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Fermentation product of RS3 inhibited proliferation and induced apoptosis in colon cancer cell HCT-116

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ABSTRACT

Cell free supernatant containing short chain fatty acid (SCFA) resulted from fermentation of resistant starch type three (RS3) by Clostridium butyricum BCC B2571 or Eubacterium rectale DSM 17629 were investigated for their ability to inhibit proliferation and induce apoptosis of human colon cancer cell line HCT-116. HCT-116 was cultured in complete medium and after 50% confluent, incubation was continued for another 48 hours in the absence or presence cell free supernatant containing SCFA mixture at butyrate levels up to 10 mM. The study revealed that the proliferation inhibition effect was higher (>80%) on HCT-116 treated with supernatant of *C. butyricum* BCC B2571 than that (<70%) of HCT-116 treated with supernatant of E. rectale DSM 17629. The cells were induced to undergo apoptosis by both supernatant. The apoptosis occured through mitochondrial pathway by changing the expression of gene Bcl-2 and Bax, thus incresed the Bax/Bcl-2 ratio by more than 3.5 fold. The protein caspase-3 was increased by more than 250% in the presence of the cell free supernatant.

Keywords: Resistant Starch; *Clostridium butyricum*; *Eubacterium rectale*; Colon; Cancer; Apoptosis

1. INTRODUCTION

Cancer is common term for a malignant cellular growth that tends to spread due to the inability of the DNA to respond to the normal physiologic stimuli. Thus, cancer is a genetic disorder. Colorectal cancer (CRC) is cancer cell that grows in colon/rectum. According to the global statistic cancer data, CRC is the third most common cancer with over 1.2 million new cases in 2008 or approximately 9.8% of the world total new cases [1]. Between 5% to 10% of colorectal cancer are consequences of recognised hereditary conditions. The remaining of CRC cases have been attributed to food, nutrition and physical activities [2]. It is suggesting that CRC is a preventable disease. Development of cancer is a complex, multi step processes that seem to progress for an extended of time. Therefore, substantial effort has been made to develop functional food ingredients such as resistant starch (RS) that could inhibit, delay or reverse the multistages carcinogenesis.

Resistant starch (RS) is defined as the sum of starch and products of starch degradation that is not absorbed in the intestine of healthy individuals. Resistant starch has been classified into four general subtypes called RS type 1 (RS1), RS type 2 (RS2), RS type 3 (RS3) and RS type 4 (RS4) [3]. RS1 is physically inaccessible, because the the starch molecules are trapped in the structural carbohydrate and this difficult to be digested. RS2 refers to native granule starch with highly dense crystalline structure that prevents enzymatic digestion. RS3 refers to non-granular starch-derived materials that resist digestion. RS3 is generally formed during the retrogradation of the starch granules, and can be produced by autoclaving and cooling in the presence of water. When starch is heated, amylose is solubilezed and a starch gel is formed. Upon cooling, the gel undergoes transformations leading to a partially crystalline structures (retrogradation). During this retrogradation, amylose is reassociates to form crystallization. RS4 is chemically modified starch, abd therefore, chemical bond other than α-(1,4) or α-(1,6) such as phosphate ester are included in this group [4].

RS is fermented in the large intestine. Mostly, colonic bacteria are sacharolytic. Carbohydrate fermentation is an important force driving microecology and physiology of large intestine. Among the genera of colonic bacteria, butyrate producing bacteria such as *Eubacterium*, *Peptostreptococci*, *Clostridia*, *Roseburia* spp. and *Butyrofibriofibrisolvens*, are thought to have beneficial effect on the human host. They produce Short Chain Fatty Acid

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(SCFA) such as acetic acid, propionic and butyrate that are greatly helpful in preventing colonic diseases [5-7]. It is reported that SCFA may modulate tissue levels and effect growth factor in the gut and so modify the gut development and reduce the risk of serious diseases, including colorectal cancer/CRC [8]. Animal study supports a direct link between dietary RS and colon cancer [9]. It was reported that hydrothermally treated RS3 prevented carcinogenesis, and that this effect was mediated by enhanced apoptosis of damaged cell accompanied by changes in parameters of differentiation in colonic mucosa.

Deregulated proliferation and inhibition of apoptosis lies at the heart of all tumor development, thus the control of the cell proliferation and apoptosis present obvious target for preventive and intervention in all cancer. Apoptosis are kept highly controlled and regulated by the cells' internal machinary. The family of Bcl-2 protein was known as the first and most widely studied regulators of apoptosis [10]. The Bcl-2 family of protein can be either pro-apoptotic or anti-apoptotic. They can determine if the cell commits to apoptosis or aborts the process.

In the previous study we developed RS3 dertived from sago and rice starch through enzyme hidrolysis process. Starch degrading enzymes: amylase and pullulanase were applied. The RS3 could be well utilized as substrate by *Clostridium butyricum* BCC B2571 or *Eubacterium rectale* DSM 17629 to produce SCFA. The study revealed that good proportion of acetate:propionate:butyrate in the cell free supernatant was produced either by *C. butyricum* BCC B2571 grown in medium supplemented with RS3 derived from sago starch treated with amylase (RSSA) or *E. rectale* DSM 17629 grown in medium supplemented with RS3 derived from rice starch treated pullulanase sago (RSSP) [11].

The objectives of the research was to investigate the effect cell free supernatant originated from the RS3 fermentation by *C. butyricum* BCC B2571 or *E. recatale* DSM 17629 on the proliferation and apoptosis of human colon cancer cell HCT-116. This study showed that SCFA in the cell free supernatant inhibited and induced apoptosis of colon cancer cell HCT-116 (ATCC CCL-247). It also provided evidence that the apoptosis occured in correlation with mitochondrial pathway, increased of the ratio of Bax gene toward the Bcl-2 gene expression, and increased caspase-3 protein.

