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Effects of Postbiotics from Food Probiotic and Protective Cultures on Proliferation and Apoptosis in HCT-116 Colorectal Cancer Cells

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Abstract

Background and Objective: Postbiotics are microbial-derived soluble products, which are released during the growth and fermentation process of beneficial microorganisms in gastrointestinal tract, food and complex microbiological culture systems (cell-free supernatant or extracellular extract) or after cell lysis (intracellular extract). Colorectal cancer is one of the most common cancers within the leading causes of cancer mortality worldwide, which can be associated with a defeated gastrointestinal barrier. In this study, potential functionality of the extracellular and intracellular extracts of probiotics (*Latilactobacillus sakei*, LS) and protective culture (FreshQ®, FQ) on proliferation and cell survival of HCT-116 colon cancer epithelial cells was investigated.

Material and Methods: Probiotic bacteria were cultivated in de Man, Rogosa and Sharpe broth and then postbiotics was isolated by centrifugation and sonication. The achieved solutions were lyophilized and stored until use. Moreover, HCT-116 cells were exposed to various concentrations of *Latilactobacillus sakei* and FreshQ® extracts (1.25-40 mg ml^{-1}) for 24 h and then effects of these products on cell cytotoxicity, proliferation and apoptosis were investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, wound healing and AO/EB assays.

Results and Conclusion: Extracellular and intracellular extracts of *Latilactobacillus sakei* and FreshQ the decreased in cell viability based on the postbiotic concentrations ($p \leq 0.05$), while cell proliferation was inhibited by extracellular and intracellular extracts of *Latilactobacillus sakei* and FreshQ® in wound healing assay. Results showed that postbiotics could induce apoptosis evidenced by acridine orange/ethidium bromide staining. In summary, *Latilactobacillus sakei* and FreshQ® postbiotics are able to decrease cell viability and proliferation and enhance apoptosis in HCT-116 colorectal cancer cells. In addition, FreshQ® postbiotics seemed more potent than that *Latilactobacillus sakei* postbiotics did.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Probiotics are defined as live and nonpathogenic microorganisms that provide health benefits to the host when given in appropriate quantities [1]. Lactic acid bacteria (LAB) and *Lactobacillus* species are well-known probiotic strains, including diverse therapeutic effects [2]. Health benefits and anticancer characteristics of probiotics have been linked to changes in the metabolism of gut microbiota, affecting physicochemical conditions of the intestine, elimination or affection of carcinogens, induction of apoptosis and production of secondary metabolites such as short-chain fatty acids that represent antimutagenic and immunomodulatory effects on the hosts [3,4]. Probiotics

seem to be one of the most important approaches in gastrointestinal disorders and biotherapy [5]. However, this approach may include limitations and disadvantages such as needs to provide appropriate temperature and pH for the optimum bacterial growth, limited shelf-life and stability, presence of antibiotic resistance factors and most importantly, ineffectiveness and hazardous in vulnerable hosts such as patients with immunodeficiency disorder [6]. Beneficial effects of probiotics on the hosts can be achieved in two ways; the first pathway depends on the living form of probiotics and its metabolic activity and the second pathway depends on the non-viable and biological compounds derived from the cells known as postbiotics [7,8]. It is described that the survival factor of probiotics may be unnecessarily linked to the probiotic beneficial effects [8,9], Therefore, probiotic-derived biological compounds (e.g., cell wall components, extracellular and intracellular biomolecules) can represent beneficial effects through similar or unsimilar metabolic pathways [8]. Despite lack of a clear and accepted definition for postbiotics, previously published reports have provided an explanation for the postbiotics as nonbiotics or all by-products derived from probiotic microorganisms that can be the source of beneficial effects for the hosts if used in adequate concentrations [10]. Constituents of the postbiotics include various intracellular and extracellular compounds (e.g., functional compounds such as enzymes, fatty acids, organic acids and bacteriocins), which are released by microbial cells during their growth in the gastrointestinal tract, microbial cultures and foods or released after cell lysis [11,12]. Released byproducts can include beneficial bioactivities such as anti-inflammatory, antiproliferative and antioxidant activities in gastrointestinal tract; however, mechanisms of these activities are not well understood [13].

Numerous advantages and distinctive biotechnological, clinical and economical characteristics are described for postbiotics in comparison to their parent probiotics; for example, longer shelf life, inability to transfer antibiotic resistance factors, inability to generate biogenic amines, described structure, relative safety, uncomplicated use and storage conditions and wide-range pH and temperature compatibilities as well as broad-range antibacterial/antifungal activities [14,15]. These illustrate benefits of using postbiotics over the live bacterial cells that produce them. Various methods have been used for the bacterial cell lysis to inactivate bacterial cell extraction of their postbiotics, including heat treatment, gamma and ultraviolet irradiations, high hydrostatic pressure, sonication and acid/enzymatic treatment [13,16]. Cancer is described as a disease characterrized by uncontrolled growth of cells and occurrence of this disease increases worldwide [17,18]. Colorectal cancer (CRC) ranks third within all forms of cancers. Regarding its prevalence, CRC is one of the leading causes of cancer-linked death worldwide [19]. The CRC treatment strategies include radiation therapy, immunotherapy, surgery and chemotherapy. However, treatment-associated challenges such as systemic toxicity, resistance to chemotherapy and long-term consequences of anticancer therapy are inevitable problems against successful cancer chemotherapy protocols [20-22]. Therefore, novel strategies and supportive treatments have been more popular in recent decades. Novel venues in CRC treatment should be oriented to use of bioactive compounds such as prebiotics and postbiotics, targeting affection of gut microbiota compo-sition and immune responses and introducing adjuvants in treatment and prevention of patients with CRC. This can improve efficiency of the treatments and decrease side effects of the available therapies [23,24].

Numerous beneficial bacteria (e.g., probiotic and protective cultures) enter the human body daily through a variety of foods. Although these microorganisms are sometimes added to foods for technological purposes, their therapeutic and nutritional effects have been interested by producers and consumers for many years. It is noteworthy that *Latilactobacillus* (*L.*) *sakei* and FreshQ® (FQ) cultures are commonly used probiotic and protective cultures that are detected in meats and yogurts and cheeses, respectively [14,25]. In a previous study, anticancer effects of viable and heat-killed forms of *L. rhamnosus* GG and *L. paracasei* IMPC2.1 in FQ were reported in colon cancer cells by decreasing cell proliferation and inducing apoptosis [26]. Although use of postbiotics has received great scientific attention in the last five years, concerns are still reported on postbiotics toxicity and their nutritional characteristics in the body. Up to date, effects of various postbiotics from *Lactobacillus* species on various cancer cell lines have been investigated. Several authors reported effects of postbiotics from beneficial microorganisms on cancer cell lines (Table 1) [4,23,27-30]. However, no comprehensive studies on use of postbiotics from protective and probiotic bacterial strains are available. Furthermore, no comparative studies on effects of a combination of lyophilized extracellular and intracellular extract (ICE) of protective and probiotic cultures on cancer cell lines have been carried out. Therefore, the aim of the current study was to compare possible effects of extracellular extract (ECE or cell-free supernatant, CFS) and ICE of LS and FQ on epithelial colon cancer cell line (HCT-116).

2. Materials and Methods

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2.1. Bacterial strains and postbiotics preparation

In this study, *L. sakei* subsp. *sakei* ATCC 15521 and FreshQ® (containing *L. rhamnosus* and *L. paracasei*) cultures were provided by the Iranian Research Organization for Science and Technology, Tehran, and Chr.

Table 1. Postbiotics derived from various lactobacillus bacteria species and their effects on cancer cells

Name of Probiotic Strain	Derived postbiotic	Type of cancer cell line	Results	Reference
Lactiplantibacillus plantarum IUL4, TL1, RS5, RI11, RG11 and RG14	Cell-free supernatant	MCF-7, HT-29 , HeLa, Hep G2,HL60 and K562	↑ Apoptosis ↓ Cell proliferation (strain-specific and cancer cell type-specific manner)	[4]
Lacticaseibacillus rhamnosus MD 14	Cell-free supernatant	Caco-2 and HT-29	Antigenotoxicity ↑ Cytotoxicity against cancer cells and inhibition of cell cycle	[27]
Lactobacillus acidophilus LA102 and *Lacticaseibacillus casei* LC232	Cell extracts	Caco-2 and HRT-18 normal vero cells	↓Cell viability of cancer cells without cytotoxic effect on normal vero cells	[28]
Lactobacillus acidophilus and *Lactobacillus delbrueckii*	Cytoplasmic extract (ICE)	HT-29	↓ Cell proliferation Induction of apoptosis	[29]
Lacticaseibacillus paracasei	cell wall protein fractions	CaCo-2	Significant decreases of cell viability, concentration- and time-dependent anti-proliferative effect Induction of apoptosis	[30]

Hansen, Horsholm, Denmark, respectively. Cultures were propagated in De Man-Rogosa-Sharpe (MRS) broth at 37 °C for 48 h using CO_2 incubator (Sina Lab, Tehran, Iran). Bacterial suspension was subsequently centrifuged at 4200× g for 10 min using centrifuge (Farzaneh Arman, Isfahan, Iran). Then, supernatant (CFS1) was collected using sterile containers and cell pellets were quickly washed twice using PBS solution. Bacterial cells were resuspended in PBS to achieve ~10^9 CFU ml^{-1} followed by ultrasonication in water bath at 40-kHz for 20 min ±1 at 4 °C with 1-min intervals [31]. Cell debris was removed by centrifugation and supernatant (CFS2) was collected using sterile containers. The CFS1 and CFS2 were filtered (0.42-µm syringe filters; Millipore, MA, USA) and freeze-dried (Zist Farayand Tajhiz Sahand, Tabriz, Iran) as ECE and ICE, respectively, and used freshly.

