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Dear Editor,

We would like to submit the revised manuscript entitled: **"Evaluating Cell Death using Cell-Free Supernatant of Probiotics in Three-Dimensional Spheroid Cultures of Colorectal Cancer Cells"** as a letter in *Journal of Visualized Experiments.*

We tried to address the reviewer's comments by correcting the manuscript and improving our results during revision duration. In addition, our manuscript underwent extensive English editing by English editing service for checking grammar, spelling and some improvement of style. In our revised manuscript, the changes of reply for reviewers were highlighted with red color. We hope that this revised manuscript will receive your kind consideration for publication in *Journal of Visualized Experiments.*

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TITLE:

Evaluating Cell Death Using Cell-Free Supernatant of Probiotics in Three-Dimensional Spheroid Cultures of Colorectal Cancer Cells

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KEYWORDS:

probiotics, *Lactobacillus fermentum*, colorectal cancer, spheroid, 3D culture, cell-free supernatant, CFS

SUMMARY:

Here methods are presented to understand anti-cancer effects of *Lactobacillus* cell-free supernatant (LCFS). Colorectal cancer cell lines show cell deaths when treated with LCFS in 3D cultures. The process of generating spheroids can be optimized depending on the scaffold and the analysis methods presented are useful for evaluating the involved signaling pathways.

ABSTRACT:

This manuscript describes a protocol to evaluate cancer cell deaths in three dimensional (3D) spheroids of multicellular types of cancer cells using supernatants from *Lactobacillus fermentum* cell culture, considered as probiotics cultures. The use of 3D cultures to test *Lactobacillus* cell-free supernatant (LCFS) are a better option than testing in 2D monolayers, especially as *L. fermentum* can produce anti-cancer effects within the gut. *L. fermentum* supernatant was

identified to possess increased anti-proliferative effects against several colorectal cancer (CRC) cells in 3D culture conditions. Interestingly, these effects were strongly related to the culture model, demonstrating the notable ability of *L. fermentum* to induce cancer cell death. Stable spheroids were generated from diverse CRCs (colorectal cancer cells) using the protocol presented below. This protocol of generating 3D spheroid is time saving and cost effective. This system was developed to easily investigate the anti-cancer effects of LCFS in multiple types of CRC spheroids. As expected, CRC spheroids treated with LCFS strongly induced cell death during the experiment and expressed specific apoptosis molecular markers as analyzed by qRT-PCR, western blotting, and FACS analysis. Therefore, this method is valuable for exploring cell viability and evaluating the efficacy of anti-cancer drugs.

INTRODUCTION:

Probiotics are the most advantageous microorganisms in the gut that improves immune homeostasis and host energy metabolism[1]. Probiotics from *Lactobacillus* and *Bifidobacterium* are the most advanced of its kind found in the intestine[2,3]. Previous investigations have shown that *Lactobacillus* has inhibitory and antiproliferative effects on several cancers, including colorectal cancer[4]. Moreover, probiotics prevent inflammatory bowel diseases, Crohn's disease, and ulcerative colitis[5,6]. However, most studies with probiotics were performed in two dimensional (2D) monolayers that are grown on solid surfaces.

Artificial culture systems lack environmental features, which is not natural for cancer cells. To overcome this limitation, three dimensional (3D) culture systems have been developed[7, 8]. Cancer cells in 3D show improvements in terms of basic biological mechanisms, such as cell viability, proliferation, morphology, cell-cell communication, drug sensitivity, and in vivo relevance[9,10]. Moreover, spheroids are made from multicellular types of colorectal cancer and are dependent on cell-cell interactions and the extracellular matrix (ECM)[11]. Our previous study has reported that probiotic cell-free supernatant (CFS) produced using *Lactobacillus fermentum* showed anti-cancer effects on 3D cultures of colorectal cancer (CRC) cells[12]. We proposed that CFS is a suitable alternative strategy for testing probiotic effects on 3D spheroids[12].

Here, we present an approach that can accommodate multicellular types of 3D colorectal cancer for the analysis of therapeutic effects of probiotic cell-free supernatant (CFS) on several 3D colorectal cancer mimicry systems. This method provides a means for the analysis of related probiotic and anti-cancer effects in vitro.

PROTOCOL:

1. Bacterial cell cultures and preparation of *Lactobacillus* cell-free supernatant (LCFS)

NOTE: Steps 1.2 – 1.9 are conducted in an anaerobic chamber.

1.1. Prepare an MRS agar plate and broth containing L-cysteine and sterilize by autoclaving.

1.2. Pre-incubate the MRS agar plate in H_2 anaerobic chamber maintained at 37 ˚C with 20 ppm

oxygen.

1.3. Thaw *Lactobacillus* bacterial stock and inoculate the agar plate with the bacterial culture (**Figure 1A (i)**).

1.5. Incubate bacteria for 2 - 3 days in H_2 anaerobic chamber at 37 ˚C and 20 ppm oxygen until single bacterial colonies are obtained.

1.6. Wash and dry the Hungate type anerobic culture tube. Autoclave the culture tube at 121 ˚C for 15 min.

1.7. Then incubate the tube in H_2 anaerobic chamber at 37 ˚C and 20 ppm oxygen to remove oxygen.

1.8. Place 2 - 3 mL of MRS broth into the tube. Seal the tube with a butyl rubber stopper and screw the cap.

1.9. Obtain a single colony with a loop and place it into the 1.5 mL culture tube with 500 µL of 1x PBS. (**Figure 1A (ii)**).

1.14. Suspend the colony using a 1 mL syringe (**Figure 1A (iii)**). Do this by, inserting the needle of the 1 mL syringe in the center of the tube lid, aspirating the suspended colony and then resuspending it back into the MRS broth media. (**Figure 1A (iv)**).

1.15. Incubate the MRS broth media in a shaker incubator for 2 days (37 °C, 5% CO_2, 200 rpm).

1.16. Measure the optical density (OD) using a spectrophotometer to monitor bacterial growth curves until the absorbance at OD_{620} reaches to 2.0.

1.17. Separate the bacterial pellets and the conditioned media by centrifuging at 1,000 x *g* for 15 min. Wash the collected bacterial pellets with 1x PBS and resuspend in 4 mL of RPMI 1640 supplemented with 10% fetal bovine serum. Do not include any antibiotics in the medium.

1.18. Maintain the bacterial pellets in RPMI and incubate in a shaker incubator for 4 h at 37 °C with 5% CO_2 at a speed of 100 rpm.

1.19. For the preparation of the probiotic supernatant, remove the bacterial pellet via centrifugation at 1000 *x g*, for 15 min at 4 °C. Sterile-filter the recovered supernatant using a 0.22 µm filter and store at −80 °C until use.

2. Generation of spheroids

2.1. Preparing colorectal cancer cell lines

2.1.1. Grow DLD-1, HT-29, and WiDr cell lines as monolayers until 70-80% confluency and incubate the plate at 37 °C in a 5% CO_2 incubator (Growth medium: RPMI containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin).

2.1.2. For cells grown in 100 mm Petri dish, wash the plate twice with 4 mL of 1x PBS. Add 1 mL of 0.25% trypsin-EDTA and incubate the Petri dish for 2 min at 37 °C in a 5% CO_2 incubator to dissociate the cells.

2.1.3. After incubation, check for the cell dissociation under a microscope and neutralize trypsin-EDTA with 5 mL of growth medium.

2.1.4. Transfer the dissociated cells to a 15 mL conical tube and centrifuge for 3 min at 300 *x g*.

2.1.5. Discard the supernatant and resuspend gently with 3 mL of growth media.

2.1.6. Count the cells with trypan blue to determine viable cells using a hemocytometer. (**Figure 1B (i)**)

2. Spheroid formation

2.2.1. In a 15 mL conical tube, dilute the cells from 2.1.5 to obtain 1 - 2 x 10^5 cells/mL (**Figure 1B (ii)**)

2.2.2. Add final concentration of 0.6% methylcellulose to the cell suspension and transfer the diluted cells to a sterile reservoir.

NOTE: For each cell line, the amount of methylcellulose needed should be titrated and determined accordingly.

2.2.3. Use a multichannel pipette to dispense 200 µL of cells to each well of an ultra-low attachment 96-well round bottom microplate. (**Figure 1B (iii)**)

2.2.4. Incubate the plate at 37 °C in a 5% CO_2 incubator for 24 - 36 h.

2.2.5. After 24 - 36 h, observe the plate under a light microscope to ensure spheroid formation.

3. Treating 3D colorectal cancer cells with LCFS

3.1. Generate spheroids as described in steps 2 and 3.

3.2. Before performing the LCFS treatment, thaw the frozen LCFS at room temperature (RT) for 10 - 20 min.

3.3 Inoculate the LCFS stock solution into a growth medium. Serially dilute to 25%, 12.5%, and

6% in the growth medium (i.e., 25% LCFS = 150 µL of growth medium + 50 µL of LCFS).

3.4. Take out the cell culture plate containing spheroids from the incubator and remove as much of the growth medium as possible from each well using a 200 µL pipette.

3.5. Add the growth media with LCFS on the cells and incubate at 37 °C in a 5% CO_2 incubator for 24 - 48 h.

NOTE: The volume to be used will depend on the plate size as follows: 2 mL for 6-well cell culture plates; 200 µL for 96-well cell culture plates.

4. Cell viability for spheroids

4.1. Prepare 8 - 10 LCFS-treated colorectal cancer spheroids in opaque-walled multi-well plates (cell viability assays are performed 48 h after LCFS treatment).

4.2. Thaw the cell viability reagent (see **Table of Materials**) at 4 °C for overnight.

4.3. Equilibrate the cell viability reagent to room temperature before use.

4.4. Before performing the assay, remove 50% of the growth media from the spheroids.

4.5. Add 100 µL of cell viability reagent to each well.

NOTE: The volume to be used will depend on the plate size as follows: 100 µL for 96-well cell culture plates.

4.5. Mix the reagent vigorously for 5 min to promote cell lysis.

4.6. Incubate for 30 min – 2 h at 37 °C.

4.7. Record the luminescence.

5. Quantitative real-time polymerase chain reaction analysis for spheroids

5.1. For each condition, prepare 10 - 15 spheroids in a 2 mL tube and centrifuge for 3 min at 400 x *g*.

5.2. Discard the supernatant and wash the spheroids twice in 1 mL of ice-cold 1x PBS.

NOTE: Avoid centrifugation, let the spheroids settle down.

5.3. Aspirate as much of the 1x PBS as possible and isolate RNA using a commercially available kit.

5.4. Synthesize cDNA from 1 µg of RNA using a commercially available kit as per the manufacturer's protocol.

5.5. Prepare a master mix to run all samples in triplicate (see **Table 1 and Table 2**).

5.6. Perform the amplification in a 20 µL of the template master mix into each qPCR plate well.

5.7. Mix reactions well and spin if necessary.

5.8. Run samples as per the recommendations of the instrument manufacturer (**Table 3**).

6. Western blotting from spheroids

NOTE: When collecting spheroids, use a 200 µL pipette and cut the end of the tips to avoid disturbing their structure.

6.1. For each condition, prepare 30 - 40 spheroids in a 2 mL tube.

6.2. Place the tube on ice and let the spheroids settle down to the bottom of the 2 mL tube.

6.3. Discard the supernatant and wash the spheroids twice in 1 mL ice-cold 1x PBS

NOTE: Avoid centrifugation, let the spheroids settle down.

6.4. Aspirate as much of the 1x PBS as possible and add RIPA buffer with a protease inhibitor cocktail (10 spheroids = 30 µL of RIPA buffer).

6.5. Lyse the cells by pipetting up and down and perform sonication for 30 s with 30 s of resting on ice for 10 cycles.

6.6. Centrifuge the protein lysates at 15000 *x g* for 15 min at 4 °C.

6.7. Determine the protein concentration for each cell lysate.

6.8. Before loading, boil each cell lysate in a sample buffer at 100 °C for 10 min.

6.9. Load equal amounts of protein into the wells of the SDS-PAGE gel and run the gel for 1 - 2 h at 100 V.

6.10. Transfer the protein from the gel to the PVDF membrane.

6.11. After transferring, block the membrane for 1 h at room temperature using a blocking buffer (5% skim milk + TBS with 0.05% Tween-20).

6.12. Incubate the membrane with 1:1,000 dilutions of primary antibody (**Table 4**) in 1x TBST with 5% BSA buffer at 4 °C overnight.