2. MATERIALS AND METHODS

2.1. Resistant Starch Type 3 (RS3)

RS3 was prepared as reported previously [11]. RS3 was prepared from amylase treated sago starch or pullulanase treated sago starch, respectively. The procedure was described as following: sago starch (50 g) was suspended in 200 mL of water, boiled and stirred for 10 min, removed from heat and cooled down to 30˚C. The gel was vacuum sealed in a retort pouch and autoclaved at 121˚C, 15 psi for 1 hour, and stored at 4˚C for 12 - 14 h, to enhance retrogradation. Retrograded starch was suspended in 1 L of water and blended in a warring blender at high speed for 2 min. The retrograded starch suspension was enzymatically hydrolyzed. The following treatments were applied for starch hydrolysis: 1) 1 mL of amylase (3.0 KNU-T/g substrate) for 3 h at 85˚C; 2) 1 mL of pullulanase (32.0 NPUN/g substrate) for 3 h at 55˚C; 3) 1 mL of amylase for 3 h at 85˚C continued with 1 mL of pullulanase for 3 h at 85˚C. Hydrolyzed starch was centrifuged for 10 min at room temperature. The supernatant was discarded and the residue was collected. The residue was stored in cool room (10˚C) overnight, suspended into water and homogenized for 2 min by using homogenizer. The suspension was loaded into the spray drier. The inlet temperature of the dryer was 160˚C.

RS contained in the product was measured according to method described by Goni *et al*. [12]. Briefly, the basic step of the analytical procedure include: removal of digestable starch' solubilization and enzymatic hydrolysis of RS and quantification of RS as glucose released. The products contained RS at approximately 32.3% and 31.7%.

2.2. Bacterial Strains and Culture Media

Pure culture of *C. butyricum* BCC-B2571 was obtained from Culture Collection of Indonesian Research Center for Veterinary Sciences (IVETRI). The basal medium for maintaining *C. butyricum* BCC-B2571 consisted of the following (in g/L): yeast extract, 3; beef powder, 10; peptone, 10; glucose, 5; soluble starch, 1; NaCl, 5; Na-acetate, 3; cysteine hydrochloride, 0.5. The pH was adjusted to 6.8.

E. rectale DSM 17629 was obtained from DSMZ, Germany and it was maintained in a medium composed of (in g/L): tryptone, 5; bacteriological peptone, 5; yeast extract, 10; beef extract, 5; glucose, 5; Tween 80 1 mL, resazurin 0.001; $CaCl_2$, 0.01; $MgSO_4$, 0.02; K_2HPO_4, 0.04, KH_2PO_4, 0.04, $NaHCO_3$, 0.4, NaCl, 0.08, Vitamin K1, 0.0002. The pH was adjusted to 7.0.

2.3. Preparation of Cell Free Supernatant and SCFA Analysis

In vitro fermentation was performed to prepare the cell free supernatant. It was carried out according to the method described previously [11]. RS3 (1%) derived from amylase treated sago was used in the fermentation medium for *C. butyricum* BCC B2571 and RS3 derived from pullulanase treated sago was added in the fermenta-

tion medium for *E. rectale* DSM 17629. It was then inoculated with 24 h of the preculture bacterial strains (at about 10^9 CFU/mL) and anaerobically incubated at 37˚C, 48 h. At the end of incubation period, the culture medium was centrifuged at 3000 g for 10 min and the supernatant was filtered through a 0.22 µm pore-size membrane filter.

The SCFA component in the cell free supernatant was analyzed by Gas Chromatography (Agilent Technologist, 7890 A GC System). The following procedure was applied. A portion of SCFA were spiked into the supernatant and then capped. Samples of 1 µl was injected into a high resolution gas chromatography (Agilent Technologist, 7890 A GC System) equipped with a flame ionization detector and a HP Innowax 19091-136 column (60 m × 0.250 mm). The carrier gas was helium with a flow rate of 1.8 mL/min, and the split ratio was 40:1. The oven temperature was maintained at 90˚C for 0.5 min, and then increased to 110˚C at a rate of 10˚C/min, increased to 170˚C at a rate of 5˚C/min and finally increased to 210˚C at a rate of 20˚C. Injector and detector temperatures were 275˚C. SCFA mixture containing acetate, propionate and butyrate at spesific concentration were used as standard.

2.4. Cells Lines

Colon cancer cell HCT-116 (ATCC CCL-247) was donated by Stem cell and Cancer Institute (SCI) Jakarta. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, and antibiotics (penicillin 100 U/mL and streptomycin 100 ug/mL) and incubated in the presence of 5% CO_2.

2.5. Cell Counting

Colon cancer cell HCT-116 were resuspended in complete medium. About 10^6 cells were seeded in tissue culture flask T75 overnight. SCFA were added into treated well and incubation was continued for another 48 h. At the end of 48 h incubation period, the medium was discarded and attached cell was then trypsinized and divided into several aliquots for assay.

Number of cells were counted and growth inhibition value was calculated as percentage of substraction of viable control cells with treated cells towards control cells.

2.6. Detection of Apoptosis

Total of 10^4 cells were plated on 8-well slide chamber for 24 h. Incubation was continued for another 48 h in the absence or presence of various concentrations of SCFA. Cells then were washed with phosphate buffered saline (PBS). Treated and untreated cells were fixed with 2% of glutaraldehyde for 1h and then stained with 10 mg/L of Hoechst 33258 fluorescent dye for 30 min. The cells were immediately washed with PBS. The stained cells were observed under a fluorescent microscope, with excitation at 365 nm and emission at 460 nm. Semi quantitatitive measurement was performed according to the following score: 0 (0 - 1 fluoresecented cell/view area), 1 (fluoresecented cell/view area 2 - 4), 2 (5 - 6 fluoresecented cell/view area) and 3 (>6 fluoresecented cell/view area).