2.2. Cell Culture

The HCT-116 cells as colon epithelial cells were seeded in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, USA) and stored in CO_2 atmosphere. Supplements in the media included 10% (v v^{-1}) fetal bovine serum (FBS) and 1% (v v^{-1}) penicillin-streptomycin (100 IU ml^{-1} and 100 µg ml^{-1}). Cells were stored in incubator until 80% confluency for the subculture was achieved using standard protocols [32]. In cytotoxicity assay, 96-well plates were used while wound healing assay and staining were carried out in 24-well plates.

2.3. Cell viability assay (MTT)

Cytotoxicity of ECE and ICE from *L. sakei* and FQ on HCT-116 cells was assessed using MTT assay, which is described as the capacity of cellular mitochondrial dehydrogenase enzymes in living cells to reduce the yellow water-soluble substrate MTT into a dark blue/purple formazan product that is insoluble in water [33]. All the chemicals used in the experiment were purchased from Sigma-Aldrich, USA. The assay was carried out with 1 × 10^4 cells in each well of 96-well plates, which were seeded for 48 h before the experiment. Cells were exposed to ECE and ICE at 1.25, 2.5, 5, 10, 20 and 40 mg ml^{-1} concentrations for 24 h. Moreover, 20 µl of MTT (3 mg ml^{-1} in PBS) were added to each well and plates were incubated for 4 h in CO_2. After incubation, MTT-containing media were discarded and 100 µl of dimethyl sulfoxide (DMSO) were added to the wells for dissolving the blue/purple formazan crystals. After 5 min of agitation at room temperature, absorbance was measured using standard protocols [33] and microplate reader (Chromate 4300 Microplate Reade; Awareness Technology, Florida, USA). Cell viability proportion was calculated using Eq. 1 [33]:

$$\text{Cell viability (\%)} = \frac{Mean\ OD\ (sample)}{Mean\ OD\ (blank)} \times 100 \qquad \text{Eq. 1}$$

2.4. Wound healing or scratch assay

The assay was carried out as an indication for quantitative and qualitative evaluation of cell migration and proliferation rate. HCT-116 cells were seeded into 24-well plates and exposed to a target medium for 24 and 48 h. The cell scratch was carried out by yellow sampler tips (200-ul pipette tips) at zero time point for creating a standard wound area in each well. The control group received a normal DMEM and the experimental groups were exposed to increasing concentrations of ECE and ICE as mentioned above. The images were achieved at final time points and the average closure area from various scratched cell borders was measured utilizing ImageJ software (ImageJ 1.52v, NIH, USA) and calculated by Eq. 2 [34]. The phase-contrast microscopy (CKX53 inverted microscope; magnification ×10, Olympus, Japan) has been used for cell migration.

$$\text{Relative area (\%) of the scratch} = \frac{Area\ of\ scratch\ at\ T\ h}{Area\ of\ scratch\ at\ 0\ h} \times 100 \qquad \text{Eq. 2}$$

2.5. Acridine orange/ethidium bromide (AO/EB) staining

Staining with AO/EB was used for the apoptosis assessment of HCT-116 cells. Briefly, 24-well plates were used for cell seeding based on standard protocols [35]. After optimal proliferation and confluency, cells were treated with ECE and ICE at 1.25-20 mg ml^{-1} for 24 h while the control cells received normal media for 24 h. Staining was carried out at the final time point using AO/EB solution (10 µl) containing 100 µg ml^{-1} AO and 100 µg ml^{-1} EB (AO/EB, Sigma, St. Louis, MO, USA) after PBS wash. To assess apoptosis rate of the cells in various treatments, cell images were captured using fluorescence microscope (ECLIPSE Ti2 fluorescence microscope, Nikon, Japan). Acridine orange includes the potential to stain live and dead cells while ethidium bromide can target cells with defeated membrane integrity. Live cells could be detected in uniform green color while apoptotic cells could be stained in orange by ethidium bromide. All experiments were carried out in triplicate and results were analyzed using ImageJ software.

2.6. Statistical analysis

Results were reported as means ±SD (standard deviation) experiments (n=3). Statistical analyses were carried out using GraphPad Prism9 software (GraphPad, La Jolla, CA, USA). One-way analysis of variance with Bonferroni post-hoc test was used as well (p<0.05).

3. Results and Discussion

3. Results and Discussion

3.1. Cell Viability

The MTT assay results showed that the ECE of the bacteria at 5, 10, 20 and 40 mg ml^{-1} significantly decreased viability of the HCT-116 cell line, compared to the control group (Figures 1a,b). Moreover, ICE showed no effects on the colon epithelial cells at the concentration range of 1.25-40 mg ml^{-1} (Figures 1c,d).

In the current study, effects of ECE and ICE from *L. sakei* and FQ (as a protective culture with *L. rhamnosus* and *L. paracasei*) were assessed on the HCT-116 colon epithelial cancer cell line for times and concentrations. The current findings indicated that ECE of the two cultures decreased mitochondrial activity and survival of HCT-116 cells at concentrations greater than 5 mg ml^{-1} after 24 h of incubation as a function of concentration (Figures 1A,B).

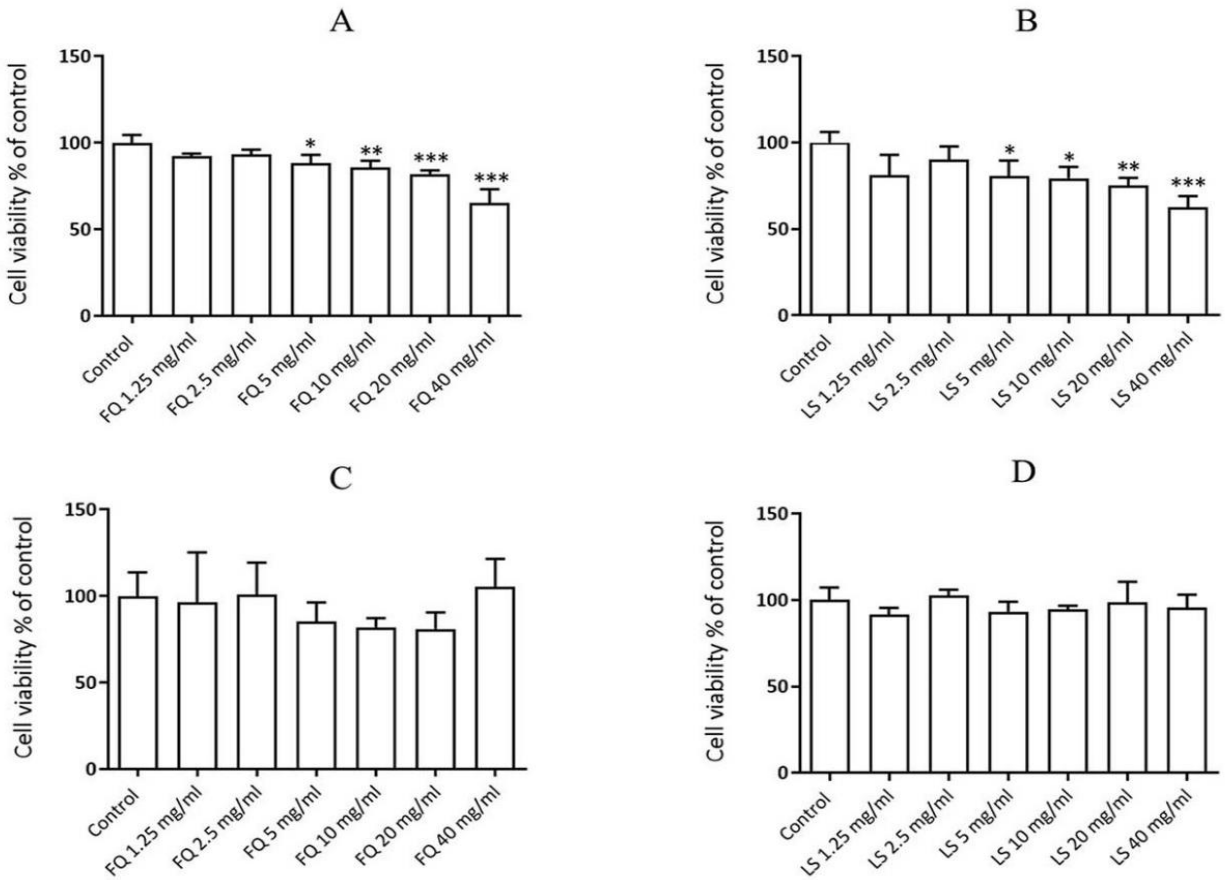

Figure 1. Proportion of HCT-116 cells viability exposed to various concentrations of *L. sakei* (LS) and FreshQ® (FQ), extracellular extract (A,B) and intracellular extract (C,D) based on MTT assay. $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.05$* are considered significant compared to the control

These findings were similar to those from previous studies, where heat-killed and ECE from *Lactiplantibacillus plantarum* A7 and *L. rhamnosus* GG at 2.5,5 and 10 mg ml^{-1} demonstrated antiproliferative effects on HT-29 and Caco-2 cancerous cell lines of the colon [36]. In another study, cytotoxic effects of cell-free supernatant (CFS) and heat-killed (HK) cells derived from three strains of *L. casei* on Caco-2 cell lines (significant decreases in cell viability at 50% (v v^{-1}) concentration of CFS and heat-killed -cells) have been reported using MTT assay [37].