6.13. Wash the membrane three times with TBST, 15 min for each wash.

6.14. Incubate the membrane with 1:2,500 dilutions of secondary antibody in the blocking buffer at room temperature for 2 h (see **Table of Materials**).

6.15. Wash the membrane three times with TBST, 15 min for each wash.

6.16. Prepare the membrane for HRP detection with a chemiluminescent substrate.

6.17. Acquire chemiluminescent images.

7. Propidium Iodide (PI) staining of spheroids

7.1. Prepare 5-10 spheroids as described in Step 4.1 and place the spheroids in an incubator at 37 °C and 5% CO_2.

7.2. Dilute a 1 mg/mL stock of PI 1:100 in 1x PBS.

7.3. Remove 50% of the medium from each well of the 96-well plate.

7.4. Add 100 µL of the PI solution to each well and place the wells in an incubator at 37 °C and 5% CO_2 for 10 - 15 min.

7.5. Wash out the PI solution with 1x PBS.

7.6. Add 200 µL of growth medium and take an image using a fluorescence microscope. Analyze the fluorescence intensity using Image J to get the viability count of the spheroid.

8. FACS analysis of spheroids

8.1. Generate spheroids as described previously.

8.2. For each condition, prepare 30 - 40 spheroids in a FACS tube and centrifuge for 3 min at 400 x *g* and RT.

8.3. Aspirate the supernatant and wash the spheroids in 3 mL of 1x PBS, then centrifuge at 400 *x g* for 3 min at 4 °C.

8.4. Aspirate the supernatant and add 200 µL of 0.25% Trypsin-EDTA, then incubate at RT for 2 - 3 min.

NOTE: The incubation time is dependent on the spheroid size and cell type.

8.5. Add 1 mL of FACS buffer and gently dissociate the spheroids using a 200 µL pipette.

8.6. Centrifuge the dissociated cells at 400 *x g* and 4 °C for 3 min.

NOTE: FACS buffer = 1x PBS + 2.5% FBS, filtered using a 0.22 µm top filter.

8.7. Discard the supernatant and add 7-AAD/Annexin V reagent (7AAD (5 µL), Annexin V (5 µL)/sample).

8.8. Gently vortex the cells and incubate for 13 - 30 min at RT in the dark.

8.9. Add 500 µL of FACS buffer and filter the cells using conical polystyrene test tubes to remove aggregate cells.

8.10. Centrifuge at 400 *x g* and 4 °C for 3 min.

8.11. Add 500 µL of Annexin V binding buffer to each tube and resuspend.

8.12. Analyze using a flow cytometer.

REPRESENTATIVE RESULTS:

We describe the protocol of obtaining spheroids from diverse colorectal cancer cell lines. Supplementation with methylcellulose was required to generate spheroids. We also present a method of LCFS preparation and present a model to study the correlation between probiotics and colorectal cancer. Spheroid formation and LCFS preparation protocols are schematically illustrated in **Figure 1A**,**B**. As shown in **Figure 2A**, methylcellulose concentration of 0.6% transforms the cancer cells into compact spheroids. This result indicates that spheroids can be generated from several types of colorectal cancer by using our methylcellulose protocol. Next, the spheroids were treated with 25% LCFS and the morphology was studied after 48 h using a light microscope. As shown in **Figure 3A**, the spheroids of the groups treated with LCFS exhibited disrupted surfaces. To investigate the anti-cancer effects of LCFS at suitable concentrations, the spheroids were treated for 48 h with various dosages of LCFS: 0 (control), 6%, 12.5%, and 25%. Disruptions in the spheroid morphology were observed in spheroids treated with 25% LCFS, as shown in **Figure 3B**. In addition, the spheroids were treated with 25% LCFS for 24 h and 48 h, and disruptions in spheroid morphology were observed after 48 h of treatment. Microscopic images of the spheroids are shown in **Figure 3C**.

After 48 h, the samples were assessed with cell viability assay, and colorectal cancer cell death was observed upon treatment with LCF5 in a dose dependent manner, higher the LCF amount higher the observed cell death (**Figure 3D**). We, then, stained the samples with propidium iodide (PI) to observe apoptosis. As expected, the induction of apoptosis was dependent on the LCFS

dose (**Figure 4A,B,C**). RT-PCR was performed to detect the changes in molecular markers of apoptosis i.e., BAX, BAK and NOXA (**Figure 5A,B**). Lastly, apoptosis markers were studied using western blotting and Annexin V/7AAD through FACS (**Figure 6A,B,C,D**). These observations show that LCFS effectively induced apoptosis in the 3D model.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of spheroid formation and LCFS preparation. (**A**) Schematic representation images of the LSFS generation protocol are marked by (i-iv) (**B**) Schematics of the methylcellulose-mediated spheroid formation are marked by (i-iii).

Figure 2: Methylcellulose-mediated spheroid formation. (**A**) Representative images of methylcellulose-mediated spheroid formation of HT-29, DLD1, and WiDr. Cells were seeded in ultra-low attachment 96-well round bottom plates with methylcellulose concentrations of 0.1-1.2% for 48 h. Scale bar 10 μm (n=3 for each experiment).

Figure 3: Evaluation of LCFS concentration and spheroid morphology. (**A**) Representative images of HT-29 treated with LCFS for 48 h. Scale bar 100 μm. (**B**) HT-29, DLD1, and WiDr spheroids treated with increasing doses of LCFS for 48 h. All spheroids had disrupted edges at 12.5-25% LCFS. Scale bar 20 μm (n=3 for each experiment) (**C**) Spheroid morphologies of HT-29, DLD1, and WiDr spheroids treated with 25 % LCFS for 24 and 48 h. Scale bar 20 μm (n = 3) (**D**) Measured cell viability, shown as mean ± SEM. ***, $P < 0.05$ (n = 3 for each experiment).

Figure 4: Propidium iodide staining of the spheroids. Representative images of PI staining in (**A**) HT-29, (**B**) DLD1, and (**C**) WiDr spheroids after 48 h of LCFS treatment. The images were acquired using a fluorescence microscope and the increase in PI intensity was measured using Image J. Scale bar 10 μm. The mean ± SEM is shown. ***, $P < 0.05$ (n = 3 for each experiment).

Figure 5: Apoptosis markers were identified using qRT-PCR. Apoptosis markers, such as BAX, BAK and NOXA, were quantified. mRNA quantification is presented as a relative expression normalized to (**A**) β-actin and (**B**) 18s rRNA. The mean ± SEM is shown. ***, $P < 0.05$ (n=3 for each experiment).

Figure 6: Apoptosis markers were determined via Western blotting and FACS analysis of the spheroids. Shown in the figure are western blots of (**A**) HT-29, (**B**) DLD1, and (**C**) WiDr cells after LCFS treatment. PARP1, BCL-XL, and p-IκBα was detected. β-actin was used as an internal control. (**D**) FACS analysis of apoptosis in HT-29, DLD1, and WiDr spheroids incubated with LCFS. Apoptotic cells were detected by the increase in the fluorescence intensity of Annexin V-FITC.

Table 1: PCR reaction mixture.

Table 2: Primer sequences used in qRT-PCR analysis.

Table 3: qRT-PCR conditions

Table 4: Antibodies used in western blot analysis.

DISCUSSION:

The tissue microenvironment, including neighboring cells and the extracellular matrix (ECM), is fundamental to tissue generation and crucial in the control of cell growth and tissue development[13]. However, 2D cultures have several disadvantages, such as the disruption of cellular interactions, as well as alterations in cell morphology, extracellular environments, and the approach of division[14]. 3D cell culture systems have been rigorously studied to better reproduce in vivo effects, and have been proven as more precise systems for in vitro cancer testing[15,16]. There is a need for model systems to more accurately predict personalized responses to chemotherapeutics[17].

3D scaffolding was developed for tissue engineering. It acts as a surrogate loss of ECM, representing the available space of tumor cells. In addition, the scaffolding provides physical interactions for cell adhesion and proliferation and causes cells to form appropriate spatial distributions and cell-ECM or cell-cell interactions[18]. The methylcellulose (MC) polymer has been continuously studied to determine its suitability in generating MC-based hydrogel systems for applications in 3D cell culture engineering[19,20]. However, the intact incorporation of these hydrogels into biomaterials like 3D cell networks remains technically challenging[21]. Therefore, the spheroid formation protocol presented here recommends the titration of MC concentrations and optimization with various time points for each CRC cell line. Cell line-specific characteristics, such as cell aggregation, viability, and death, can significantly affect each of the conditions we tested. This method can provide a means of generating uniform spheroids for testing LCSF on cancer cells.

Probiotics, which are beneficial bacteria, produce active metabolites that can potentially mimic anti-cancer effects. Thus, our study was designed to isolate lactic acid bacteria (LAB) and test the anti-cancer effects of their metabolic extracts from cell-free supernatants (CFS). Our studies provided a method for observing the effects of *L. fermentum* cell-free supernatants that induces apoptotic cell death in colorectal cancer cells in a 3D system. The mRNA levels of apoptosis markers involved in apoptotic pathways are dramatically induced after LCFS exposure in 3D conditions. Moreover, decreased levels of PARP1 and BCL-XL were expressed in the LCFS-treated 3D spheroid control compared to the control in **Figure 4C,D**,**E**. Inhibition of NF-κB activation was, also, observed in 3D cultures after treatment with LCSF. Taken altogether, the advantages of culturing cells in 3D include increasing cell-cell interactions and responses to signaling molecules to better mimic in vivo systems. Western blotting using spheroids can lead to quantitative insights into the state of various signaling molecules.

Cell lines have certain limitations as preclinical models of cancer research. Recently, cancer organoids have been utilized in the modeling of personalized anti-cancer therapy[22,23]. The treatment of LCFS with probiotics in organoids is expected to be used as a powerful platform to test anti-cancer effects. Moreover, we only tested one of the *Lactobacillus* species among the various probiotics in the cancer model. Various probiotics are, also, being tested for the

prevention of metabolic syndrome, immunological, and neurological disorders[24–26]. LCFS from *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, and *Akkermansia* species are potential candidates for the testing of health benefits through various types of disease models[27–29].

Based on this study, it can be concluded that the understanding of signaling in spheroids and the various responses to LCFS treatment in 3D models may be beneficial for testing anti-cancer effects using the method that we proposed. Additionally, 3D cancer models can provide several advantages that are not possible with traditional 2D monolayers.

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The authors have no relevant financial disclosures.

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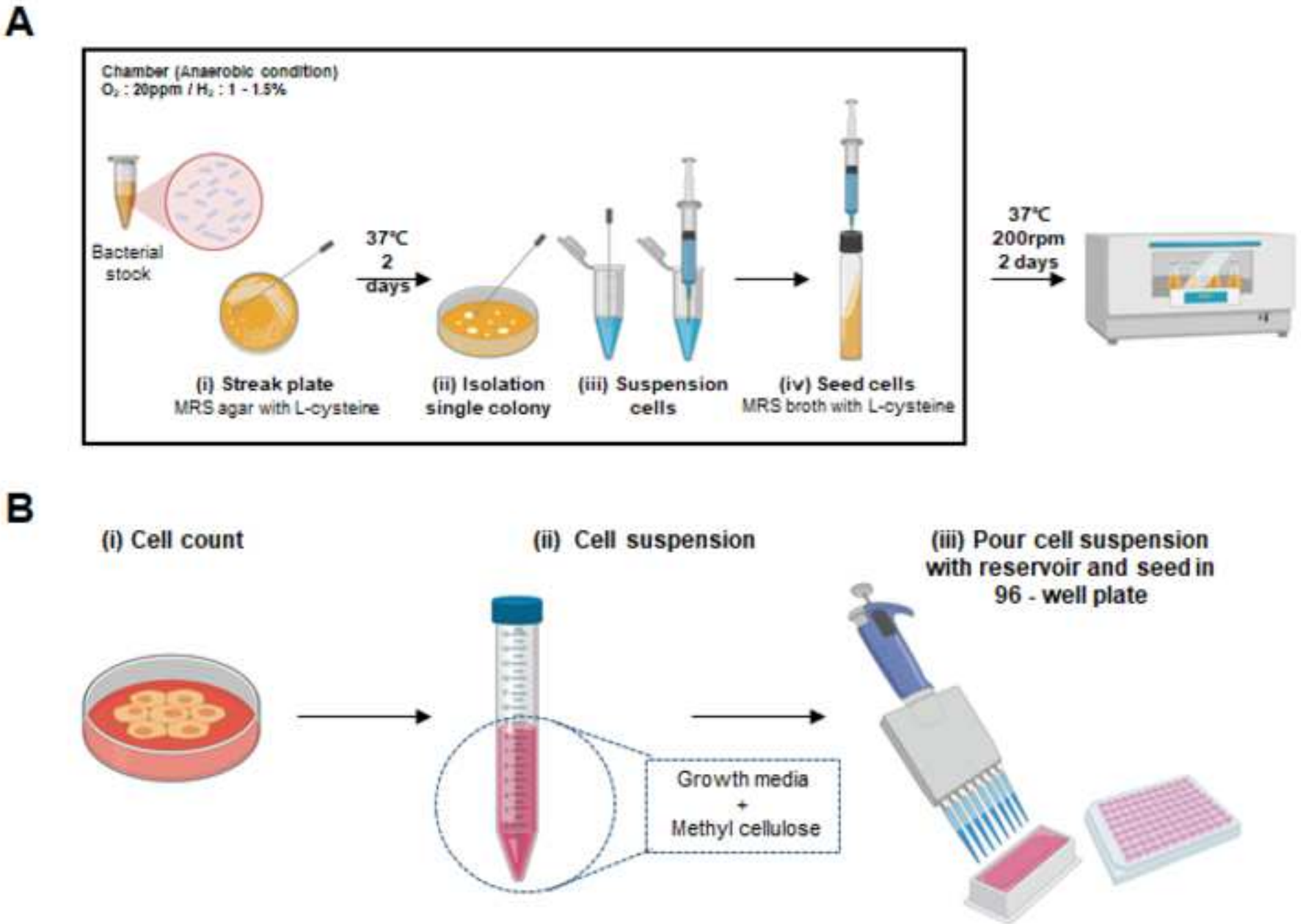
530