2.7. RNA Isolation and Real-Time PCR Assay

Total RNA was isolated from the treated and untreated cell HCT-116 using RNeasy Kit (Qiagen) according to the manufacturer's instruction. Concentration of the RNA was measured by spectrophotometer (SmartSpec-Plus, Biorad) at 260 nm.

Real-time PCR (RT PCR) was performed on a IQ5 Multicolor Real Time PCR Detection System (Biorad) to analyze the expression level of Bax and Bcl-2 relative to the housekeeping gene GAPDH (Gyceraldehyde-3-Phosphate Dehydrogenase). The primer sets used for the amplification of the target gene are listed in **Table 1** as described by Nohara *et al*. [13].

RT-PCR analysis was performed in a final volume of 25 µl containing 5 µl RNA template (equivalent to 400 mg of RNA), 1 µl of each forward/reverse primer, 12.5 µl of 2 × SYBR Green RT-PCR reaction mix, 0.5 µl of reverse transcriptase and 5 µl of nuclease free water. The cycling condition were: 50˚C, 10 min for activation of reverse transcriptase, 95˚C, 5 min for inactivation of reverse transcriptase and 40 cycles at 95˚C for 10 s, 52˚C for 10 s and 72˚C for 10 s. The baseline and the threshold were automatically set by the software. The crossing point of the amplification curve with the threshold represents the cycle threshold (Ct). Relative gene expression was calculated [14]. All samples were normalized into equivalent level of GAPDH.

2.8. Caspase-3 Assay

Caspase-3 was quantified using Human Caspase-3 Instant ELISA BMS2012INST (Bender MedSystem), according to the manufacturer's instruction. Cells were lysed in the lysis buffer and the concentration of caspase-3 in the lysates was measured.

Table 1. Primer for amplification of Bax, Bcl-2 and GAPDH gene.

Gene	Sequen
Bax	Reverse primer: 5'-GCCTTGAGCACCAGTTTG-3'
	Forward primer: 5' -CCCGAGAGGTCTTTTTCC-3
Bcl-2	Reverse primer: 5'-CCTCTCCATCATCAACTT-3'
	Forward primer: 5'-GCTCTAAAATCCATCCAG-3'
GAPDH	Reverse primer: 5'-TCAAAGGTGGAGGAGTGG-3'
	Forward primer: 5'-CGGATTTGGTCGTATTGG-3'

2.9. Statistical Analysis

Data were analyzed by One Way Analysis of Variance followed by Tukey HSD test to compare the mean value. Data on gene expression was subjected to descriptive analysis. SPSS 10.0 software was applied to analyze the data.

3. RESULTS

3.1. SCFA Proportion in the Cell Free Supernatant

As expected, RS3 was shown as powerful butyrate producing substrates for the two bacteria. Quantification of SCFA in the bacterial supernatant is presented in **Table 2**. Generally, *C. butyricum* BCC B2571 produced more acetate, propionate and butyrate in comparison to *E. rectale* DSM 17629. The proportion of propionate in the bacterial supernatant of *C. butyricum* BCC B2571 was slightly lower than that in bacterial supernatant of *E. rectale* DSM 17629. *C. butyricum* BCC B2571 produced 218 mM of acetate, 192 mM of propionate and 170 mM of butyrate or molar ratio around 1.3:1.1:1. *E. rectale* DSM 17629 produced 117 mM of acetate, 134 mM of propionate and 115 mM of butyrate or molar ration around 1:1.2:1.

3.2. Cell Number and Proliferation Inhibition

For experimental cell cultures, the SCFA in the cell free supernatant was diluted with DMEM until the butyrate concentration was <10 mM. Prior to this experiment we run toxicity test. Vero cell line was used as non-cancerous control cells. The SCFA mixture at butyrate concentration levels up to 10 mM was observed to be non-toxic to Vero cell (data not shown). The colon cancer cell HCT-116 treated with SCFA appeared dead after 48 hours incubation when examined under inverted microscope. Therefore, concentrations of less than 10 mM were regarded as reasonable range to test the biological effect in this study.

To investigate the role of the SCFA as anticancer agent, HCT 116 cells were treated in the absence or presence of SCFA in the cell free supernatant. The cell free supernatant containing SCFA decreased the viability, total cell number and inhibited growth of cancer cell line HCT-116 in a dose dependent manner ($p < 0.05$). Viable and total number of the untreated and treated HCT-116 is presented in **Figure 1**, while the inhibition effects of SCFA mixture on HCT-116 is presented in **Figure 2**. As shown in **Figure 2**, the inhibition effect was observed at butyrate concentration as low as 2.6 mM (Treatment 1 of SCFA mixture from *C. butyricum BCC* B2571). The maximum inhibition was noted by treatment with SCFA containing 7.2 mM butyrate (Treatment 2 of SCFA produced from *E. rectale* DSM 17629).

Table 2. Short Chain Fatty Acid (SCFA) proportion in cell free supernatant.