Considering previous investigations, postbiotics achieved from *Lactobacillus* spp., including ECE, sonicated-cell suspension and cell extracts, have shown specific anti proliferative effects on Caco-2, HT-29 as CRC models and other types of cancers. This might be linked to proapoptotic (e.g., BAX and caspase-3) signaling pathways activation, which has resulted in cell death or immune-modulatory and anti-inflammatory effects by upregulating certain anti-inflammatory agents (TGF-β2 and IL-10) and decreasing in pro-inflammatory cytokines (TNF-a, TNF-β and IL-6) [27,38]. Previous investigations demonstrated that postbiotics of *Lactobacillus* strains could be identified as antiproliferative substances when exposed to cancerous cells and this effect is believed to be associated to the production of organic acids. Differences between the antiproliferative effects in various species of these bacteria might be linked to differences in concentrations of these organic acids in various species [39]. In recent years, postbiotics have attracted significant interests due to their effects on the modulation of microbiome composition, as well as modulatory effects on the immune system and antiproliferative effects and as a promising strategy for the promotion of CRC treatment efficacy [8]. Awaisheh et al. reported significant cytotoxic effects of the cell extracts of two *Lactobacillus* strains against two colorectal cancer cell lines (Caco-2 and HRT-18) using MTT and trypan blue assays. They reported that cell-free supernatants from *L. casei* LC232 and *L. acidophilus* LA102 decreased the cell viability up to 48 and 47.5% of *L. casei* and 37 and 68.5% of *L. acidophilus* against HRT-118 and Caco-2 cell lines, respectively [28].

Moreover, a study by Kim et al. on *in vitro* and *in vivo* human colorectal carcinoma RKO cell xenograft models using MTT and flow cytometry cytotoxicity methods showed that a combination of heat-killed *Limosilactobacillus reuteri* MG5346 and *L. casei* MG4584 decreased cell proliferation in these cells and inhibited tumor growth in xenograft models through increasing expression of cleaved caspases 3, 7 and 9 and PARP in tumor tissues [40]. Finding of a study by Nozari et al. revealed that treatment of human CRC cells (Caco-2 cell line) with cell wall proteins from *L. paracasei* decreased significantly their proliferation in concentration and time-dependent manners using MTT and annexin V-FITC/PI staining cell viability assays [30] .

3.2. Cell migration rate based on wound healing assay

Effects of ECE and ICE of *L. sakei* and FQ on the migration rate of HCT-116 cells were assessed. Effects of these postbiotics at 1.25-20 mg ml^{-1} for ECEs and 1.25-40 mg ml^{-1} for ICEs at 0, 24 and 48 h were assessed. Results showed that by increases in postbiotic concentrations, rates of cell migration and scratched area closures significantly decreased, as shown by decreases in the distance at the edges compared to the beginning of the experiment between various treatments and the control group (Figures 2a, b, c and 3a, b, c). Results of other studies showed that the antiproliferative effects of postbiotics derived from *Lactobacillus* spp. in ECE and heat-killed cell forms on Caco-2, HT-29 and normal (L-929) cell lines could include strain and concentration-dependent functions [36]. The antiproliferative effects of ECE of *L. rhamnosus* MD have been reported in HT-29 and Caco-2 cells [27]. Results demonstrated that 0.005-5 μg ml^{-1} concentrations included minimal inhibitory effects (18.5%) on cell proliferation at 8 and 24 h, while increased concentrations up to 500 μg ml^{-1} demonstrated inhibitory effects on Caco-2 cell proliferation up to 50% [41]. It is known that Wnt/β-catenin signaling pathway plays essential roles in pathogenesis and development of CRC and compounds with the potency of inhibiting or modulating the pathway could play important roles in CRC treatment [42].

A study by Chen et al. has shown that *Clostridium butyricum* derived short-chain fatty acids are able to inhibit Wnt/β-catenin pathway in HCT-116, Caco-2 and HCT-8 CRC cell models, which is an indication of potency in the decrease of cell proliferation and cell migration [43]. Furthermore, results from the scratch test (wound healing) in the current study showed that ECE and ICE from of *L. sakei* and FQ Included the potency to decrease cell proliferation and migration of HCT-116 cell time and concentration-dependently, compared to the control cells (Figures 2a, b, c and 3a, b, c). Anti-proliferative effects of ICE from *L. casei* ATCC 393 on HT-29 colorectal adenocarcinoma cells have been demonstrated in previous studies [44]. In the present study, wound healing assay indicated that the rate of migration and a similar proliferation rate decreased after 24 and 48 h of incubation with ECE and ICE, compared to the control treatment (Figures 2a, b, c and 3a, b, c).

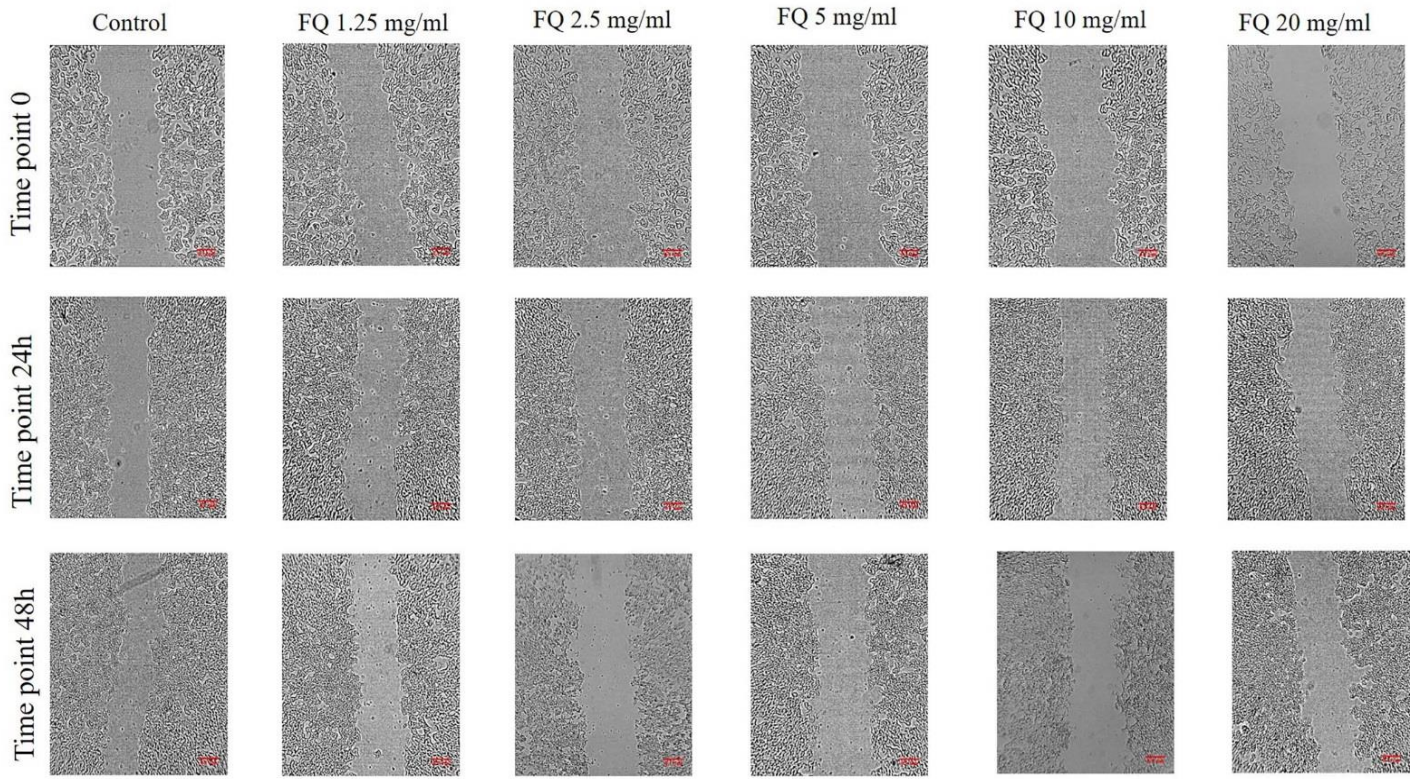

Figure 2a. Wound healing assay in HCT-116 cells treated by FreshQ® (FQ) ECE with various concentrations at 24 and 48 h and its comparison with zero time and distance changes in the control group