Figure 1



Click here to access/download;Figure;Figure 1.tif

Figure 2

Click here to access/download;Figure;Figure 2.tif

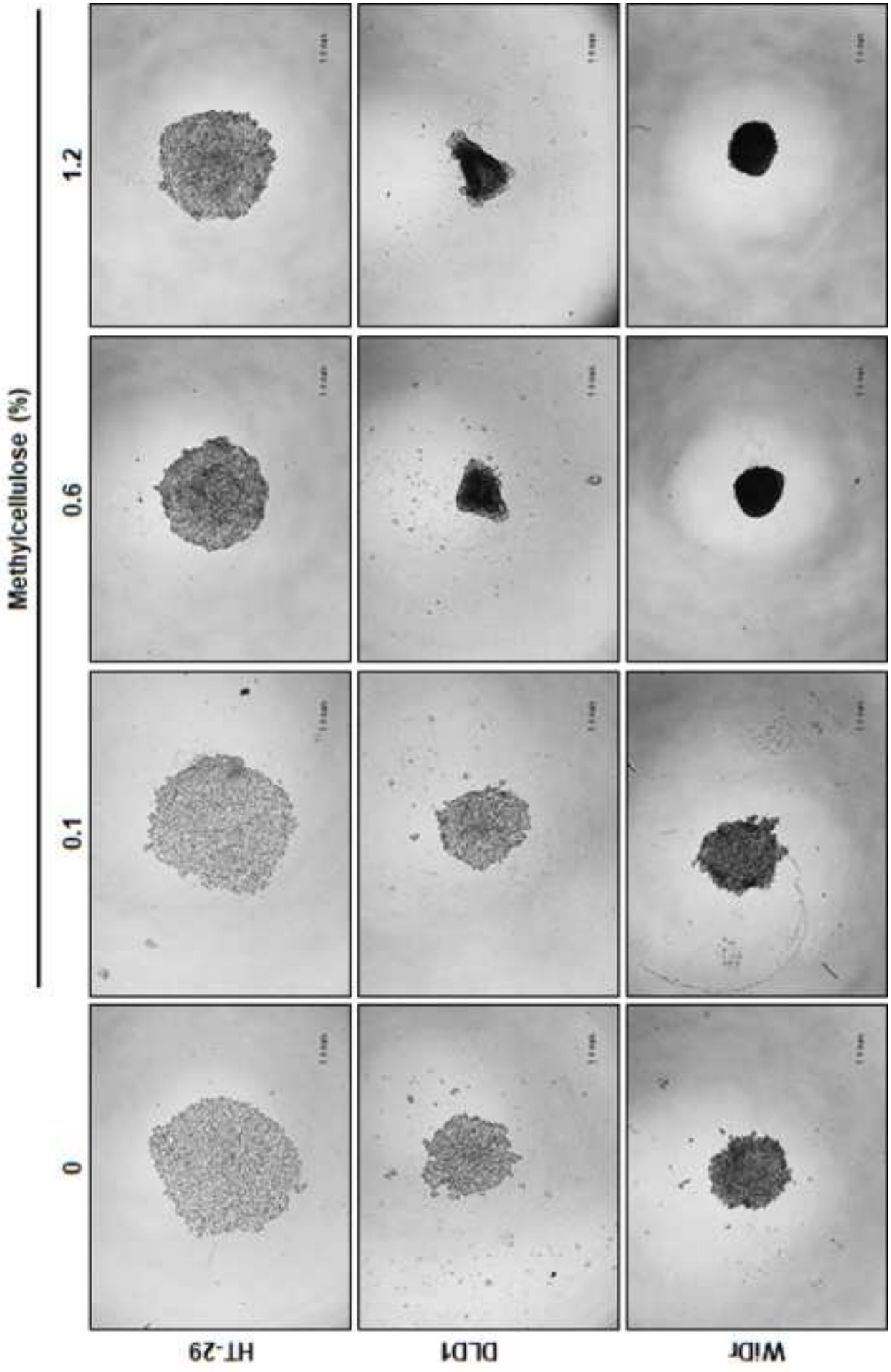


Figure 3

Click here to access/download;Figure;Figure 3.tif

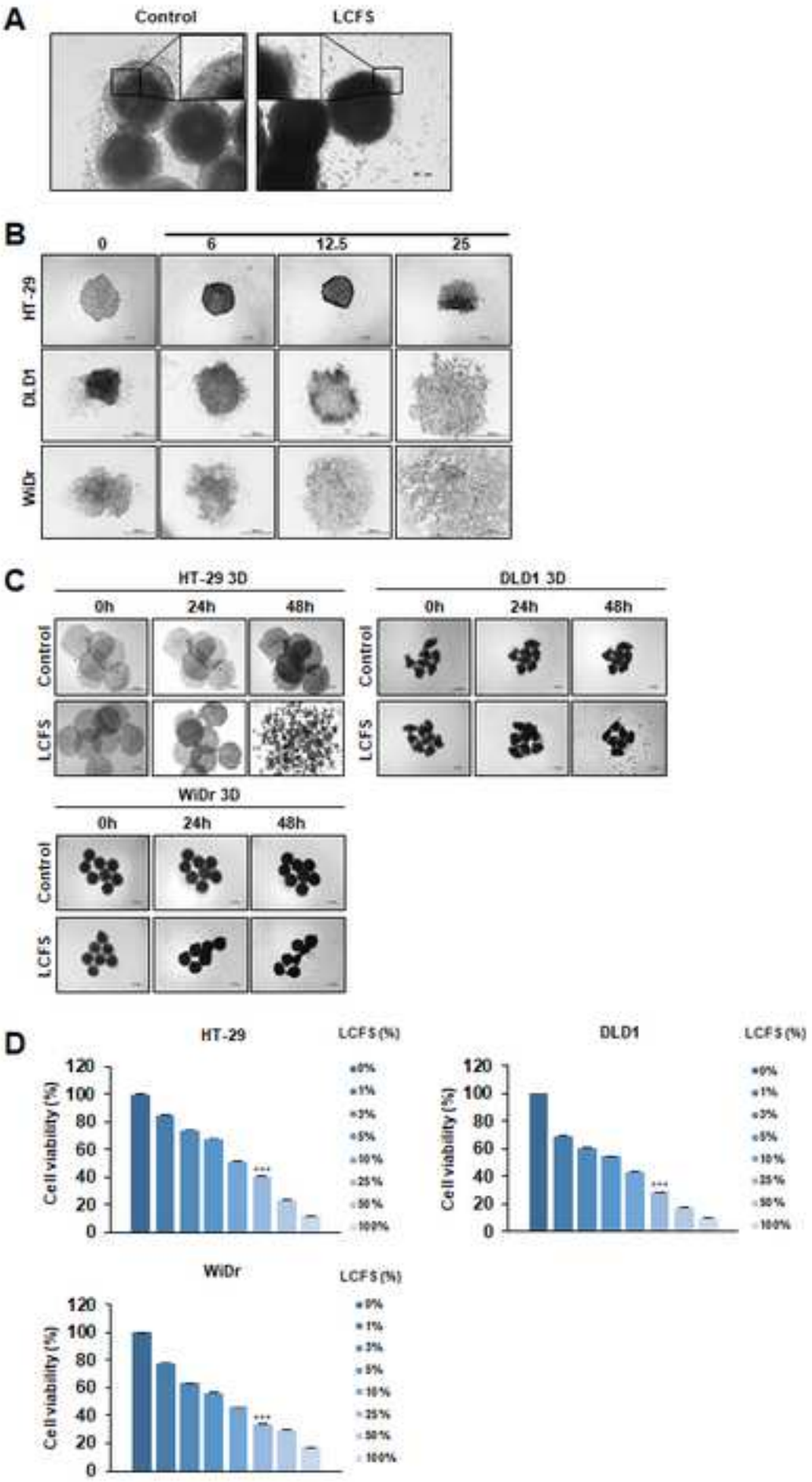


Figure 4

Click here to access/download;Figure;Figure 4.tif

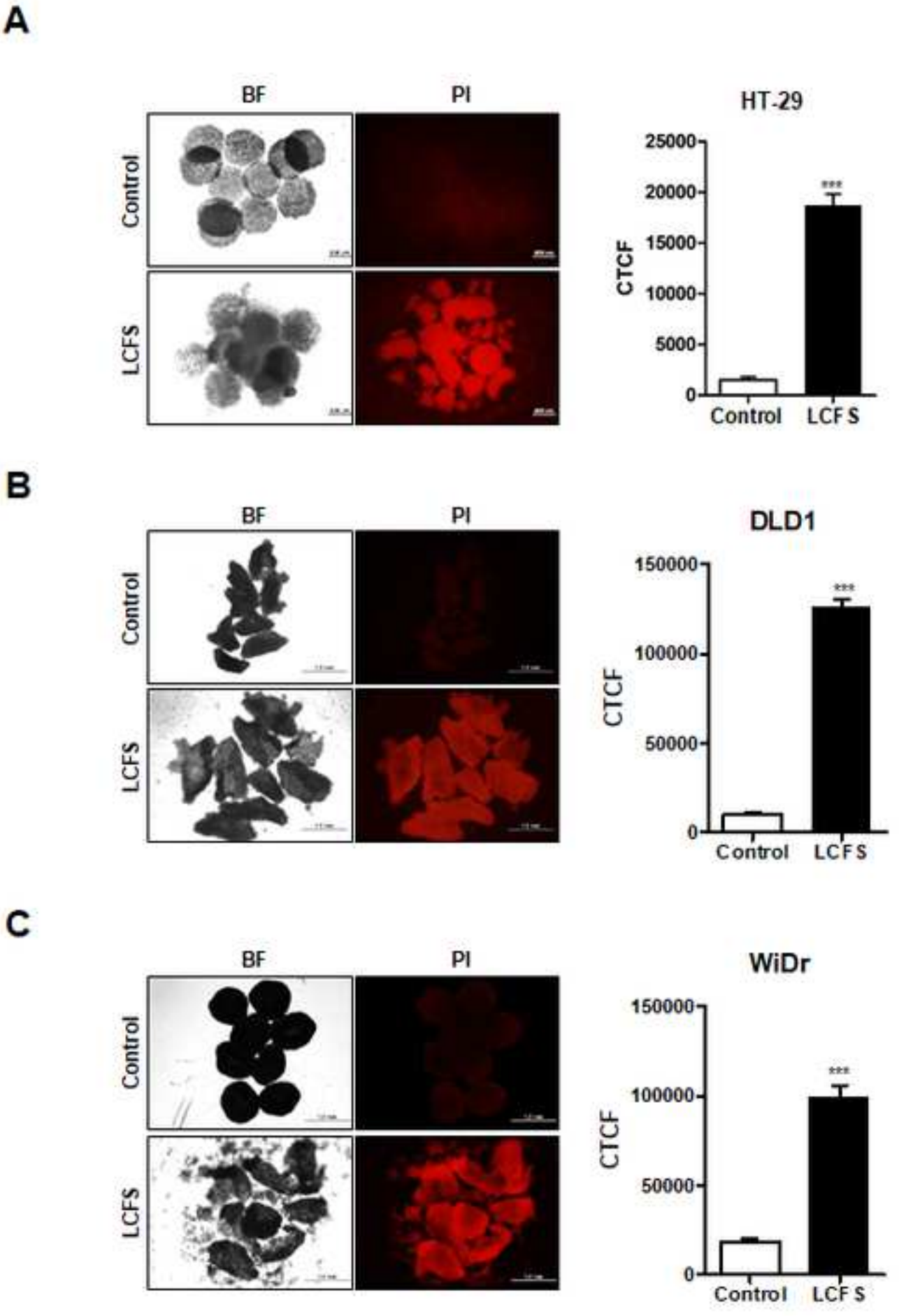


Figure 5

Click here to access/download;Figure;Figure 5.tif

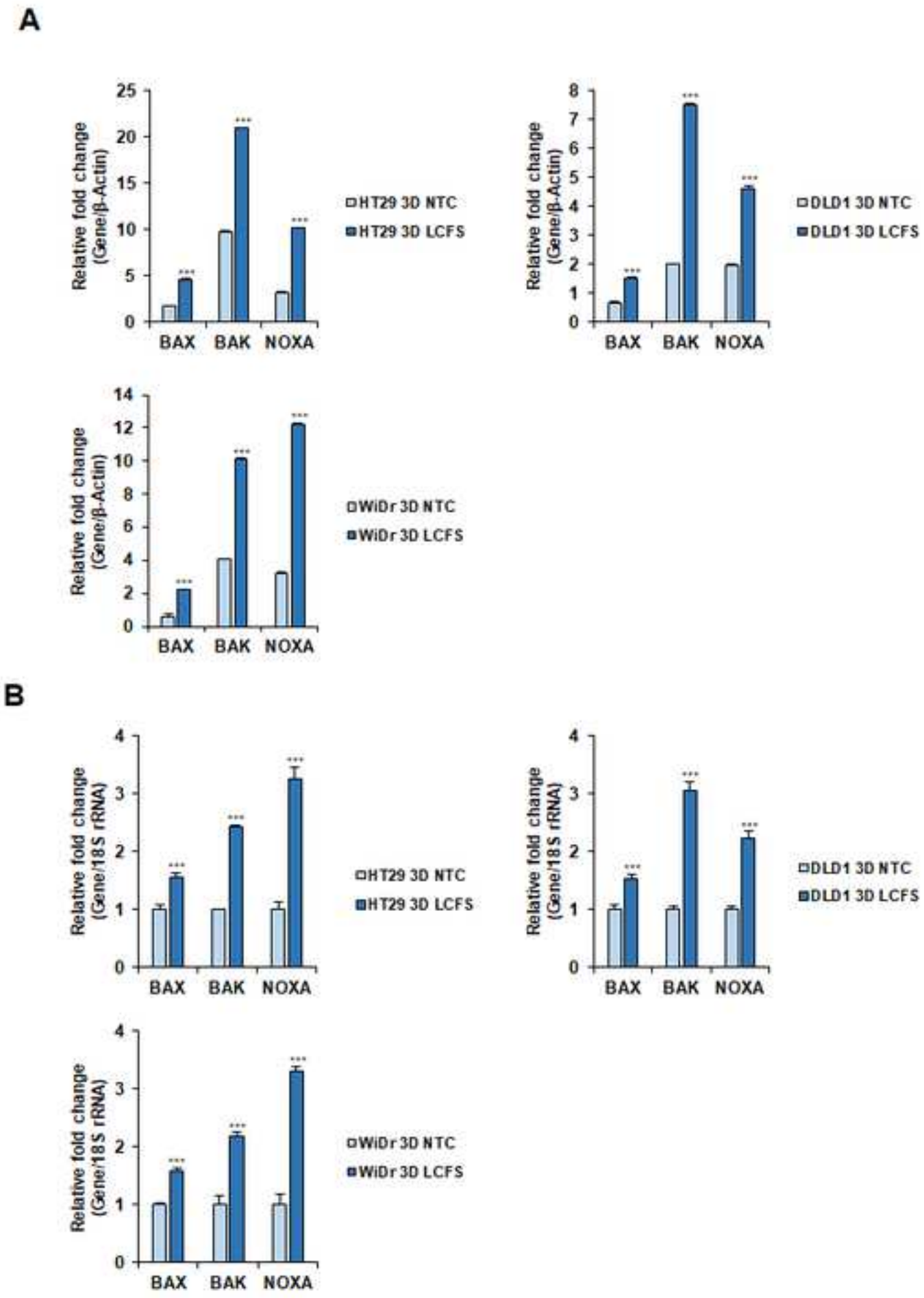


Figure 6

Click here to access/download;Figure;Figure 6.tif

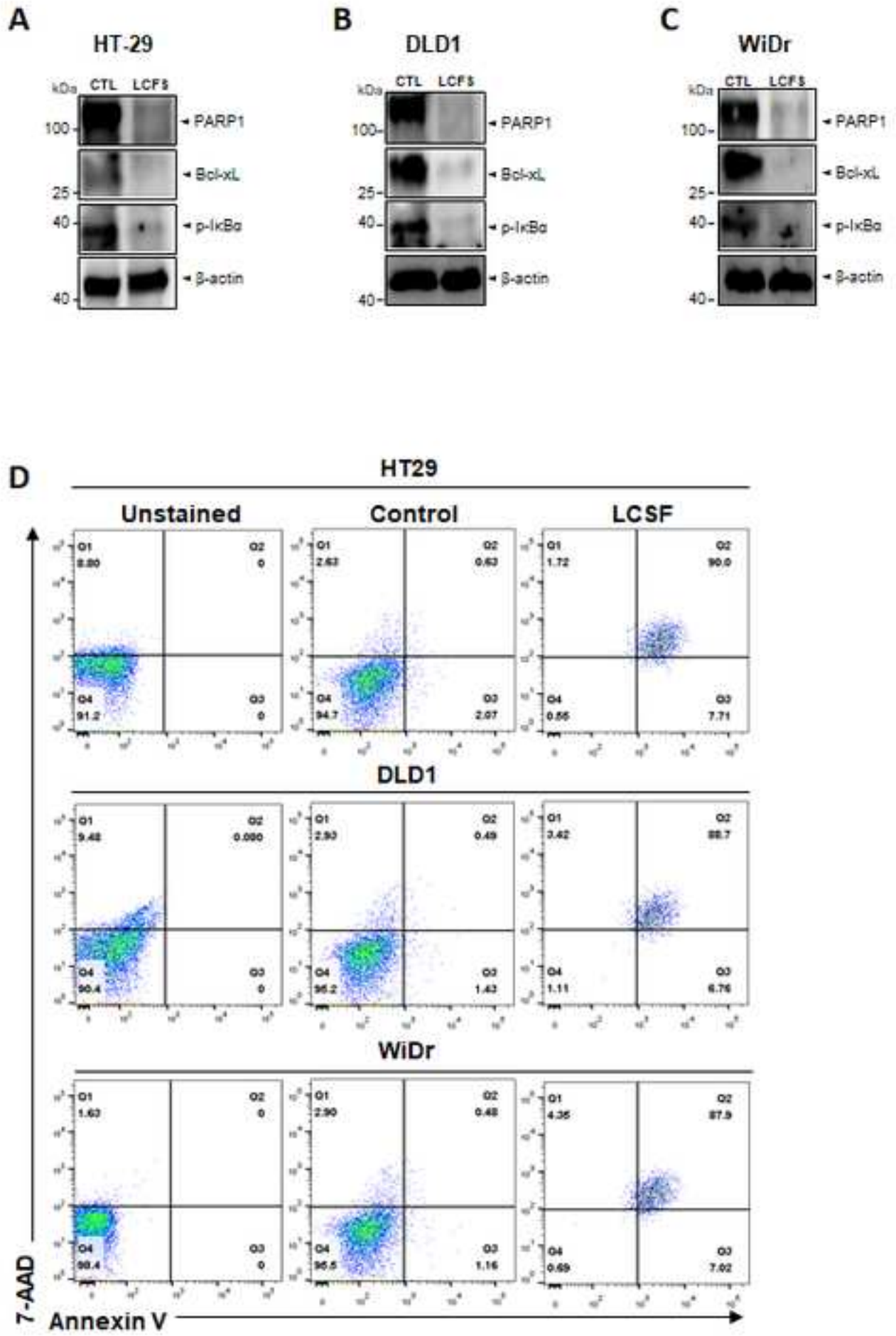


Table 1

Click here to access/download;Table;Table 1.xlsx

Reaction	Volume per single 20 µL
2X qPCR mix	10 µL
Forward primer (10 pm	1 µL
Reverse primer (10 pm	1 µL
cDNA (50 ng/µl)	1 µL
PCR grade water	7 µL

Table 2

Click here to access/download;Table;Table 2.xlsx

Primer	Sequence
BAX-Forward	CCCGAGAGGTCTTTTTCCGAG
BAX-Reverse	CCAGCCCATGATGGTTCTGAT
BAK-Forward	ATGGTCACCTTACCTCTGCAA
BAK-Reverse	TCATAGCGTCGGTTGATGTCG
NOXA-Forward	ACCAAGCCGGATTTGCGATT
NOXA-Reverse	ACTTGCACTTGTTCCTCGTGG
18s rRNA-Forward	GATGGGCGGCGGAAAATAG
18s rRNA-Reverse	GCGTGGATTCTGCATAATGGT
β-Actin-Forward	TCCTGTGGCATCCACGAAACT
β-Actin-Reverse	GAAGCATTTGCGGTGGACGAT

Table 3

Click here to access/download;Table;Table 3.xlsx

Stage	Temp (ºC)	Time
Initial denaturation	95	10 min
40 cycles:		
Step 1	95	15 sec
Step 2	60	60 sec
Melting curve stage	95	15 sec
	60	60 sec
	95	15 sec

Table 4

Click here to access/download;Table;Table 4.xlsx

Antibody	Dilution
PARP 1 (C2-10)	1:1000
BclxL (H-5)	1:1000
p-IκBα (B-9)	1:1000
β-actin (C4)	1:1000
Goat Anti-Mouse IgG (H+L)	1:2500
Goat Anti-Rabbit IgG (H+L)	1:2500

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of kits/Solution