Bacterial supernatant	SCFA		
	Acetate (mM)	Propionate (mM)	Butyrate (mM)
C. butyricum BCC B2571	218	192	170
E. rectale DSM 17629	117	134	115

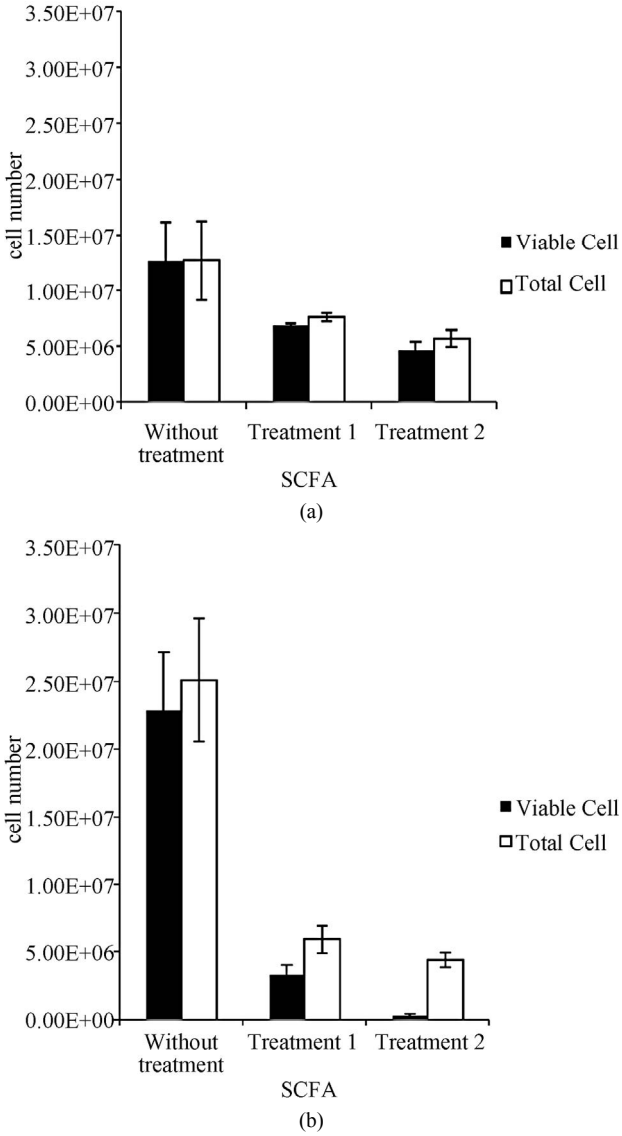

Figure 1. Effect of SCFA in cell free supernatant on viable and total cell number of HCT 116 cell. (a) HCT-116 was untreated or treated with SCFA in cell free supernatant of *C. butyricum* BCC B2571. Treatment 1 consisted of acetate 3.4 mM, propionate 3.0 mM, butyrate 2.6 mM and Treatment 2 consisted of acetate 6.8 mM, propionate 6.0 mM, butyrate 5.3 mM; (b) HCT-116 was untreated or treated with SCFA in cell free supernatant of *E. rectale* DSM 17629. Treatment 1 consisted of acetate 3.6 mM, propionate 4.2 mM, butyrate 3.6 mM and Treatment 2 consisted of acetate 7.3 mM, propionate 8.4 mM, butyrate 7.2 mM.

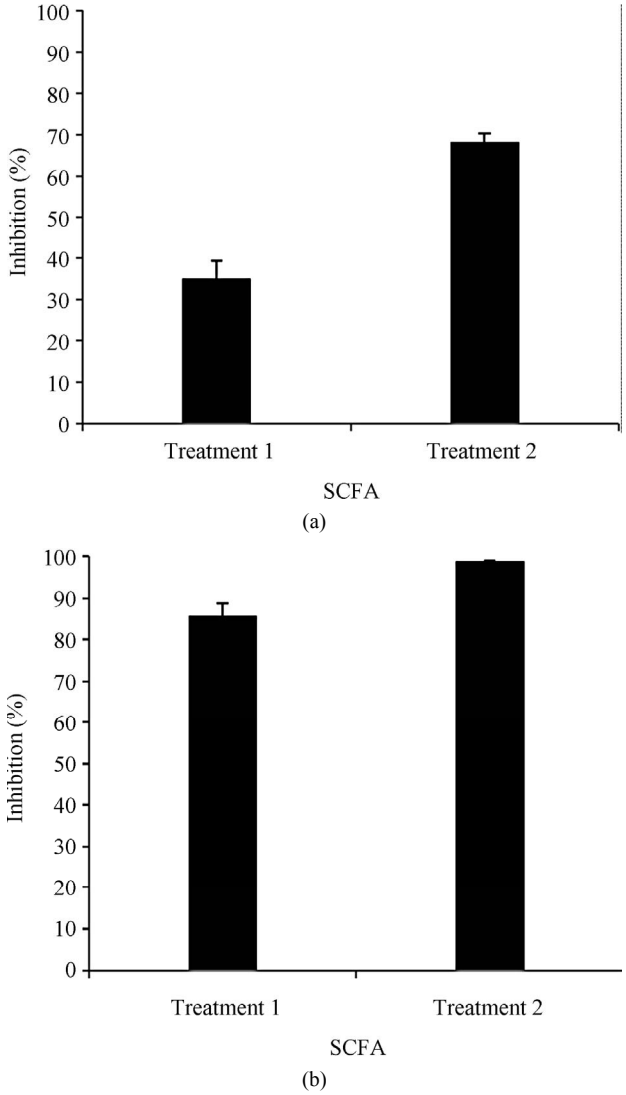

Figure 2. Effect of SCFA in cell free supernatant on growth inhibition of HCT-116 cell. (a) HCT-116 was treated with SCFA in cell free supernatant of *C. butyricum* BCC B2571; (b) HCT-116 was treated with SCFA in cell free supernatant of *E. rectale* DSM 17629. Treatment 1 & Treatment 2 was described as in previous **Figure 1**.