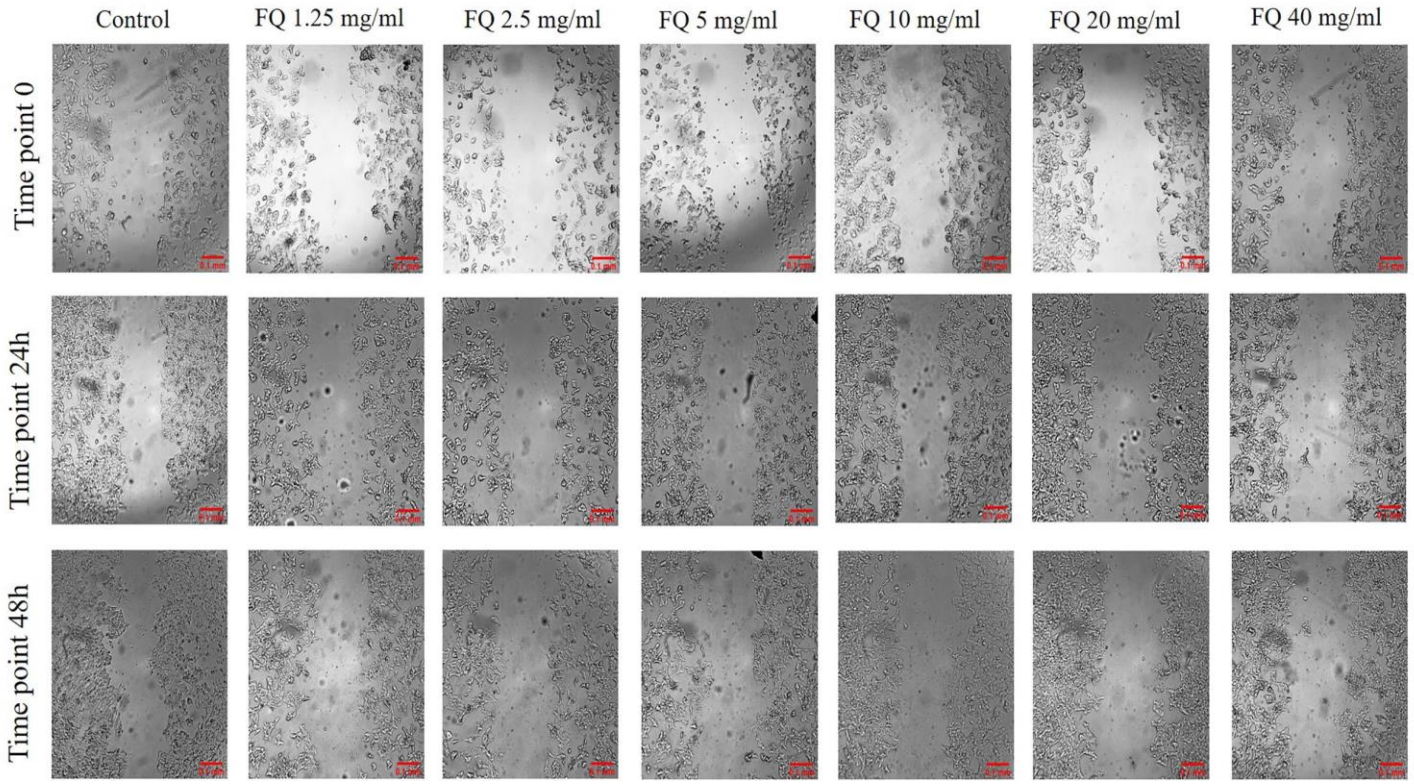

Figure 2b. Wound healing assay in HCT-116 cells treated by FreshQ® (FQ) ICE with various concentrations at 24 and 48 h and its comparison with zero time and distance changes in the control group

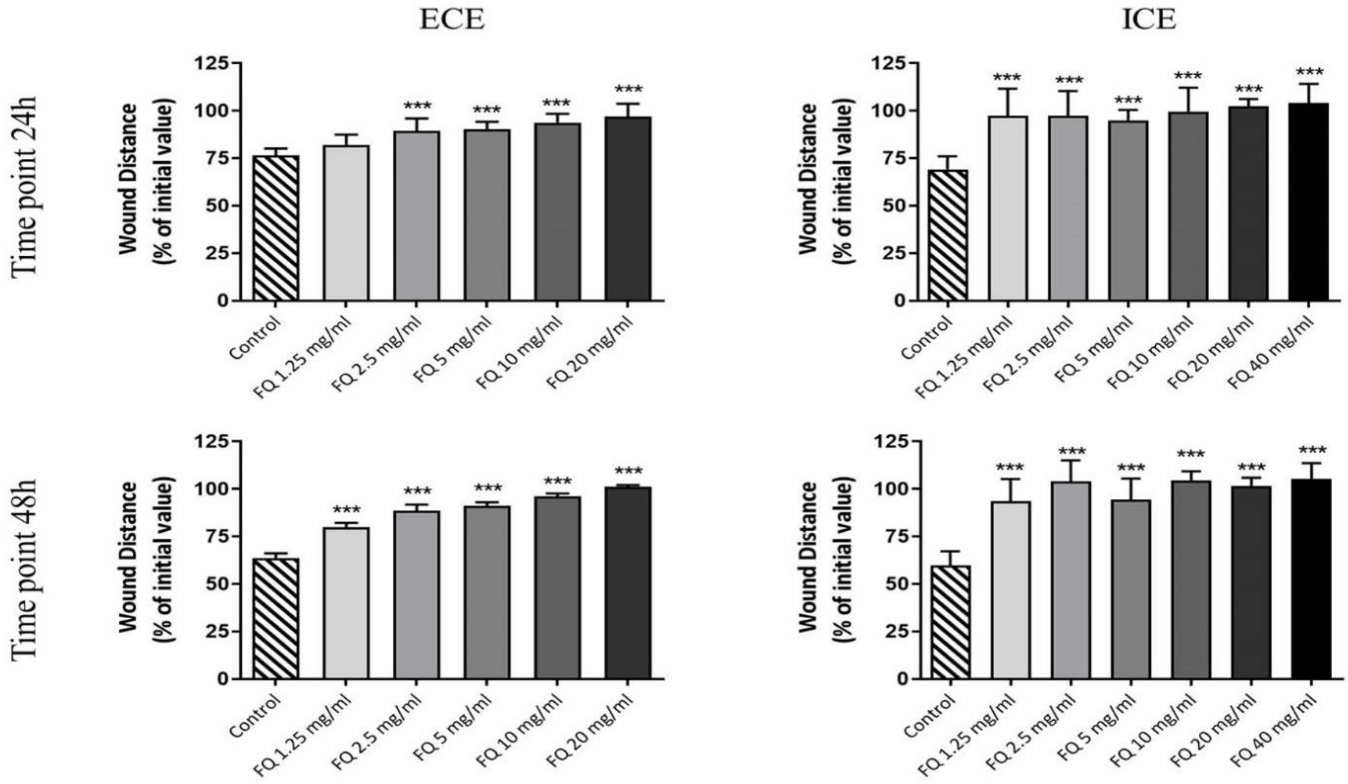

Figure 2c. FreshQ® (FQ) ICE and ECE attenuated the epithelial wound healing process. The 24-well plates were used for scratch and the cells were exposed to postbiotics for 24 and 48 h after scratching. Images were captured directly after scratching of 0, 24 and 48 h. Widths of wound areas were calculated in comparison to the time zero area in percentage as mean ±SD of triplicate experiment conditions. $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.05$* were considered significant compared to the control