10% Mini-PROTEAN TGX Precast Protein Gels, 15-well, 15 µl
Applied Biosystems MicroAmp Optical Adhesive Film
10x transfer buffer
10X Tris-Glycine (W/SDS)
Axygen 2.0 mL MaxyClear Snaplock Microcentrifuge Tube, Polypropylene, Clear, Nonsterile, 500 Tubes/Pack, 10 Packs
BD Difco Bacto Agar
BD Difco Lactobacilli MRS Broth
CellTiter-Glo 3D Cell viability assay
cOmplete Protease Inhibitor Cocktail
Corning Phosphate-Buffered Saline, 1X without calcium and magnesium, PH 7.4 ± 0.1
EMD Millipore Immobilon-P PVDF Transfer Membranes
Falcon 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap
Fetal Bovine Serum, certified, US origin
iScript cDNA Synthesis Kit, 25 x 20 µl rxns #1708890
iTaq Universal SYBR Green Supermix
Lactobacillus fermentum
L-Cysteine hydrochloride monohydrate
Methyl Cellulose (3500-5600mPa·s, 2% in Water at 20°C)
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL
Millex-GS Syringe Filter Unit, 0.22 µm, mixed cellulose esters, 33 mm, ethylene oxide sterilized
PE Annexin V Apoptosis Detection Kit with 7-AAD
Penicillin-Streptomycin (10,000 U/mL)
Propidium Iodide
RIPA Lysis and Extraction Buffer
RNeasy Mini Kit (250)
RPMI-1640
Trypsin-EDTA (0.25%), phenol red

Name of Materials/Equipment/Software
anti - p-IκBα (B-9)
anti-BclxL (H-5)
anti-PARP 1 (C2-10)
anti-β-actin (C4)
BD FACSVerse
Synergy HTX Multi-Mode Microplate Reader
CO2 incubator
Conical tube 15 ml
Conical tube 50 ml
Corning Costar Ultra-Low Attachment Multiple Well Plate
Corning Costar Ultra-Low Attachment Multiple Well Plate
Costar 50 mL Reagent Reservoirs, 5/Bag, Sterile
Countess Cell Counting Chamber Slides
Countess II FL Automated Cell Counter
EnSpire Multimode Reader