3.3. Apoptosis and Alteration of Bcl-2 and Bax Expression

Fluorescence microscopy after stained with Hoechest 33258 DNA dye showed the specific features of apoptotic cells, including nuclear shrinkage, condensation and fragmentation of treated cells (**Figure 3**).

This effects was detected either at low SCFA concentration (2.6 mM of butyrate in SCFA from *C. butyricum* BCC B25671) or high SCFA concentration (7.2 mM of butyrate in SCFA from *E. rectale* DSM 17629). On the other hand, untreated cells showed uniformly, regular and oval shape of nuclei. Semi quantitative measurement of apoptotic cell was presented in **Table 3**.

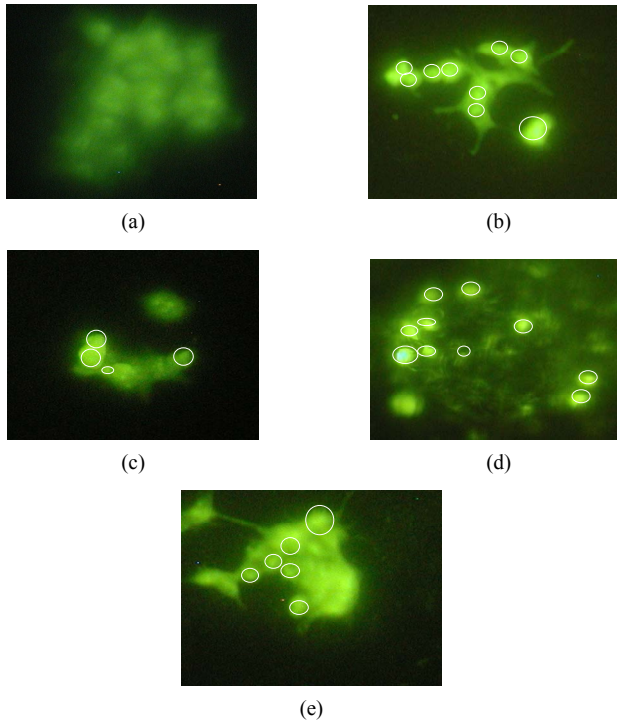

Figure 3. Fluoresence microscopy appearance of HCT 116 cell treated or untreated with SCFA in cell free supernatant. Cells was stained with Hoechst 33258. (a) Untreated cell; (b) Cell treated with Treatment 1 of SCFA in cell free supernatant of *C. butyricum* BCC B2571; (c) Cell treated with Treatment 2 of SCFA in cell free supernatant of *C. butyricum* BCC B257; (d) Cell treated with Treatment 1 of SCFA in cell free supernatant of *E. rectale* DSM 17629; (e) Cell treated with Treatment 1 of SCFA in cell free supernatant of *E. rectale* DSM 17629. Treatment 1 & Treatment 2 was described previously as in **Figure 1**.

Table 3. Effect of cell free supernantant on apoptosis of cancer cell HCT-116.

Treatment	Score
Control	0
Treatment 1 HCT-116 + Supernatant *C. butyricum* BCC B2571	3
HCT-116 + Supernatant *C. butyricum* BCC B2571	2
HCT-116 + Supernatant *E. rectale* DSM 17629 Treatment 2	2
HCT-116 + Supernatant *E. rectale* DSM 17629	2

Note: Treatment 1 & Treatment 2 was as previously described.

To investigate the role of SCFA in cell free supernatant in inducing apoptosis through mitochodrial pathway, we measured the Bcl-2 and Bax mRNA level by using Real-Time PCR. **Figure 4** shows that SCFA in cell free supernatant of *C. butyricum* BCC 2571 (with butyrate at 2.6 and 5.3 mM) increased Bax mRNA level 7 - 9 fold change in HCT-116 compare to that of the untreated cell, without significant alteration of Bcl-2 mRNA level.

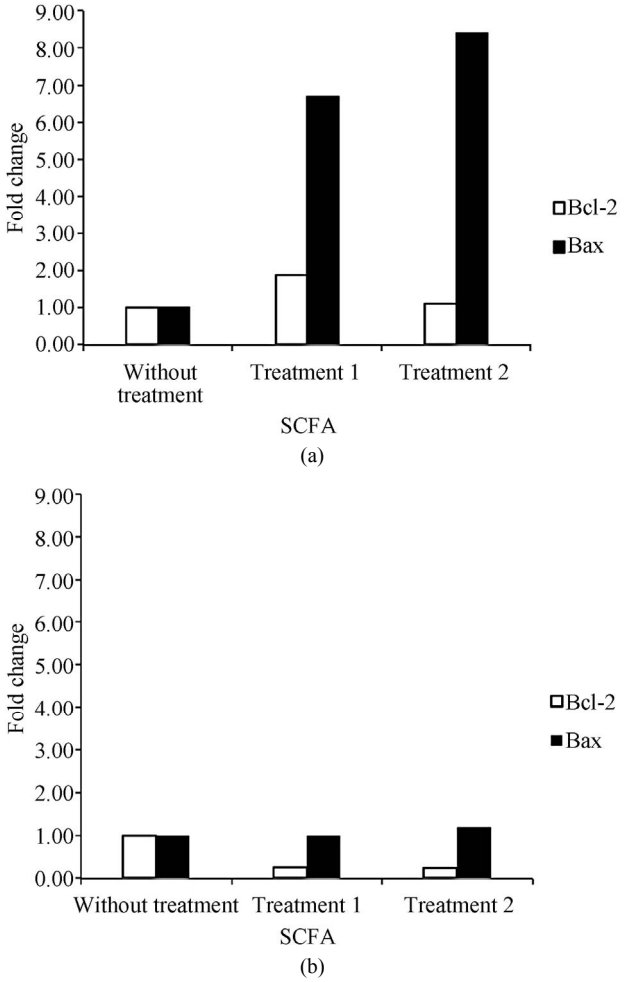

Figure 4. Real-time semi quantitative measurement of Bcl-2 and Bax mRNA level in HCT-116. (a) HCT-116 was untreated or treated with SCFA in cell free supernatant of *C. burtyricum* BCC B2571; (b) HCT-116 was untreated or treated with SCFA in cell free supernatant of *E. rectale* DSM 16729. Treatments were described previously as in **Figure 1**. The fold increases are based on treatment without fermentation product consist of SCFA.