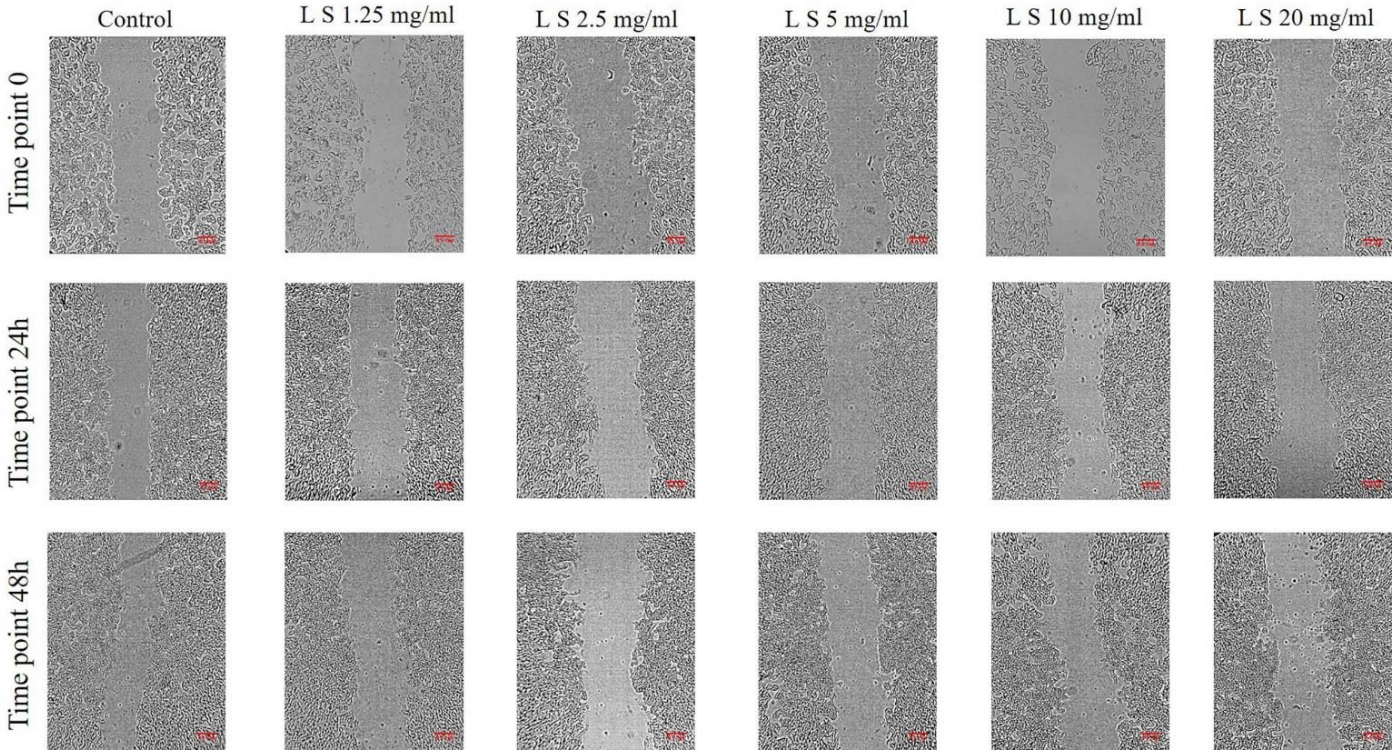

Figure 3a. Wound healing assay in HCT-116 cells treated by *Lactobacillus sakei* (LS) ECE with various concentrations at 24 and 48 h and its comparison with zero time and distance changes in the control group

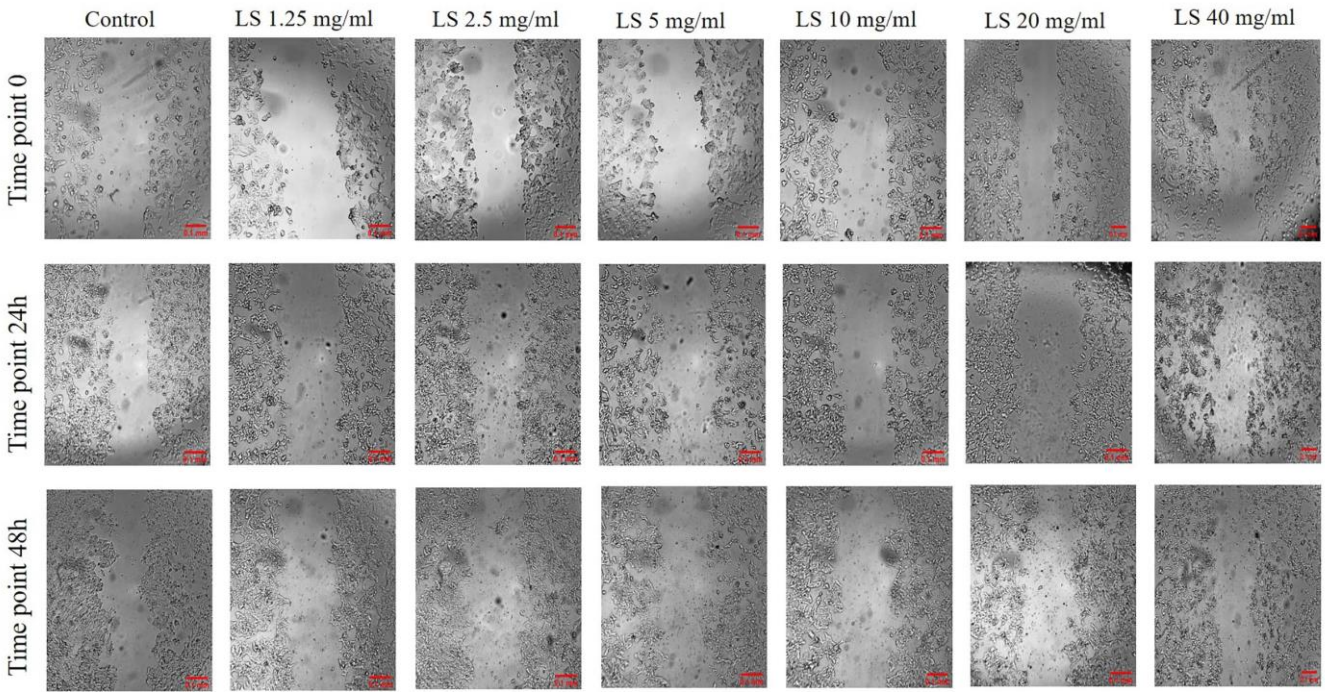

Figure 3b. Wound healing assay in HCT-116 cells treated by *Lactobacillus sakei* (LS) ICE with various concentrations at 24 and 48 h and its comparison with zero time and distance changes in the control group

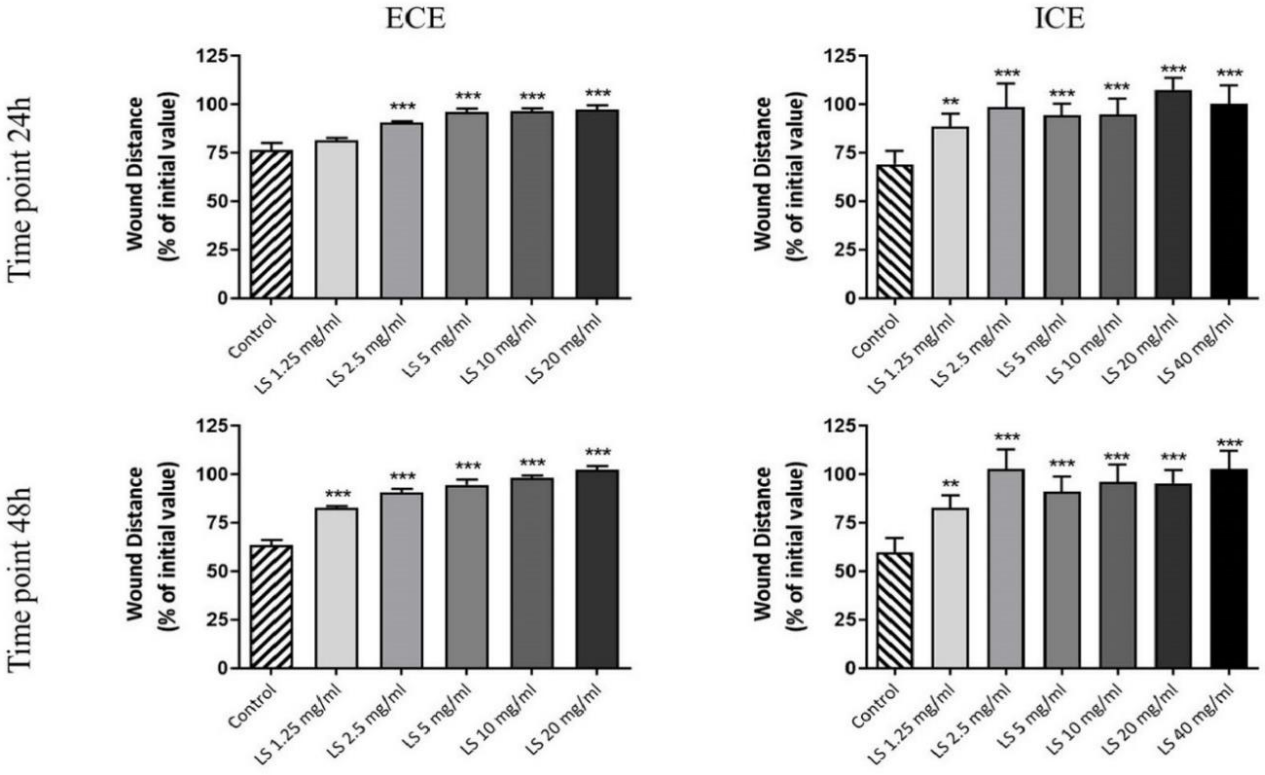

Figure 3c. The *Lactobacillus sakei* (LS) ICE and ECE attenuated the epithelial wound healing process. The 24-well plates were used for scratch and the cells were exposed to postbiotics for 24 and 48 h after scratching. Images were captured directly after scratching of 0, 24 and 48 h. The widths of wound areas were calculated in comparison to the time zero area in percentage as mean ±SD of triplicate experiment conditions. $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.05$* were considered significant compared to the control

A study on cervical cancer cell lines (HeLa) showed that postbiotics derived from *Lactobacillus* spp. from human milk increased the expression of genes such as Bax, Bad, and caspase-3, 8 and 9 involved in apoptosis, while downregulated the expression of antiapoptotic genes such as BCL-2 [45].