Eppendorf Research Plus Multi Channel Pipette, 8-channel
FlowJo software
Goat Anti-Mouse IgG (H+L)
Goat Anti-Rabbit IgG (H+L)
GraphPad Prism 5
ImageJ
ImageQuant LAS 4000 mini
Incubated shaker
Multi Gauge Ver. 3.0,
Optical density (OD)LAMBDA UV/Vis Spectrophotometers
Phase-contrast microscope
SPL microcentrifuge tube 1.5mL
SPL Multi Channel Reservoirs, 12-Chs, PS, Sterile
StepOnePlus Real-Time PCR system
Vibra-Cell Ultrasonic Liquid Processors
Vinyl Anaerobic Chamber

Company	Catalog Number
Biorad	4561036
Thermo Fisher Scientific	4311971
Intron	IBS-BT031A
Intron	IBS-BT014
Corning	SCT-200-C
BD	214010
BD	DF0881-17-5
Promega	G9681
Sigma-Aldrich	11697498001
Corning	21-040-CV
fisher Scientific	IPVH00010
Corning	352235
Thermo Fisher Scientific	16000044
Biorad	1708890
Biorad	1725121
Korean Collection for Type Cultures	KCTC 3112
Sigma-Aldrich	C6852-25G
TCI	M0185
Applied Biosystems	4346906
Millipore	SLGS033SB
Biolegend	640934
Thermo Fisher Scientific	15140122
Introgen	P1304MP
Thermo Fisher Scientific	89901
Qiagen	74106
Gibco	11875-119
Thermo Fisher Scientific	25200056

Company	Catalog Number
Santa cruze	sc-8404
Santa cruze	sc-8392
Santa cruze	sc-53643
Santa cruze	sc-47778
BD Biosciences	
BioT	S1LFA
Thermo fisher	HERAcell 150i
SPL	50015
SPL	50050
Sigma-Aldrich	CLS7007
Sigma-Aldrich	CLS3471
Costar	4870
Thermofisher	C10228
invitrogen	AMQAF1000
Perkin Elmer	

Eppendorf	3122000051
TreeStar	
Jackson immunoresearch	115-035-062
Jackson immunoresearch	111-035-144
GraphPad Software	
NIH	
Fujifilm	
Lab companion	SIF-6000R
Fujifilm	
Perkin Elmer	
Olympus	
SPL	60015
SPL	21012
Thermo Fisher Scientific	
SONICS-vibra cell	VC 505
COY LAB PRODUCTS	

Comments/Description
Pkg of 10
100 covers
1 L
1 L
500 Tubes/Pack, 10 Packs/Case
500 g
500 g
100µl/assay in 96-well plates
vial of 20 tablets
500 mL
26.5cm x 3.75m roll; Pore Size: 0.45um
25/Pack, 500/Case
500 mL
25 x 20 µL rxns
5 x 1 mL
25 g
500 g
20 plates
250
100 tests
100 mL
100 mg
250 mL
250
500 mL
100 mL

Comments/Description
200 µg/mL
200 µg/mL
50 µl ascites
200 µg/mL
San Diego, CA, USA
Enspire 2300

Ashland, OR, USA
1.5 mL
2.0 mL
Inc., San Diego, CA, USA
Tokyo, Japan
Tokyo, Japan
Waltham, MA, USA
Tokyo, Japan
Waltham, MA, USA
500 Watt ultrasonic processor

Rebuttal Letter

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Journal: Journal of Visualized Experiments (ISSN 1940-087X)

Manuscript ID: JoVE61285

Type: Article

Title: Evaluating Cell Death using Cell-Free Supernatant of Probiotics in Three-Dimensional Spheroid Cultures of Colorectal Cancer Cells

Authors: Jina Lee[1,*], Joo-Eun Lee[2,*], Seil Kim[1,3], Dukjin Kang[1] and Hee Min Yoo[1]

We thank the Reviewers for their thoughtful comments. We tried to address the comments with additional experimental data and our manuscript underwent extensive English editing. We sincerely hope that the revisions will render this manuscript suitable for publication in *Journal of Visualized Experiments.*

Response to Editorial Comments

We are profoundly grateful for your valuable comments on our manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Our manuscript underwent extensive English editing by English editing service during revision for checking grammar, spelling and some improvement of style. We appreciate the kind comments made by the editor.

2. Please revise lines 259-261, 265-267, and 286-287 to avoid textual overlap with previously published work.

We corrected the sentence in the manuscript according to your kind comments

Lines 291-293: However, 2D cultures have several disadvantages, such as the disruption of cellular interactions as well as alterations in cell morphology, extracellular environments, and the approach of division.

Lines 293-300: 3D scaffolding was developed for tissue engineering. It acts as a surrogate loss of ECM, representing the available space of tumor cells. In addition, the scaffolding provides physical interactions for cell adhesion and proliferation and causes cells to form appropriate spatial distributions and cell-ECM or cell-cell interactions.

Lines 319-320: Western blotting using spheroids can lead to quantitative insights into the state of various signaling molecules.

3. Keywords: Please provide at least 6 keywords or phrases.

We added the keywords in the manuscript according to your kindly comments.

Keywords: Probiotics, *Lactobacillus fermentum*, Colorectal cancer, Spheroid, 3D culture, Cell-free supernatant (CFS)

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Perkin Elmer, LAMBDA, falcon, CellTiter 96 Aqueous, EnSpire, iTaq™ Universal SYBR®, StepOnePlus, etc.

We removed all commercial language from our manuscript according to your comments.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have now added more details to our protocol steps based on your kind comments and suggestions.

6. 1.2, 1.5: Please specify the incubation temperature.

- 1.2: "Pre-incubate the MRS agar plate in an anaerobic chamber at 20 ppm Oxygen, H2." has been changed to "Pre-incubate the MRS agar plate in a H_2 anaerobic chamber at 37 ℃ and 20 ppm oxygen."
- 1.5: "Incubate the bacteria for 4-6 days in an anaerobic chamber and passage every 2 days until single bacteria colonies are obtained." has been changed to "Incubate the bacteria for 2-3 days in a H_2 anaerobic chamber at 37 ℃ and 20 ppm oxygen and passage every 2 days until single bacteria colonies are obtained."

7. 1.5: Is the chamber the same as that used in step 1.4?

We used the same anaerobic chamber as described in 1.4. In addition, we have been changed the sentence as "Incubate the bacteria for 2-3 days in a H_2 anaerobic chamber at 37 ℃ and 20 ppm oxygen and passage every 2 days until single bacteria colonies are obtained."

8. 1.6: Please describe how to sterilize the tube.

Thank you for the valuable comments. As suggested, we added more detailed information.

Additions

1.6. Wash and dry the hungate tube
1.7. Incubate the hungate tube in a H_2 anaerobic chamber at 37 ℃and 20 ppm oxygen to exclude oxygen
1.8. Place 2-3 mL of MRS broth into the tube
1.9. Seal the tube with a buthyl rubber stopper and screw the cap
1.10. Autoclave the hungate tube at 121 ℃ for 15 min.
1.11. After autoclaving, incubate the hungate tube in a H_2 anaerobic chamber at 37 ℃ and 20 ppm oxygen

9. 1.11: What volume of RPMI 1640 is used to resuspend the pellet?

1.17: Separate the bacterial pellets and the conditioned media using a centrifuge for 15 min at 1000 x g. Wash the collected bacterial pellets (with PBS) and resuspend in 4 mL RPMI 1640 containing 10 % fetal bovine serum without antibiotics.

10. 3.4: Each well of what? Please specify.

- 3.4: "Remove as much of the growth medium as possible from each well using a 200p pipette." has been changed to "Remove as much of the growth medium as possible from each of the wells containing the spheroids using a 200 µL pipette."

11. 5.4-5.6: Please provide more details here. For PCR, please specify primers and conditions used.

Thank you for the valuable comments. As suggested, we added more detailed information

5. Quantitative real-time polymerase chain reaction analysis for spheroids.

5.1. For each condition, prepare 10-15 spheroids in a 2 mL tube and centrifuge for 3 min at 400 x g
5.2. Discard the supernatant and wash the spheroids twice in 1 mL of ice-cold 1X PBS (note: avoid centrifugation, let the spheroids settle down).
5.3. Aspirate as much of the 1X PBS as possible and isolated the RNA
5.4. Synthesize cDNA from 1 µg of RNA
5.5. Prepare a master mix to run all samples in triplicate (refer to the Table of Materials)

5.6. Perform the cDNA synthesis by placing 20 µL of the template master mix into each qPCR plate well.

5.7. Mix reactions well and spin if necessary.
5.8. Run samples as per the recommendations of the instrument manufacturer.

Table 1. PCR reaction mixture

Reactions	Volume per single 20 µL
2X qPCR mix	10 µL
Forward primer (10 pmols/µl)	1 µL
Reverse primer (10 pmols/µl)	1 µL
cDNA (50 ng/µl)	1 µL
PCR grade water	7 µL

Table 2. Primer sequences used in qRT-PCR analysis.

Primer	Primer sequences (5' -> 3')
BAX-Forward	CCCGAGAGGTCTTTTTCCGAG
BAX-Reverse	CCAGCCCATGATGGTTCTGAT
BAK-Forward	ATGGTCACCTTACCTCTGCAA
BAK-Reverse	TCATAGCGTCGGTTGATGTCG
NOXA-Forward	ACCAAGCCGGATTTGCGATT
NOXA-Reverse	ACTTGCACTTGTTCCTCGTGG
18s rRNA-Forward	GATGGGCGGCGGAAAATAG
18s rRNA-Reverse	GCGTGGATTCTGCATAATGGT
β-Actin-Forward	TCCTGTGGCATCCACGAAACT
β-Actin-Reverse	GAAGCATTTGCGGTGGACGAT

Table 3. qRT-PCR conditions

Stage	Temp (ºC)	Time
Initial denaturation	95	10 min
40 cycles:		
Step 1	95	15 sec
Step 2	60	60 sec
Melting curve stage	95	15 sec
	60	60 sec
	95	15 sec

12. 2.1.4, 5.1, 6.1, 6.6, 8.6, 8.10, etc.: Please list all centrifugation speeds in terms of centrifugal g-force (x g) instead of rpm.

We have all changed as recommended centrifugation speed in terms of centrifugal force g- (x g).

-2.1.4: Transfer the dissociated cells to a 15 mL conical tube and centrifuge for 3 min at 300 x g.
-5.1: For each condition, prepare 10-15 spheroids in a 2 mL tube and centrifuge for 3 min at 400 x g.
-6.1: For each condition, prepare 30-40 spheroids in a 2 mL tube.
-6.6: Centrifuge the protein lysates for 15 min at 15000 x g and 4 °C.
-8.2 : For each condition, prepare 30-40 spheroids in a FACS tube and centrifuge for 3 min at

400 x *g* and RT.
- 8.6: Centrifuge the dissociated cells at 400 x g and 4 °C for 3 minutes (note: FACS buffer = 1X PBS + 2.5% FBS, filtered using a 0.22um top filter).
-8.10: Centrifuge the dissociated cells at 400 x *g* for 3 min

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

We are grateful for your comments. As suggested, we highlighted the protocol text to be featured in the video in yellow.

14. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We are grateful for your comments. As suggested, we uploaded each Figure individually as .tiff files.

15. Figures: Please include a space between all numbers and the corresponding unit: 20 ppm, 37 °C, 24 h, etc.

We thank the reviewer for their helpful comment. As suggested, we included a space between all numbers and the corresponding unit.

16. Figure 4: Please submit multipanel figures (A, B, C, etc.) as a single image file that contains the entire figure.

We are grateful for your comments. As suggested, we submitted a single image file

17. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

We are grateful for your comments. As suggested, we removed symbols from the table.

Response to Reviewer 1 Comments

We are profoundly grateful for your valuable comments on our manuscript.

Reviewer #1:
Manuscript Summary:
Anew approach to cancer cell and probiotic bacteria association in 3D models, Good working

Major Concerns:
N/A
Minor Concerns:
Maybe it can be tried a bifidobacterium culture models next time

Thank you for the valuable comments. As you recommended, we will develop a *bifidobacterum* culture model for the next study.