Different pattern was observed when SCFA in the cell free supernatant of *E. rectale* DSM 17629 was applied to treate the HCT-116. In this case, the level of Bcl-2 mRNA decreased upon SCFA treatment, without significant change of Bax mRNA level. The level of Bcl- mRNA in HCT-116 treated cells decreased by 0.2 - 0.3 fold compared to the untreated cells. Overall, these data shows that SCFA in the cell free supernatant increased the ratio of Bax over Bcl-2 (**Figure 5**). It indicates that there was an excess of pro-apoptotic protein resulted by Bax-mRNA and thus cells were more sensitive to apoptosis with the applied treatment.

3.4. Caspase-3 Concentration

In our study, caspase-3 was measured by using Instant ELISA Kitt. The effect of SCFA in cell free supernatant on production of Caspase-3 is presented in **Figure 6**. Caspase-3 was significantly increased ($p < 0.05$) after the cells were treated with fermentation product consisted of SCFA. The treatment consisted of butyrate at concentration level of more than 5 mM (Treatment 2 of SCFA either from *C. butyricum* BCC2571 or *E. rect*ale 17629). It was observed that caspase-3 produced by HCT-116 treated with SCFA in cell free supernatant of *C. butyricum* BCC B2571 was much lower compared to that of treated cell with SCFA in bacterial supernatant of *E. rectale* DSM 17629. The caspase concentration in HCT-116 treated cells increased by >250%.

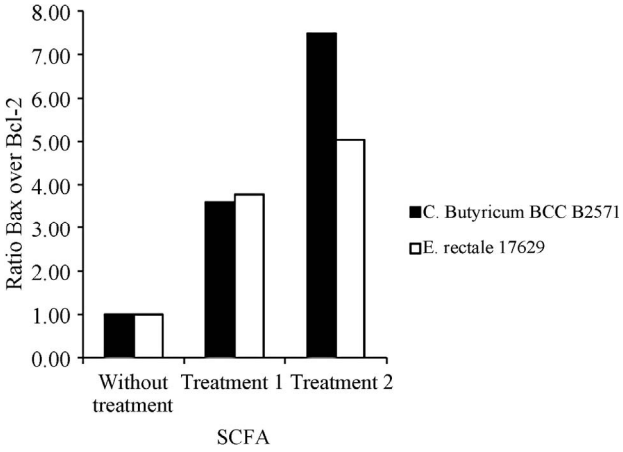

Figure 5. Ratio of bax/Bcl-2 mRNA in HCT-116 treated with SCFA in cell free supernatant of *C. butyricum* BCC B2571 and *E. rectale* DSM 17629.

4. DISCUSSION

Currently, it has been well documented that lipid has functions not only as energy stores but also as signaling molecules. They play important role in various cellular processes. As a special group of lipid, SCFA have been investigated and reviewed intensively [15-20]. SCFA are organic acid produced by anaerobic fermentation of dietary fiber (including RS3) in gastrointestinal tract of mammalian species and then directly absorbed at the site of production. The major fatty acid in large intestine are acetate, propionate and butyrate.

As expected, RS3 showed powerful butyrate producing substrate. Quantification of SCFA in bacterial supernatant is presented in **Table 2**. Generally, *C. butyricum* BCC B2571 produced more acetate, propionate and butyrate in comparison with *E. rectale* DSM 17629. The proportion of propionate in the bacterial supernatant of *C. butyricum* BCC B2571 was slightly lower than that in bacterial supernatant of *E. rectale* DSM 17629. *C. butyricum* BCC B2571 produced 218 mM of acetate, 192 mM of propionate and 170 mM of butyrate or molar ratio around 1.3:1.1:1. *E. rectale* DSM 17629 produced 117