In a study by Nedzvetsky et al., peptidoglycan fractions enriched with muramyl pentapeptide (MPP) as a postbiotic component from *L. bulgaricus* decreased U373MG glioblastoma cell migration capability and tumor progression in a dose-dependent manner *via* upregulating parp1 and NF-κB levels using cellular migration scratch and Western blot assays [46]. Based on the results of this study, it was revealed that NADH levels in exposed glioblastoma cells (25, 50, 100 and 200 μg ml^{-1} MPP) decreased, resulting in decreases of metabolic energy level and cell viability [46]. Possible mechanisms of action from various soluble factors derived from probiotics such as short-chain fatty acids on the NF-κB signaling pathways and their downstream genes that are playing essential roles in inflammatory diseases such as inflammatory bowel disease as a risk factor in development of CRC can be further studied [47,48]. In another study on cervical cancer cells (HPV16 and HPV18), it was reported that *L. plantarum* metabolites (CFS) included dose, strain and cell line-depended antiproliferative and antimetastatic effects on cervical cancer cells through upregulating E-cadherin and downregulating matrix metalloproteinase-9 (MMP9) modes of action [49].

3.3. Apoptosis assessment in HCT-116 cells based on dual AO/EB staining

Effects of ECE and ICE from *L. sakei* and FQ in a concentration range of 1.25-20 mg ml^{-1} on apoptosis were assessed by AO/EB staining. Results indicated that untreated (control) cells included uniform green color depicted as color intensity using ImageJ software with no fragmented nuclei. Apoptotic cells were reddish-orange and included orange spots in their cytoplasm, which were compressed with fragmented chromatin. Results of the AO/EB staining showed that the rate of apoptosis might be induced by the postbiotics as a function of concentration (Figures 4a, b and 5a, b).

It is noteworthy that the cell population decreased in microscopic assessments by increasing postbiotics concentrations, which FQ ECE included the most decreasing effects on cell populations and proapoptotic effects, while *L. sakei* ICE included the minimum effects (data not shown). Results from AO/EB staining in the current experimental setting (Figures 4a, b and 5a, b) were similar to those of earlier studies concerning antiproliferative and apoptosis-inducing effects of ICE or cytoplasmic extracts derived from two species of *L. acidophilus* and *L. delbrueckii*, showing that cytoplasmic extracts of the two bacteria induced intrinsic pathway-dependent apoptosis in HT-29 cells assessed by Hoechst staining and flow cytometry assays [29].

Lee et al. investigated effects of ECE of *L. fermentum* on HCT-116 and HT-29 cells and reported that the ECE included the ability to induce apoptosis by upregulating caspase-3, Bax, Bak, Noxa and Bid mRNA expressions [38].

Furthermore, results of AO/EB staining showed that increasing ECE concentrations of *L. sakei* and FQ included apoptosis-inducing effects verified by increases in cells with orange-red color as a sign of apoptosis.

This finding was similar to previously published findings, showing that postbiotics could induce apoptosis in cancerous cells [50]. Chiu et al. demonstrated that soluble compounds secreted by *L. casei* and *L. rhamnosus* induced apoptosis in monocytic leukemia cells, hence postbiotics could be addressed as safe agents against cancers because they did not affect intestinal barrier epithelial cells and could be used as adjuvants in chemotherapy [51]. Important mechanisms that contributed to the biological function of postbiotics in improving cancer prevention or treatment, especially in colorectal cancer, include negative effects on tumorous cell viability, improving immune responses, eliminating carcinogens and mutagens agents, activating cell death pathways (proapoptotic activity) such as intrinsic and extrinsic pathways of apoptosis and inducing TNF-related apoptosis inducing ligand, decreasing pathogenic bacteria colonization and inflammation mediated by these bacteria, decreasing metalloproteinase-9 activity and having antiproliferative characteristics against colon cancer cells. These effects have been shown to vary in various cell lines depending on the type of postbiotics [23,44]. Antiproli-ferative effects of exopolysaccharides from four strains of *L. casei* (K11, M5, SB27 and X12) on epithelial CRC cells (HT-29) showed that the exopolysaccharides of *Lactobacillus* included inhibitory effects on G0/G1 stages in cell cycle and viability as well as ability to induce expression of apoptosis-associated genes such as caspase-3 [52]. Tukenmez et al. demonstrated that exopolysaccharides from several strains of *L. plantarum GD2*, *L. rhamnosus* E9 and *Levilactobacillus brevis* LB63) included apoptotic effects on HT-29 CRC cell line by increasing expression of Bax, caspase-3 and caspase-9 and decreasing expression of Bcl-2 and survivin [53]. In a study by Chuah et al., it was revealed that postbiotics derived from six strains of *L. plantarum* included selective pronounced cytotoxicity *via* antiproliferative and proapo-ptotic effects against various tissue malignant cancer cells (breast, colorectal, cervical, liver and leukemia cancer cell lines) and normal cells in a strain-specific and cancer cell type-specific manner whilst sparing the normal cells. Moreover, it was indicated that ECE of *L. plantarum* UL4 and *L. plantarum* RG14 induced apoptotic cell death at 15-30% (v v^{-1}) concentrations in MCF-7 and HT-29 cells, respectively, verified *via* fluorescent microscopy and AO/PI staining [54].

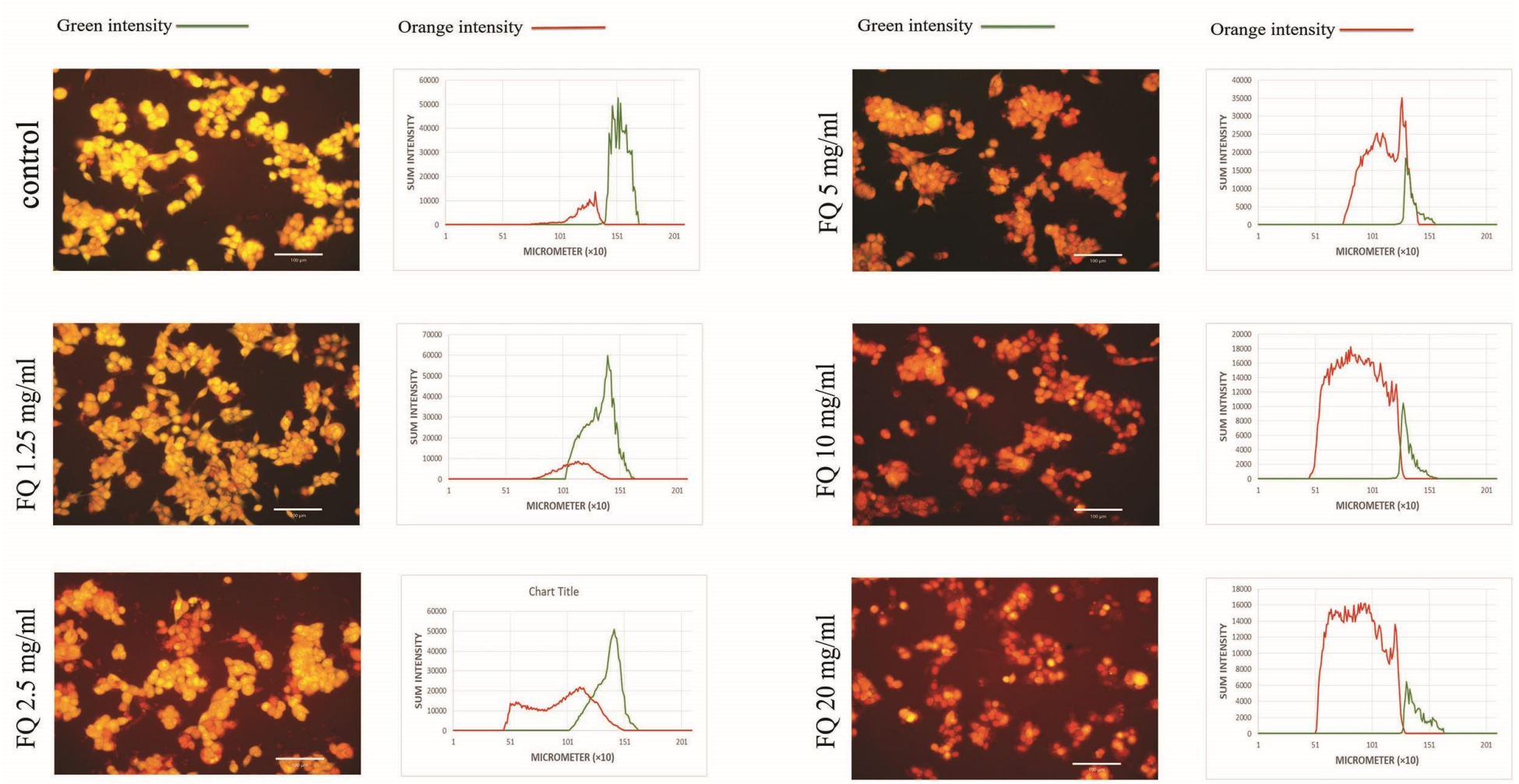

Figure 4a. Staining with AO/EB in HCT-116 cells treated with FreshQ® (FQ) ECE at 1.25–20 mg ml^{-1}