Response to Reviewer 2 Comments

We are profoundly grateful for your valuable comments on our manuscript.

Reviewer #2:
Manuscript Summary:
ABSTRACT
L 36-37 "The use of Lactobacillus cell-free supernatant (LCFS) could potentially be a better option", …The sentence needs to be rephrased as it is missing the meaning…..LCFS IS A BETTER OPTION THAN WHAT?

We corrected the sentence in the manuscript according to your kind comments

Lines 37-39: The use of Lactobacillus cell-free supernatant (LCFS) in spheroids could potentially be a better option than testing in 2D monolayers, especially as *L. fermentum* is able to produce anti-cancer effects within the gut.

L 43 it is not clear what abbreviation CLC stands for

We truly apologize for using incorrect abbreviation. We have corrected our English la nguage errors based on your kind comments and suggestion

-Lines 43: “CLCs” has been changed to “CRCs (colorectal cancer cells).”

PROTOCOL
L 78 misspelled word "Lactobacillus"

We truly apologize for using incorrect wording. We have corrected our English langua ge errors based on your kind comments and suggestion

-Lines 7: “Lactibacillus” has been changed to “Lactobacillus”

L 79 the growing condition is not clear: it should be overnight or for 2 days?
Why is so important to have 20ppm O2, H2? Lactobacillus strains don't need strict anaerobic conditions. They will grow in CO2 incubator and there is no need to pre-incubate MRS plate in anaerobic chamber.

We sincerely appreciate the reviewer's thoughtful comments. As the reviewer pointed out, Lactobacillus strains are facultative anaerobic bacteria that do not require strict anaerobic conditions. Oxygen concentrations in the lumen of the human intestine are known to affect the spatial distribution and metabolism of gut flora [1]. Most intestinal bacteria are obligate anaerobes, many of which fail to grow at oxygen concentrations greater than ~0.5% [2]. Lactic acid bacteria (LAB) are anaerobic, aerotolerant (not aerophilic) microorganisms that lack the capability to synthesize active electron transport chains [3]. Therefore, a more in vivo-like intestinal tissue microenvironment including anaerobes significantly influences the composition of microbial metabolism [4]. Interestingly, lactic acid bacteria are generally used in the food industry for anaerobic fermentation to produce anaerobic metabolite [5,6]. In summary, we used anaerobic conditions when growing Lactobacillus to mimic in the vivo-like microenvironment.

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L81 now it is even more confusing. In line 79 we should incubate for 2 days, in next step author suggests incubation for another 4-6 days? Why? To obtain single colonies there is no need to passage bacteria for almost 6 days, but using streaking microbiological technique it can be obtain even after first incubation period.

We truly apologize for using an inconsistent sentence that could confuse respectful readers. We have corrected the sentence as follows.

Lines 82: "Incubate the bacteria for 4-6 days in an anaerobic chamber and passage every 2 days until single bacteria colonies are obtained." has been changed to "Incubate the bacteria for 2-3 days in a H_2 anaerobic chamber at 37 ℃ and 20 ppm oxygen and passage every 2 days until single bacteria colonies are obtained."

L88 I would suggest placing the bacteria to CO2 incubator.

We sincerely appreciate the reviewer's thoughtful comments.
-Lines 97: " Incubate the MRS broth media in a shaker incubator for 2 days (37°C, 200rpm)" has been changed to "Incubate the MRS broth media in a shaker incubator for 2 days (37 °C, 5 % CO_2, 200 rpm)"

L89-91 measurement of OD is not clear, author suggest monitoring bacterial growth curves AFTER incubation?

We sincerely appreciate the reviewer's thoughtful comments. We suggest monitoring bacterial growth curves before and after incubation to obtain a consistent amount of LCFS.

L103 missing information about cultivating temperature (presence /absence CO2)

Thank you for the valuable comments.

-Lines 111: " Grow DLD-1, HT-29, and WiDr cell lines as monolayers until 70-80 % confluency RPMI containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin." has been changed to "Grow DLD-1, HT-29, and WiDr cell lines as monolayers until 70-80 % confluency and incubate the plate at 37 °C in a 5 % CO_2 incubator (growth medium: RPMI containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin)."

L110 the sentence needs to be rephrased PLACE THE TUBE WHERE?
We truly apologize for using an inconsistent sentence that could confuse respectful readers.
- Lines 119: " Place the 15 ml conical tube containing the dissociated cells and centrifuge for 3 min at 1200 rpm. " has been changed to " Transfer the dissociated cells to a 15 mL conical tube and centrifuge for 3 min at 300 x g."

L117 wrong sentence from gramma point of view
We truly apologize for using the incorrect sentence.

Lines 125: Dilute the cells in a 15 mL conical tube to obtain 1-2 x 10^5 /mL.

L118-120 the procedure is not clear at all. Do we need to determine methylcellulose density? Methylcellulose must be 0,2 % in each well? I don't understand this step. Please make it more clear.

Thank you for your comments. We apologize for using incorrect methylcellulose concentration. We have corrected the sentence.
.
Lines 126: "Add 0.2 % methylcellulose and transfer the diluted cells to a sterile reservoir. Use a multichannel pipette to dispense 200 µl/well into an ultra-low attachment 96-well round bottom microplate. (Each cell line and methylcellulose density should be determined. (Figure 1B (iii))" has been changed to "Add final concentration of 0.6 % methylcellulose and transfer the diluted cells to a sterile reservoir. Use a multichannel pipette to dispense 200 µL/well into an ultra-low attachment 96-well round bottom microplate. Each cell line and methylcellulose density should be determined (Figure 1B (iii))"

L130 /L194 200p pipette - what is „p"?

We truly apologize for using incorrect abbreviation. We have corrected our English la nguage errors based on your kind comments and suggestion

- Lines 138: "Remove as much of the growth medium as possible from each well using a 200p pipette." has been changed to " Remove as much of the growth medium as possible from each

well using a 200 µL pipette."

- Lines 139: "Add 1 ml of FACS buffer and gently dissociate the spheroids using a 200p pipette." has been changed to " Add 1 mL of FACS buffer and gently dissociate the spheroids using a 200 µL pipette."

L131 no information about volume of added growth media with LCFS / need more detailed specification. It is also not clear how long was the incubation time for different analyses.

Thank you for the valuable comments. As suggested, we added more detailed information.

Additions

-Lines 139: " Add the growth media with LCFS and incubate at 37 °C in a 5 % CO_2 incubator for 24-48 h. NOTE: The volume to be used will depend on the plate size as follows: 2 mL for 6-well cell culture plates; 200 µL for 96-well cell culture plates.

L135 incubation time in step 3.5 is stated 24-48h. Here we should perform viability assay after 24-72h (?) Please clarify this.

Thank you for your comments. We apologize for using the incorrect incubation time for the experiments. To investigate whether the cells underwent apoptosis, LCFS-treated cells and untreated cells were incubated at 37 °C for 48 hours before a significant portion of the cell populations died.

Correction

-Lines 143: "Prepare 8-10 LCFS-treated colorectal cancer spheroids in an ultra-low attachment 96-well round bottom microplate (cell viability assays are performed 24-72 hours after LCFS treatment)" has been changed to "Prepare 8-10 LCFS-treated colorectal cancer spheroids in an ultra-low attachment 96-well round bottom microplate (cell viability assays are performed 48 hours after LCFS treatment)"

L153 What was measured by RT-qPCR - not stated

Thank you for the valuable comments. We measured BAX, BAK and NOXA. To clarify, we added more detailed information

5. Quantitative real-time polymerase chain reaction analysis for spheroids.

5.1. For each condition, prepare 10-15 spheroids in a 2 mL tube and centrifuge for 3 min at 400 x g
5.2. Discard the supernatant and wash the spheroids twice in 1 mL of ice-cold 1X PBS (note: avoid centrifugation, let the spheroids settle down).
5.3. Aspirate as much of the 1X PBS as possible and isolated the RNA
5.4. Synthesize cDNA from 1 µg of RNA
5.5. Prepare a master mix to run all samples in triplicate (refer to the Table)
5.6. Perform the cDNA synthesis as a 20 µL of the template master mix into each qPCR plate

well.
5.7. Mix reactions well and spin if necessary.
5.8. Run samples as per the recommendations of the instrument manufacturer.

Table 1. PCR reaction mixture

Reactions	**Volume per single 20 µL**
2X qPCR mix	10 µL
Forward primer (10 pmols/µl)	1 µL
Reverse primer (10 pmols/µl)	1 µL
cDNA (50 ng/µl)	1 µL
PCR grade water	7 µL

Table 2. Primer sequences used in qRT-PCR analysis.

Primer	**Primer sequences (5' -> 3')**
BAX-Forward	CCCGAGAGGTCTTTTTCCGAG
BAX-Reverse	CCAGCCCATGATGGTTCTGAT
BAK-Forward	ATGGTCACCTTACCTCTGCAA
BAK-Reverse	TCATAGCGTCGGTTGATGTCG
NOXA-Forward	ACCAAGCCGGATTTGCGATT
NOXA-Reverse	ACTTGCACTTGTTCCTCGTGG
18s rRNA-Forward	GATGGGCGGCGGAAAATAG
18s rRNA-Reverse	GCGTGGATTCTGCATAATGGT
β-Actin-Forward	TCCTGTGGCATCCACGAAACT
β-Actin-Reverse	GAAGCATTTGCGGTGGACGAT

Table 3. qRT-PCR conditions

Stage	**Temp (ºC)**	**Time**
Initial denaturation	95	10 min
40 cycles:		
Step 1	95	15 sec
Step 2	60	60 sec
Melting curve stage	95	15 sec
	60	60 sec
	95	15 sec

L161 missing the information about time of incubation on ice. However, spheroids are after centrifugation, aren't they settled down? What is the point of this step?

We truly apologize for using an incorrect sentence. We have corrected the sentence.

Correction

-Lines 167-168
6.1. For each condition, prepare 30-40 spheroids in a 2 mL tube.
6.2. Place the tube on ice and let the spheroids settle down to the bottom of the 2 mL tube.

L168 Is the centrifugation step final step in Western blotting procedure?

Thank you for your comments. More detailed method was described as bellow;

Addition

-6.7. Determine the protein concentration for each cell lysate
-6.8. Boil each cell lysate in the sample buffer at 100 ˚C for 10 min.
-6.9. Load equal amounts of protein into the wells of the SDS-PAGE gel and run the gel for 1-2 h at 100V.
-6.10. Transfer the protein from the gel to the PVDF membrane.
-6.11. After transferring, block the membrane for 1 h at room temperature using a blocking buffer (5 % skim milk + TBS with 0.05 % Tween-20).
-6.12. Incubate the membrane with 1:1000 dilutions of primary antibody in TBST with 5 % BSA buffer at 4 ˚C overnight. Refer to the Table of Materials.
-6.13. Wash the membrane three times with TBST, 15 min for each wash.
-6.14. Incubate the membrane with 1:2500 dilutions of secondary antibody in the blocking buffer at room temperature for 2 h. Refer to the Table of Materials.
-6.15. Wash the membrane three times with TBST, 15 min for each wash.
-6.16. Prepare the membrane for HRP detection with a Chemiluminescent substrate. Refer to the Table of Materials.
-6.17. Acquire chemiluminescence images.

L208 "several groups" …does it mean each spheroid was prepared by different group?

We truly apologize for using incorrect word. We have corrected our English language errors based on your kind comments and suggestion

“In the present study, several groups developed spheroid cultures from diverse cancer cell types” has been changed to “In the present study, several research groups developed spheroid cultures from diverse cancer cell types”

L213-L214 in the section 2.2.3 is stated to use 0,2 % methylcellulose, but now it is between 0 and 1,2%. So again, very confusing.

Thank you for your comments. We apologize for using an incorrect methylcellulose concentrations. We have corrected the sentence.

.

2.2.3.: “Add 0.2 % methylcellulose and transfer the diluted cells to a sterile reservoir. Use a multichannel pipette to dispense 200 µl/well into an ultra-low attachment 96-well round bottom microplate. (Each cell line and methylcellulose density should be determined. (Figure 1B (iii))” has been changed to “Add final concentration of 0.6 % methylcellulose and transfer the diluted cells to a sterile reservoir. Use a multichannel pipette to dispense 200 µL/well into an ultra-low

attachment 96-well round bottom microplate. Each cell line and methylcellulose density should be determined (Figure 1B (iii)).”