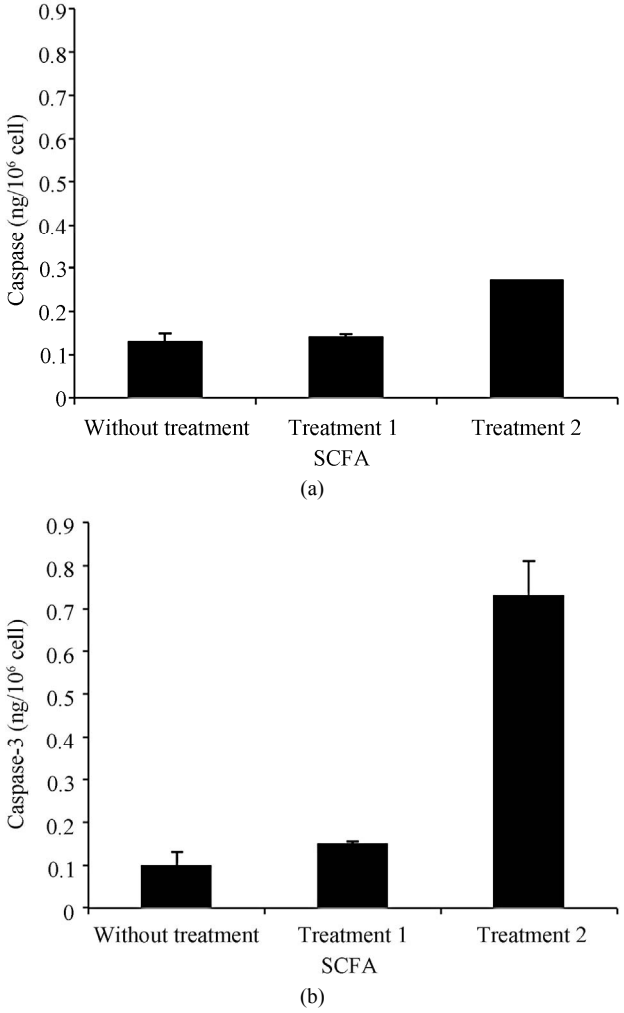

Figure 6. Effect of SCFA in bacterial supernatant on caspase-3 production in HCT-116. (a) The cell of HCT-116 was untreated or treated with SCFA in cell free supernatant of by *C. butyricum* BCC B2571; (b) The cell of HCT-116 was untreated or treated with SCFA in cell free supernatant of *E. rectale* DSM 17629. Treatment 1 & Treatment 2 was described as in previous **Figure 1**.

mM of acetate, 134 mM of propionate and 115 mM of butyrate or molar ration around 1:1.2:1. It was better than our study earlier [11]. This result was comparable with those reported by Guo-Qing *et al*. [20] and was higher than those reported by Reid *et al*. [22] or Lesmes *et al*. [23].

When the cell free supernatant was exposed to either non cancerous or cancerous cell such as VERO, HCT-116 etc., the SCFA including butyrate would enter into the cell through transport system. Several studies have been focused on SCFA transport system [24,25]. SCFA are transported into colonic epithelial cells by two different mechanisms, *i.e*. passive non ionic diffusion (unspecific component) of the undissociated SCFA form and specific carrier-mediated transport of SCFA anion. Although butyrate should be ionized at the physiological pH of the colonic lumen, the non ionic form can exist due to luminal acidic microclimate generated in the apical region of colonocytes by H+-secreting system, as the Na^+/H^+ exchanger. Carrier mediated transport of the ionized form of butyrate has been described as: 1) A non-electrogenic $SCFA^-/HCO_3^-$ antiporter; 2) An electroneutral H^+-coupled MCT (monocarboxylate transporter) and 3) An electrogenic Na^+-coupled transporter for MCT. Different cellular process might exist between cancerous and non cancerous cell. For non cancerous cell, SCFA espicially butyrate was metabolized as energy source. As for cancerous cell, transported butyrate inside the cell affected/changed the cellular process. As the result showed, the supernatant containing SCFA less than 10 mM was found toxic for HCT-116 colon cancer cell.

The result of our study was in agreement with the study conducted by other researchers. Reummele *et al*. [18] found that butyrate inhibited Caco-2 colon cancer cell proliferation. The inhibition effect was observed at concentration as low as 0.1 mM. Maximal inhibition (approximately 30% - 50%) by butyrate was observed at of 10 mM. Hatayama *et al*. [26] reported that butyrate treatment inhibited proliferation of LS174T colon cancer cells in a dose dependent manner. Butyrate concentration of either 1 or 2 mM markedly reduced cell number in the culture in comparison with the non butyrate treatment.

Apoptosis is a defined form of cell death, which plays an important role in the development of multi cellular organism and in the regulation and maintenance of the population in tissues under physiological and pathological conditions. Apoptosis has quickly surfaced as a potential target for cancer prevention/treatment at various stages of carcinogenesis. Therefore, induction of apoptosis by fermentation product of RS3 such as SCFA in bacterial supernatant provide a mean of protection against carcinogenesis by removing genetically damaged cells before they change to pre-cancerous lesions.

Our observation was in agreement with the study conducted by other researchers. Avivi-Green *et al*. [27] found that incubation of colon cancer cell line of $CaCO_2$ or RSB with butyrate at varied concentration levels of 2 - 10 mM induced apoptosis. Studies conducted by using animal model showed similar phenomenon. Le-Leu *et al*. [28] reported that SCFA and butyrate level in the rats feces correlated positively with the acute apoptotic response in the distal colonic crypt. It was also reported that feeding RS significantly increased SCFA level in the rat caecum and colon, thus reducing intestinal neoplasm and colorectal adenocarcinomas. Apoptosis was also observed in HCT-116 colon cancer cell treated with biological compound such as 3,3'-diindolylmethane (DIM) [29].

There are two different apoptosis pathways. Firstly,

apoptosis is activated by binding of TNF (Tumor Necrosis Factor) or Fatty acid synthetase ligand (FasL) to the cell surface receptors. It is known as extrinsic or death receptor pathway. Secondly, intrinsic stress responses induced several factors (for example limitation of growth factor or DNA damage), which leads to release of cytochrome c from mitocondria. It is known as mitochodrial pathway. This mitochodrial pathway of apoptosis is regulated by genes expression. One of these genes is a member of Bcl-2 (B-cell lymphoma-2) family, which consists of two groups namely anti-apoptotic genes and pro-apoptotic genes. Bcl-2 is an anti-apoptotic gene which protects the cell death. Bax (Bcl-2 associated X protein) is the second members of this group which is a pro-apoptotic gene. It promotes cell death and it is expressed abundantly and selectively during apoptosis [10].

The ratio of pro- to anti-apoptotic of Bcl-2 proteins is an important regulator of a cell's susceptibility to undergo apoptosis upon a specific apoptotic stimulus [10]. Bax protein resides in the cytosol. Bax, together with other pro-apoptotic family member, promotes apoptosis by formation of homodimeric and heterodimeric complexes. Furthermer, these complexes form channels or pores in the mitochondrial membrane to facilitate the release of cytochrome c and apoptosis inducing factor (AIFs) from mitocondrial intermembrane spaces into the cytosol. Consequently, it cause in the loss of the selective ion permeability across the mitochondrial membrane. The intracisternal contents that are released into the cytosol triggers a cascade of caspase reaction and finally execute apoptosis [10].