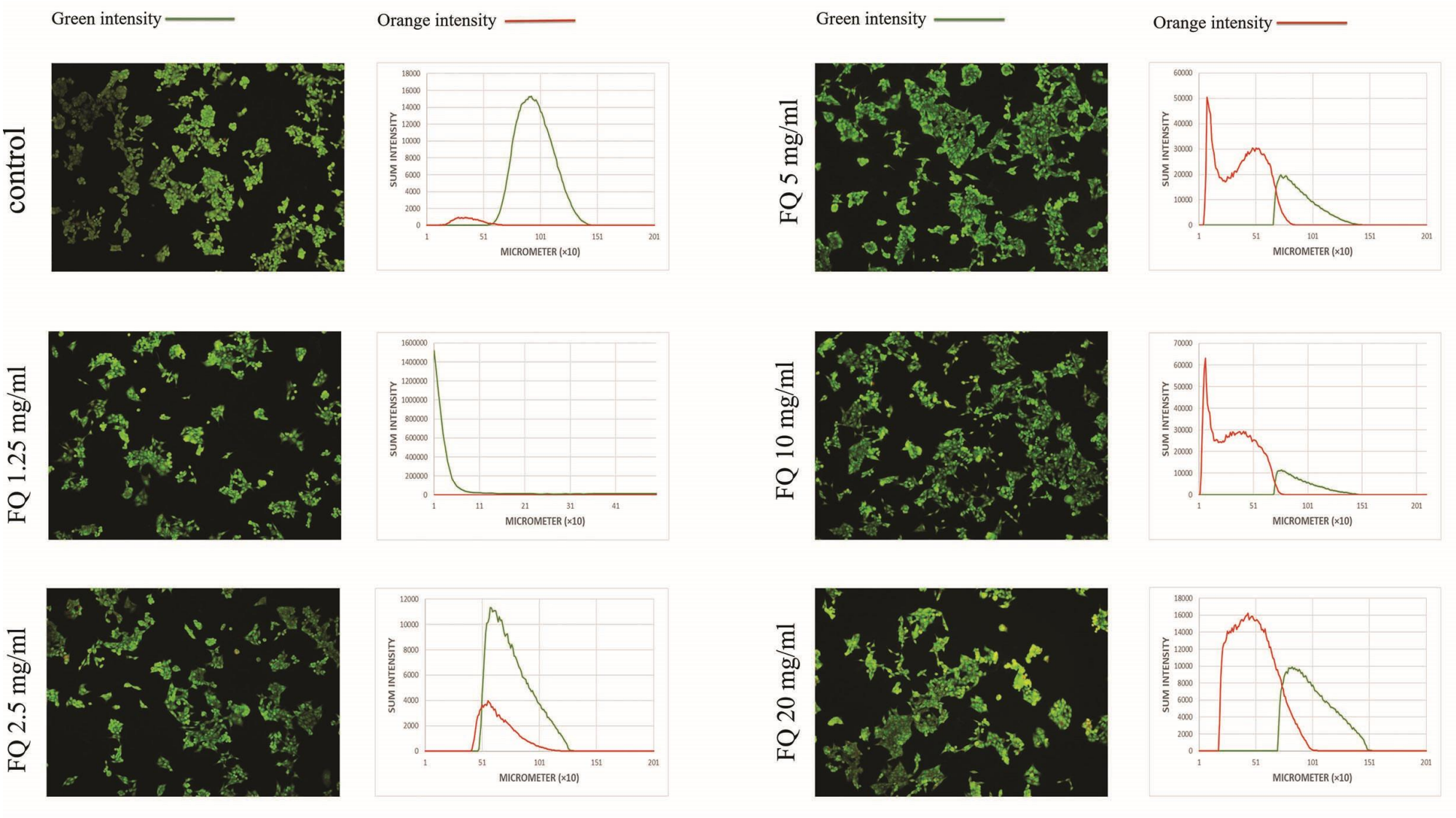

Figure 4b. Staining with AO/EB in HCT-116 cells treated with FreshQ® (FQ) ICE at 1.25–20 mg ml^{-1}

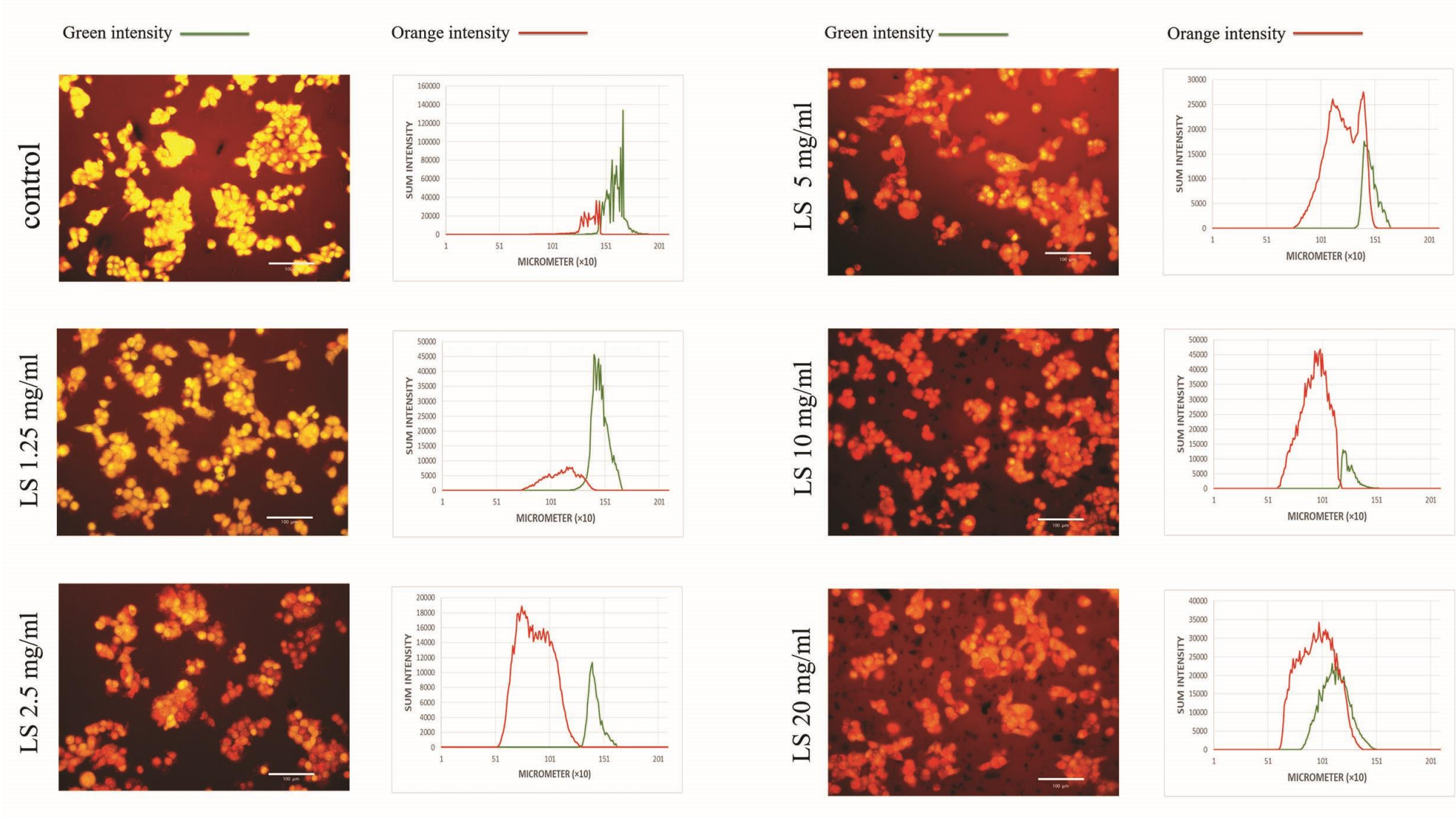

Figure 5a. Staining with AO/EB in HCT-116 cells treated with *L. sakei* (LS) ECE at 1.25-20 mg ml^{-1}