L235-238: “ As shown in Figure 2A, concentrations of methylcellulose between 0 and 1.2 % transform the compactness and morphology of the spheroids. This result indicates that our methylcellulose protocol may be capable of obtaining consistent spheroids from several types of colorectal cancer.” has been changed to “As shown in Figure 2A, a methylcellulose concentration of 0.6 % transforms the compactness and morphology of the spheroids. This result indicates that our methylcellulose protocol may be capable of obtaining consistent spheroids from several types of colorectal cancer.”

L217 "we treated the spheroids with LCFS" ..... spheroids treated with what concentration of methylcellulose - how did you obtain spheroids?

Thank you for your comments. When we generated spheroids we added 0.6 % methylcellulose in the growth media that contained 1-2 x 10^5ml diluted cells. We then dispensed 200 µl/well into an ultra-low attachment 96-well round bottom microplate and incubated the plate at 37 °C in a 5 % CO2 incubator for 24-36 hours. After 24-36 hours, the generated spheroid was treated with 25 % LCFS and we measured the morphology after 48 hours using a light microscope. To clarify, we corrected the sentence as below:

Line 238-239:
“Next, we treated the spheroids with LCFS and measured the morphology after 48 hours using a light microscope.” has been changed to “Next, we treated the spheroids with 25 % LCFS and measured the morphology after 48 h using a light microscope.”

L220 "dosages of 0,6, 12.5, 25"….missing the unit. In step 3.3 is stated also dosage of 50. It will not be used?

We truly apologize for the confusion. Although the LCFS experiment was performed with dosages of 50 %, only 25 % LCFS was determined as there was no difference in cell death between 50 % and 25 % LCFS. To clarify the method, we deleted the 50 % LCFS part in Step 3.3.

Correction

-Lines 136. Inoculate the LCFS stock solution into a growth medium. Dilute to 25 %, 12.5 %, and 6 % in the growth medium (i.e., 25 % LCFS = 150 µL growth medium + 50 µL LCFS)

L222-224 in 4.1 is stated that viability was tested after 24-72 hours. Here after 48 hours only. Please clarify this.

Thank you for your comments. We apologize for using the incorrect incubation time for the experiments. To investigate whether the cells underwent apoptosis, LCFS-treated cells and untreated cells were incubated at 37 °C for 48 hours before a significant portion of the cell populations died.

Correction

-Lines 143: “Prepare 8-10 LCFS-treated colorectal cancer spheroids in an ultra-low attachment 96-well round bottom microplate (cell viability assays are performed 24-72 hours after LCFS treatment)” has been changed to “Prepare 8-10 LCFS-treated colorectal cancer spheroids in opaque-walled multi-well plates (cell viability assays are performed 48 h after LCFS treatment).”

L228 "including BAX, BAK, and NOXA" authors did not state in the methods that they detected these markers.

Thank you for the valuable comments. To clarify, we added more detailed information

Additions

5. Quantitative real-time polymerase chain reaction analysis for spheroids.

5.1. For each condition, prepare 10-15 spheroids in a 2 mL tube and centrifuge for 3 min at 400 x g
5.2. Discard the supernatant and wash the spheroids twice in 1 mL of ice-cold 1X PBS (NOTE: avoid centrifugation, let the spheroids settle down).
5.3. Aspirate as much of the 1X PBS as possible and isolated the RNA
5.4. Synthesize cDNA from 1 µg of RNA
5.5. Prepare a master mix to run all samples in triplicate. Refer to the material table.
5.6. Perform the cDNA synthesis as a 20 µL of the template master mix into each qPCR plate well.
5.7. Mix reactions well and spin if necessary.
5.8. Run samples as per the recommendations of the instrument manufacturer..

Table 1. PCR reaction mixture

Reactions	**Volume per single 20 µL**
2X qPCR mix	10 µL
Forward primer (10 pmols/µl)	1 µL
Reverse primer (10 pmols/µl)	1 µL
cDNA (50 ng/µl)	1 µL
PCR grade water	7 µL

Table 2. Primer sequences used in qRT-PCR analysis.

Primer	Primer sequences (5' -> 3')
BAX-Forward	CCCGAGAGGTCTTTTTCCGAG
BAX-Reverse	CCAGCCCATGATGGTTCTGAT
BAK-Forward	ATGGTCACCTTACCTCTGCAA
BAK-Reverse	TCATAGCGTCGGTTGATGTCG
NOXA-Forward	ACCAAGCCGGATTTGCGATT
NOXA-Reverse	ACTTGCACTTGTTCCTCGTGG
18s rRNA-Forward	GATGGGCGGCGGAAAATAG
18s rRNA-Reverse	GCGTGGATTCTGCATAATGGT
β-Actin-Forward	TCCTGTGGCATCCACGAAACT
β-Actin-Reverse	GAAGCATTTGCGGTGGACGAT

Table 3. qRT-PCR conditions

Stage	Temp (°C)	Time
Initial denaturation	95	10 min
40 cycles:		
Step 1	95	15 sec
Step 2	60	60 sec
Melting curve stage	95	15 sec
	60	60 sec
	95	15 sec

Addition

6.6. Centrifuge the protein lysates for 15 min at 15000 x g and 4 °C.
6.7. Determine the protein concentration for each cell lysate
6.8. Boil each cell lysate in a sample buffer at 100 °C for 10 min.
6.9. Load equal amounts of protein into the wells of the SDS-PAGE gel and run the gel for 1-2 h at 100V.
6.10. Transfer the protein from the gel to the PVDF membrane.
6.11. After transferring, block the membrane for 1 h at room temperature using a blocking buffer (5 % skim milk + TBS with 0.05 % Tween-20).
6.12. Incubate the membrane with 1:1000 dilutions of primary antibody in TBST with 5 % BSA buffer at 4 °C overnight. Refer to the Table of Materials.
6.13. Wash the membrane three times with TBST, 15 min for each wash.
6.14. Incubate the membrane with 1:2500 dilutions of secondary antibody in the blocking buffer at room temperature for 2 h. Refer to the Table of Materials.
6.15. Wash the membrane three times with TBST, 15 min for each wash.
6.16. Prepare the membrane for HRP detection with a Chemiluminescent substrate. Refer to the Table of Materials.
6.17. Acquire chemiluminescent images..

Table 4. Antibodies used in western blot analysis.

Antibody	Dilution
PARP 1 (C2-10)	1:1000
BclxL (H-5)	1:1000
p-IκBα (B-9)	1:1000
β-actin (C4)	1:1000
Goat Anti-Mouse IgG (H+L)	1:2500
Goat Anti-Rabbit IgG (H+L)	1:2500

L238/L242 (n=3) what „n" stands for? Is it the number of experiments or number of wells per one experiment. Please clarify it.

We sincerely appreciate the reviewer's thoughtful comments. We apologize for using i ncorrect nomenclature. It refers to the number of experimental replicates for each exp eriment. To clarify, we edited the sentence as follows.

Additions

- "n=3" has been change to " n=3 for each experiment"

L244 Fig 3D ht-29 cells: how can be viability more than 100%? different blue colours are percentage of what?? also data are represented as MEAN?? +/- SD? SEM? Moreover, MTS assay is not mentioned anywhere apart from line 222 (but NOT in methods)

We sincerely appreciate the reviewer's thoughtful comment. As recommended by the r eviewer, we represented the LCFS % in blue colors, and the statistical analysis within the figure legend and MTT was replaced with a cell viability assay.

Additions

- Figure 3: Evaluation of LCFS concentration and spheroid morphology. (A) Representative images of HT-29 treated with LCFS for 48 h. Scale bar 100 µm. (B) HT-29, DLD1, and WiDr spheroids treated with increasing doses of LCFS for 48 h. All spheroids had disrupted edges at 12.5-25 % LCFS. Scale bar 20 µm (n=3 for each experiment) (C) Spheroid morphologies of HT-29, DLD1, and WiDr spheroids treated with 25 % LCFS for 24 and 48 h. Scale bar 20 µm (n = 3) (D) Measured cell viability, shown as mean ± SEM. ***, P < 0.05 (n=3 for each experiment).
- Lines 246: After 48 h, the samples were assessed through a cell viability assay, and we found that apoptosis in spheroid colorectal cancers depended on the dosage of L CFS (Figure 3D).

L246 why authors did not show PI staining in spheroids from other cell types?

Thank you for your comments. As suggested, we performed PI staining in spheroids from other cell types. The data indicated that LCFS induced apoptosis not only in HT-29 spheroids but also in DLD1 and WiDr spheroids. We added these results in Figure 4.

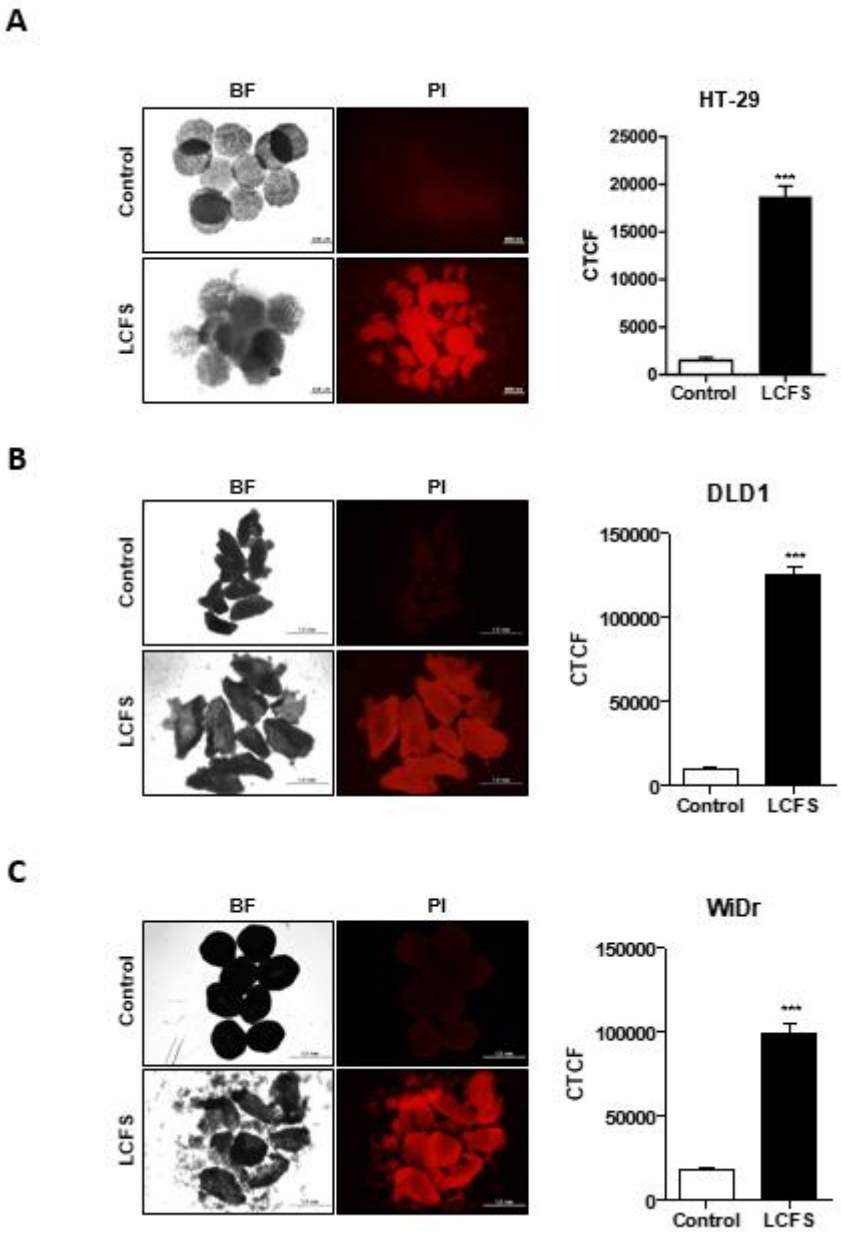

Figure 4: Propidium iodide staining of the spheroids. Representative images of PI staining in (A) HT-29, (B) DLD1, and (C) WiDr spheroids after 48 h of LCFS treatment. The images were identified using a fluorescence microscope and the increase in PI intensity was measured using Image J. Scale bar 10 µm. The mean ± SEM is shown. ***, $P < 0.05$ (n=3 for each experiment).

Additions

- Description of the results in the text: line 247-250
- Data: Figure 4A, B, C

L248/249 „Quantification of apoptosis markers, measured through qRT-PCR". data are represented as MEAN?? +/- SD? SEM? statistical analysis? *** is p < ????

