco-2 cell line as a result of caspase-3 and caspase-1 activation [46]. Therefore, it can be suggested that synthetic SCFAs and SCFAs from *L. reuteri* cause apoptosis via inducing of intrinsic apoptotic pathway. According to previous findings, the induction of cancer cells with butyrate, a fermentation product in the colon during apoptosis, is considered to be an important mechanism for protection against colorectal cancer. An important effect of butyrate is to inhibit histone deacetylase (which inhibits chromatin relaxation and epigenetically

leads to the suppression of apoptotic gene expression), while butyrate may induce apoptosis by releasing specific cell death genes by inhibiting this enzyme [47]. It has been shown that butyrate can induce apoptosis in colorectal cancer cells, leading to conversion of procaspase-3 to active caspase-3 [47]. Moreover, it has been shown in previous studies that butyrate, which is from SCFAs, causes apoptosis in colon cancer cells but not in apoptosis in normal cells [28,29,48]. Therefore, the anti-cancer activity of synthetic SCFAs and SCFAs from *L. reuteri* can be varied in relation to the content.

In summary, we clearly found that, synthetics SCFAs and SCFAs from *L. reuteri* induce oxidative damage in HT-29 cells via increasing ROS and LPO production. Additionally, Bax/Bcl-2 ratio and the expression level of caspase-9 and -3, which are linked with the intrinsic pathway of apoptosis were also increased. We demonstrated that synthetics SCFAs and SCFAs from *L. reuteri* induce over-producing of ROS, LPO and finaly apoptosis that led to cytotoxicity in HT-29 cells. As a novelty of our study, it was first time represented that SCFAs from *L. reuteri*-induced oxidative stress and apoptosis in HT-29 cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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