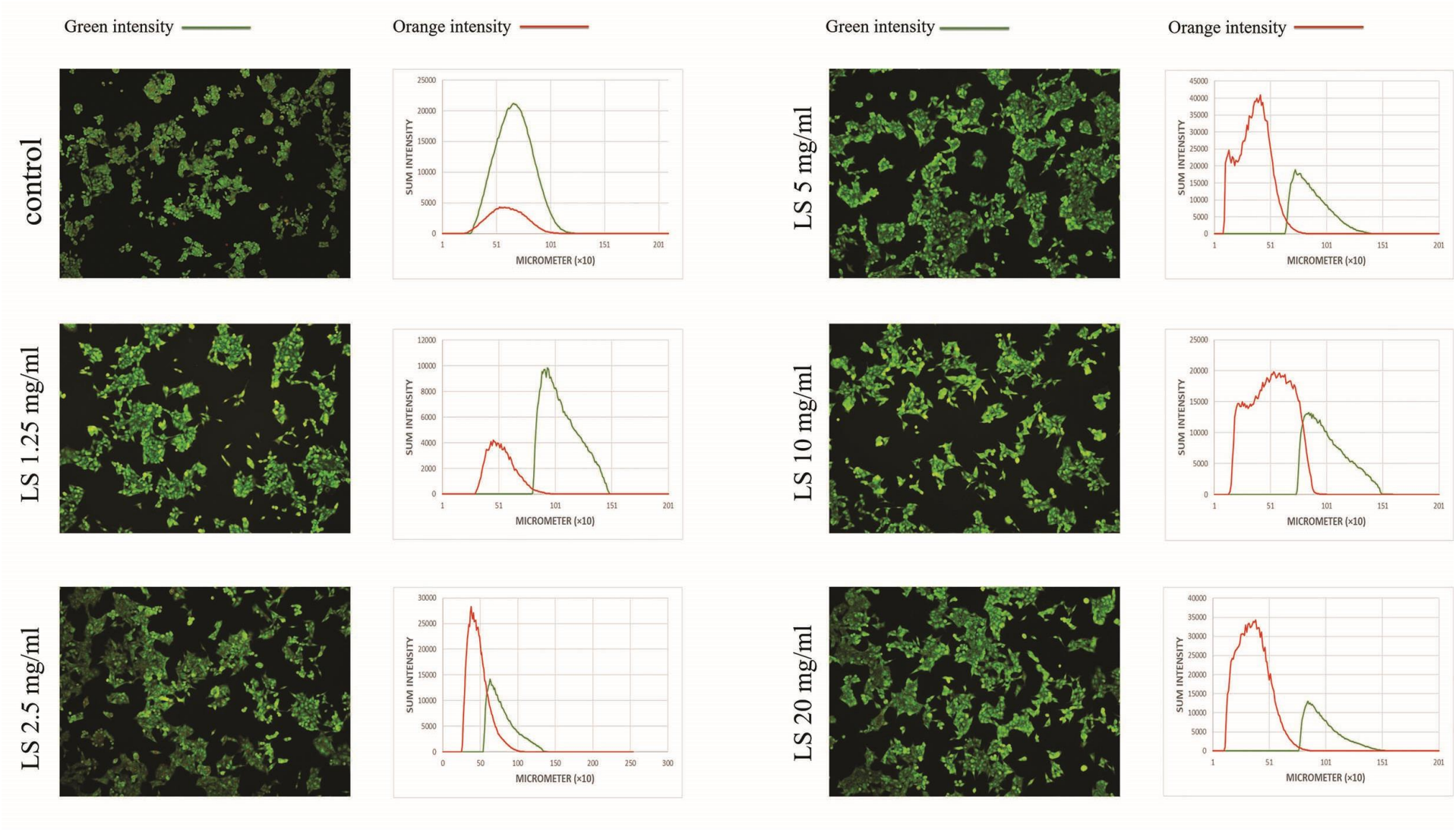

Figure 5b. Staining with AO/EB in HCT-116 cells treated with *L. sakei* (LS) ICE at 1.25–20 mg ml^{-1}

Results of a study by Hadad et al. revealed that a combination of postbiotic (CFS or ECE) produced by *L. acidophilus* with the chemotherapy drug (Methotrexate or MTX) included significant effects on the induction of apoptosis in human T-acute lymphoblastic leukemia (T-ALL cell line) through increasing transcription levels of Bax, NF-κB, Notch1 and Notch2 genes (pro-apoptotic genes) as well as decreasing transcription levels of JAG1 and JAG2 genes (anti-apoptotic genes) [55]. In another study by Maleki et al., it was reported that postbiotics produced by *L. sakei* included proapoptotic effects on Ht-29 colon cancer cells in a dose-dependent manner, verified using annexin V-FITC/PI kit [56]. Based on the finding of this study and other studies, it can be concluded that postbiotics are promising tools in combination therapy for combating cancers and decreasing doses and adverse effects of common chemotherapy agents.

4. Conclusion

In general, it can be concluded that postbiotics (ICE and ECE) derived from FQ and *L. sakei* can decrease survival (possibly by inducing apoptosis) of HCT-116 colon cancer cells and inhibit their proliferation. Moreover, potency of postbiotic (ECE and ICE) derived from FQ protective cultures seems to be higher than that of *L. sakei* (LS) postbiotics. Further investigations are necessary to clarify possible mechanisms of action and signaling pathways behind the induced effects, which may offer support options in cancer prevention, control and treatment protocols. Moreover, investigation of the highlighted effects in non-cancerous cell lines could present better insights into the associated effects. Based on the available information on the benefits and drawbacks of probiotics, postbiotics can be considered as promising novel approaches in a wide range of pharmaceuticals and food products to prevent and improve cancer treatment methods. However, effects of postbiotics can vary with the type of probiotic and methods of fermentation and extraction. Results can serve as the preliminary data for further investigations in the field of cancer treatments.

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6. Conflict of Interest

The authors report no conflicts of interest.

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اثر پست‌بیوتیک‌های حاصل از پروبیوتیک غذایی و کشت‌های محافظ بر تکثیر و مرگ سلول‌های سرطانی روده بزرگ HCT-116

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واژگان کلیدی
- آپوپتوز
- سرطان روده بزرگ
- کشت محافظ
- *لاکتوباسیلوس*
- پست بیوتیک
- تکثیر

***نویسنده مسئول**
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چکیده

سابقه و هدف: پست‌بیوتیک‌ها فرآورده‌های محلول مشتقات میکروبی هستند که در طی فرآیند رشد و تخمیر میکروارگانیسم‌های مفید در دستگاه گوارش، غذا یا سامانه‌های پیچیده کشت میکروبیولوژیکی (مایع رویی فاقد سلول یا عصاره خارج سلولی) یا متعاقب تخریب[۱] سلولی (عصاره داخل سلولی) آزادسازی می‌شوند. سرطان کولورکتال یکی از شایع‌ترین سرطان‌ها در میان علل اصلی مرگ و میر ناشی از سرطان در جهان محسوب می‌گردد که می‌تواند با سد معیوب دستگاه گوارش ارتباط داشته باشد. در مطالعه حاضر، اثر بالقوه عصاره‌های خارج سلولی (ECE) و داخل سلولی (ICE) حاصل از پروبیوتیک (*لاکتوباسیلوس ساکای*؛ LS) و کشت محافظ (FreshQ®, FQ) بر تکثیر و بقای سلولی سلول‌های اپیتلیالی سرطان کولون HCT-116 مورد بررسی قرار گرفت.

مواد و روش ها: پست‌بیوتیک‌ها در محیط کشت میکروبی MRS براث[۲] با سانتریفیوژ و سونیکاسیون جداسازی شدند. محلول‌های به‌دست‌آمده به‌روش انجمادی خشک و تا زمان مصرف ذخیره شدند. سلول‌های HCT-116 به مدت ۲۴ ساعت در تماس با غلظت‌های گوناگون عصاره‌های *لاکتوباسیلوس ساکای* و FreshQ® (۱/۲۵-۴۰mg ml^{-1}) قرار داده شدند و سپس اثرات ناشی از این فرآورده‌ها بر سمیت سلولی، تکثیر و مرگ سلول‌ها با استفاده از روش‌های MTT، آزمون خراش و رنگ‌آمیزی AO/EB مورد بررسی قرار گرفتند.

یافته‌ها و نتیجه‌گیری: عصاره‌های خارج و داخل سلولی *لاکتوباسیلوس ساکای* و FreshQ® موجب کاهش زنده‌مانی سلولی بر اساس غلظت پست‌بیوتیک شدند ($P\leq$۰/۰۵)، در حالی‌که تکثیر سلولی توسط عصاره‌های خارج و داخل سلولی حاصل از *لاکتوباسیلوس ساکای* و FreshQ® در روش آزمون خراش مهار شد. نتایج همچنین نشان داد که پست‌بیوتیک‌ها می‌توانند مرگ سلولی[۳] را القا کنند که با رنگ‌آمیزی آکریدین نارنجی/اتیدیوم بروماید مشهود است. به طور خلاصه، پست‌بیوتیک‌های حاصل از *لاکتوباسیلوس ساکای* و FreshQ® توانایی کاهش زنده‌مانی و تکثیر سلولی و افزایش مرگ سلولی در سلول‌های سرطانی روده بزرگ (کولورکتال) HCT-116 را دارند. علاوه بر این، به نظر می‌رسد پست بیوتیک‌های حاصل از FreshQ® قدرت و اثرگذاری بیشتری از پست‌بیوتیک‌های *لاکتوباسیلوس ساکای* دارند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] lysis
[۲] de Man, Rogosa and Sharpe broth
[۳] apoptosis