The Bcl-2 gene encodes a 26 kDa protein which is localised in the outer mitochodrial membrane. It stabilizes the integrity of the mitochondrial membrane, thus mediates its anti-apoptotic effect. The stable mitochondrial membrane leads to the inhibition of the permeability transtition pore and the release of cytrochrome c, and this is achieved by forming the heterodimers with pro-apoptotic members such as Bax and Bak, thus neutralising their activity. Thus, the sensitivity of cells to apoptotic stimuli will depend upon the relative ratios of Bax/Bax homodimers, Bax/Bcl-2 heterodimers and Bcl-2/Bcl-2 homodimers.

The pattern of caspase-3 seems to relate with the proportion of acetate, propionate and butyrate in the cell free supernatant. It was found that proportion of propionate in cell free supernatant of *E. recatale* 17629 was slightly higher than that in bacterial supernatant of *C. butyricum* BCC B2571. According to the study reported by Jan *et al*. [30], pure propionate as well as propionate in the Propionibacterium extra cellular fluid supernatant induced the cleavage of procaspase-3 resulting in the active form of caspase-3.

The caspases are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Caspase-3 in particular is believed to be most commonly involved in the execution of apoptosis in various cell types. The caspase play an important role in apoptosis by activating DNAse, inhibiting DNA repair enzymes and breaking down structural protein in the nucleus [10]. It was reported that the crucial step in apoptosis of colorectal cancer cell induced by SCFA or bioactive compund such as oligomeric proanthocyanidin, grape seed procyanidine is the activation of caspase-3 [27,29,31].

Taken together, the data showed that SCFA in cell free supernatant of *C. butyricum* BCC B2571 and *E. rectale* DSM 17629 not only inhibited proliferation but also induced apoptosis in colon cancer cell HCT-116. It provided evidence that the apoptosis occured through mitocondrial pathway in diversely mode of action. SCFA in cell free supernatant of *C. butyricum* BCC B2571 induced expression of the Bax gen, while those of *E. rectale* DSM 17629 repressed expression of the Bcl-2 gene. Finally, it increased the ratio of Bax gene over Bcl-2 gene and lead to caspase-3 production. The SCFA proportion in cell free supernatant seems to have contribution in increasing of the caspase-3 produced by colon cancer cell HCT-116 to intensify apoptosis.

Currently, there is an increasing interest in the use of foods that are capable of modulating the composition of human colonic microflora in a way that is beneficial to health. Our study indicated that beneficial effect may be obtained espicially when RS3 is consumed because of SCFA production in the colon. *In vitro* study confirm that the SCFA is capable of altering the expression of apoptosis related gene in colorectal cancer cell. This study supportutilization of sago starch into high valuable product of RS3 which can be applied as functional food ingredient.

5. CONCLUSIONS

SCFA in bacterial supernatant either from *C. butyricum* BCC B2571 or *E. rectale* DSM 17629 inhibited proliferation of human colorectal cancer cell line HCT-116. The inhibition was lower when HCT-116 was treated with SCFA in bacterial supernatant of *C. butyricum* BCC B 2571 compare with that treated with SCFA in bacterial supernatant of *E. rectale* DSM 17629.

The SCFA in bacterial supernatant induced apoptosis and change the expression of apoptosis related genes such as Bcl-2 and Bax. The Bcl-2 and Bax mRNA were expressed in different pattern by HCT-116 upon treatment with the bacterial supernatant consisted of SCFA. When HCT-116 was treated with SCFA from *C. bu-*

tyricum BCC B 2571, the expression of Bcl-2 mRNA was not changed significantly and the expression of Bax mRNA was increased by 7 - 9 fold of the untreated cell. When HCT-116 was treated with SCFA from of *E. rectale* DSM 17629, the level of Bcl-2 mRNA was significantly decreased by 20% - 30% of the untreated cell without significant alteration in the level of Bax mRNA. These data revealed that treatment of bacterial supernatant consisted of SCFA increased the expression ratio of pro-apoptosis gene (Bax) over anti-apoptosis gen2 (Bcl-2). The synthesis of caspase-3 was also increased (70%) after HCT-116 was treated with bacterial supernatant consisted of SCFA. Finally, it was concluded that fermentation product of RS3 either by *C. butyricum* BCC B 2571 or *E. rectale* DSM 17629 induced apoptosis through mitochondrial pathway in human colorectal cancer cell line HCT-116.

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ABBREVIATIONS

Bcl-2: B-lymphoma Cell 2, Bax: B-associated X protein, CRC: Colorectal Cancer, DMEM: Dulbescco's Modified Eagle's Media, DNA: Deoxyribonucleic Acid, ELISA: Enzyme Linkage Immuno Assay, FaSL: Fatty Acid Synthase Ligand, FBS: Fetal Bovine Serum, MCT: Monocarboxylate Transporter, mRNA: massenger Ribonucleic Acid, GAPDH: Gyceraldehy-3-Phosphate Dehydrogenase, RS1: Resistant starch type 1, RS2: Resistant starch type 2, RS3: Resistant starch type 3, RS4: Resistant starch type 4, RSSA: Resistant starch type 3 derived from sago, treated with amylase, RSSP: Resistant starch type 3 derived from sago, treated with pullulanase, RT-PCR: Real Time Polymerase Chain Reaction, SCFA: Short Chain Fatty Acid.