We sincerely appreciate the reviewer's thoughtful comment regarding this important st atistical issue. As recommended by the reviewer, we added this information within the figure legend.

Additions

Figure 5: Apoptosis markers were identified using qRT-PCR. Apoptosis markers, such as BAX, BAK and NOXA, were quantified. mRNA is presented as a relative expressi on normalized to (A) β-actin and (B) 18s rRNA. The mean ± SEM is shown. ***, P < 0.05 (n=3 for each experiment).

L252 Why authors don't show FACS analysis of apoptosis in spheroids of other cell types?

Thank you for your comments. As suggested, we performed FACS analysis of apoptosis for DLD1 and WiDr spheroids.

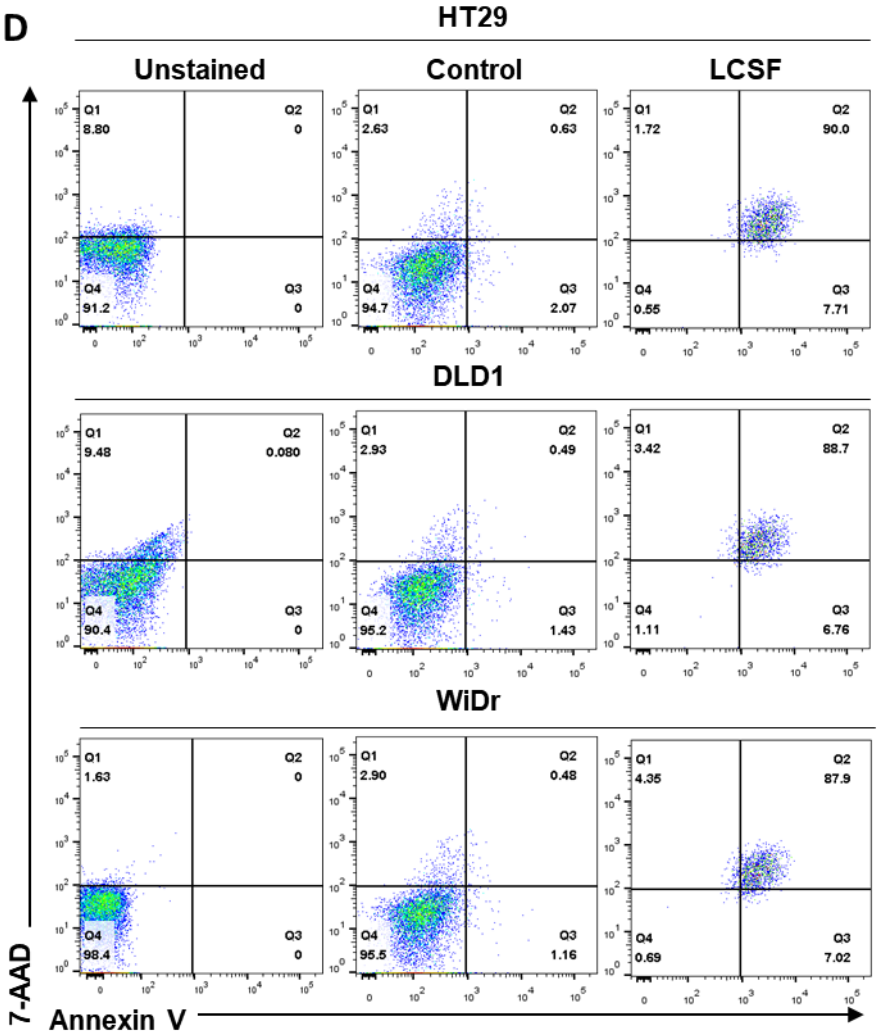

Figure 6. Apoptosis markers were determined via Western blotting and FACS analysis of the

spheroids. Shown in the figure are Western blots of (A) HT-29, (B) DLD1, and (C) WiDr cells after LCFS treatment. The antibodies that were detected were PARP1, Bcl-xL, and p-IκBα. β-actin expression was used as an internal control. (D) FACS analysis of apoptosis in HT-29, DLD1, and WiDr spheroids incubated with LCFS. Apoptotic cells were detected by the increase in the fluorescence intensity of Annexin V-FITC.

Additions

- Description of the results in the text: line 251-252
- Data: Figure 6D

Major Concerns:
Majority of steps listed in the procedure are not clearly explained and thus very confusing.

We thank the Reviewer for their thoughtful comments. We tried to clearly explain the majority of steps with additional experimental data and reviewer comments, and the manuscript underwent extensive English editing.

Minor Concerns:
English language need to be improved.

Our manuscript underwent extensive English editing by English editing service during revision for checking grammar, spelling and some improvement of style. We appreciate for reviewer kindly comments.

Response to Reviewer 3 Comments

We are profoundly grateful for your valuable comments on our manuscript.

Reviewer #3:
Manuscript Summary:
The manuscript aim the generation of a protocol to study the impact of probiotics in cancer spheroids. I recommended the following improvements.

Major Concerns:
1. The authors used MTS as viability assay (Figure 3). MTS is commonly used, however MTS has many limitations, I'd recommended additionally the use of CellTiter-Glo® Luminescent Cell Viability Assay optimized for spheroid analysis ratifying the findings of MTS assay.

We sincerely appreciate the reviewer's comments. As pointed out by the reviewer, we performed viability assay for HT-29, DLD1, and WiDr spheroids using the CellTiter-Glo® 3D Cell Viability assay to examine the anti-cancer effects of *Lactobacillus fermentum*. The CellTiter-Glo® 3D Cell Viability assay kit is more suitable for determining the number of

viable cells in 3D cell cultures as it depends on the quantitation of ATP present and is based on the original CellTiter-Glo® Luminescent Cell Viability Assay chemistry suggested by the reviewer. We have changed the results of the cell viability assessment to those obtained using the CellTiter-Glo® 3D Cell Viability assay in Figure 3D.

4. Cell viability assay for spheroids

4.1. Prepare 8-10 LCFS-treated colorectal cancer spheroids in opaque-walled multi-well plates (cell viability assays are performed 48 h after LCFS treatment).

4.2. Thaw the cell viability reagent at 4 °C overnight. Refer to the material table.

4.3. Equilibrate the cell viability reagent to room temperature before use.

4.4. Before observing the assay, remove 50 % of the growth media from the spheroid s.

4.5. Add 100 µL of cell viability reagent to each well.

NOTE: The volume to be used will depend on the plate size as follows: 100 µL for 96-well cell culture plates.

4.5. Mix the reagent vigorously for 5 min to promote cell lysis.

4.6. Incubate for 30 min–2 h at 37 °C.

4.7. Record the luminescence.

Additions

- Description of the method in the text: line 141-150

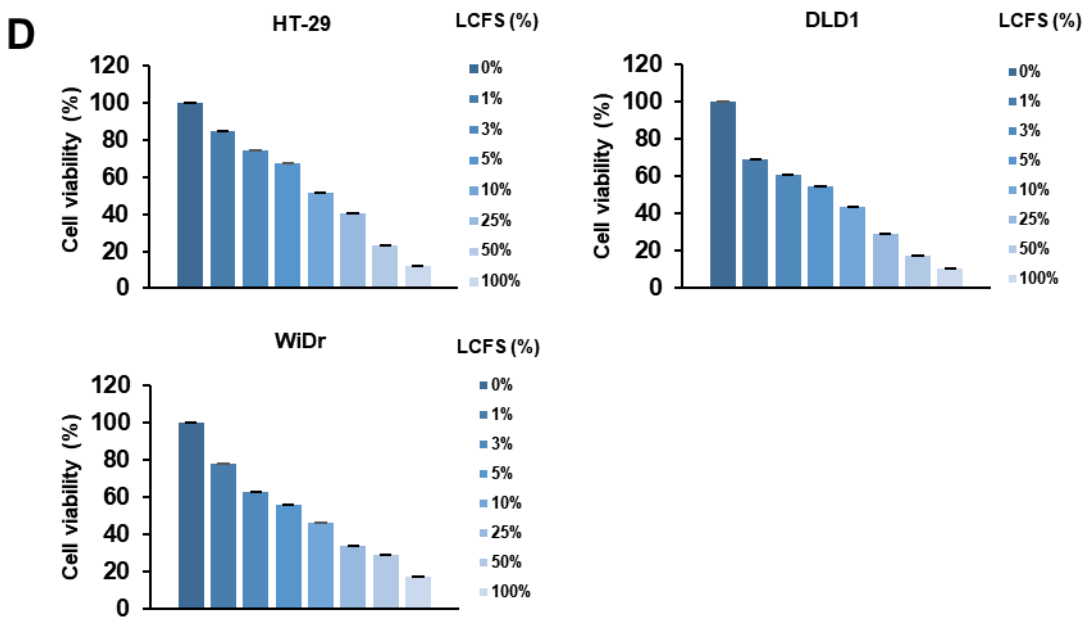

Figure 3: Evaluation of LCFS concentration and spheroid morphology. (A) Representative images of HT-29 treated with LCFS for 48 hours. Scale bar 100 µm. (B) HT-29, DLD1, and WiDr spheroids treated with increasing doses of LCFS for 48 h. All spheroids had disrupted edges at 12.5-25 % LCFS. Scale bar 20 µm (n=3 for each experiment) (C) Spheroid morphologies of HT-29, DLD1, and WiDr spheroids treated with 25 % LCFS for 24 and 48 h. Scale bar 20 µm (n = 3) (D) Measured cell viability, shown as mean ± SEM. ***, P < 0.05 (n=3 for each experiment).

Changes

- Description of the results in the text: line 246-247
- Data: Figure 3D

2. The authors does not describe the cat#, company and sequence of BAX, BAK, NOXA primers. Please provide full details of the primers used in the study.

Thank you for the valuable comments. To clarify, we added more detailed information

5. Quantitative real-time polymerase chain reaction analysis for spheroids.

5.1. For each condition, prepare 10-15 spheroids in a 2 mL tube and centrifuge for 3 min at 400x g
5.2. Discard the supernatant and wash the spheroids twice in 1 mL of ice-cold 1X PBS (note: avoid centrifugation, let the spheroids settle down).
5.3. Aspirate as much of the 1X PBS as possible and isolated the RNA
5.4. Synthesize cDNA from 1 µg of RNA
5.5. Prepare a master mix to run all samples in triplicate (refer to the Table)

5.6. Perform the cDNA synthesis as a 20 µL of the template master mix into each qPCR plate well.
5.7. Mix reactions well and spin if necessary.
5.8. Run samples as per the recommendations of the instrument manufacturer.

Table 1. PCR reaction mixture

Reactions	**Volume per single 20 µL**
2X qPCR mix	10 µL
Forward primer (10 pmols/µl)	1 µL
Reverse primer (10 pmols/µl)	1 µL
cDNA (50 ng/µl)	1 µL
PCR grade water	7 µL

Table 2. Primer sequences used in qRT-PCR analysis.

Primer	**Primer sequences (5' -> 3')**
BAX-Forward	CCCGAGAGGTCTTTTTCCGAG
BAX-Reverse	CCAGCCCATGATGGTTCTGAT
BAK-Forward	ATGGTCACCTTACCTCTGCAA
BAK-Reverse	TCATAGCGTCGGTTGATGTCG
NOXA-Forward	ACCAAGCCGGATTTGCGATT
NOXA-Reverse	ACTTGCACTTGTTCCTCGTGG
18s rRNA-Forward	GATGGGCGGCGGAAAATAG
18s rRNA-Reverse	GCGTGGATTCTGCATAATGGT
β-Actin-Forward	TCCTGTGGCATCCACGAAACT
β-Actin-Reverse	GAAGCATTTGCGGTGGACGAT

Table 3. qRT-PCR conditions

Stage	**Temp (°C)**	**Time**
Initial denaturation	95	10 min
40 cycles:		
Step 1	95	15 sec
Step 2	60	60 sec
Melting curve stage	95	15 sec
	60	60 sec
	95	15 sec

3. The Authors does not describe the housekeeping genes used to normalize the qPCR data. Please provide at least two housekeeping genes.

We sincerely appreciate the reviewer's thoughtful comments and helpful suggestions. We normalized qPCR using Actin and 18s rRNA. LCFS-treated spheroids exhibited similar effects

when normalized with Actin or 18s RNA. Therefore, LCFS effectively induced apoptosis in the 3D model. We added the results of the cell death assessment by qPCR in Figure 5.

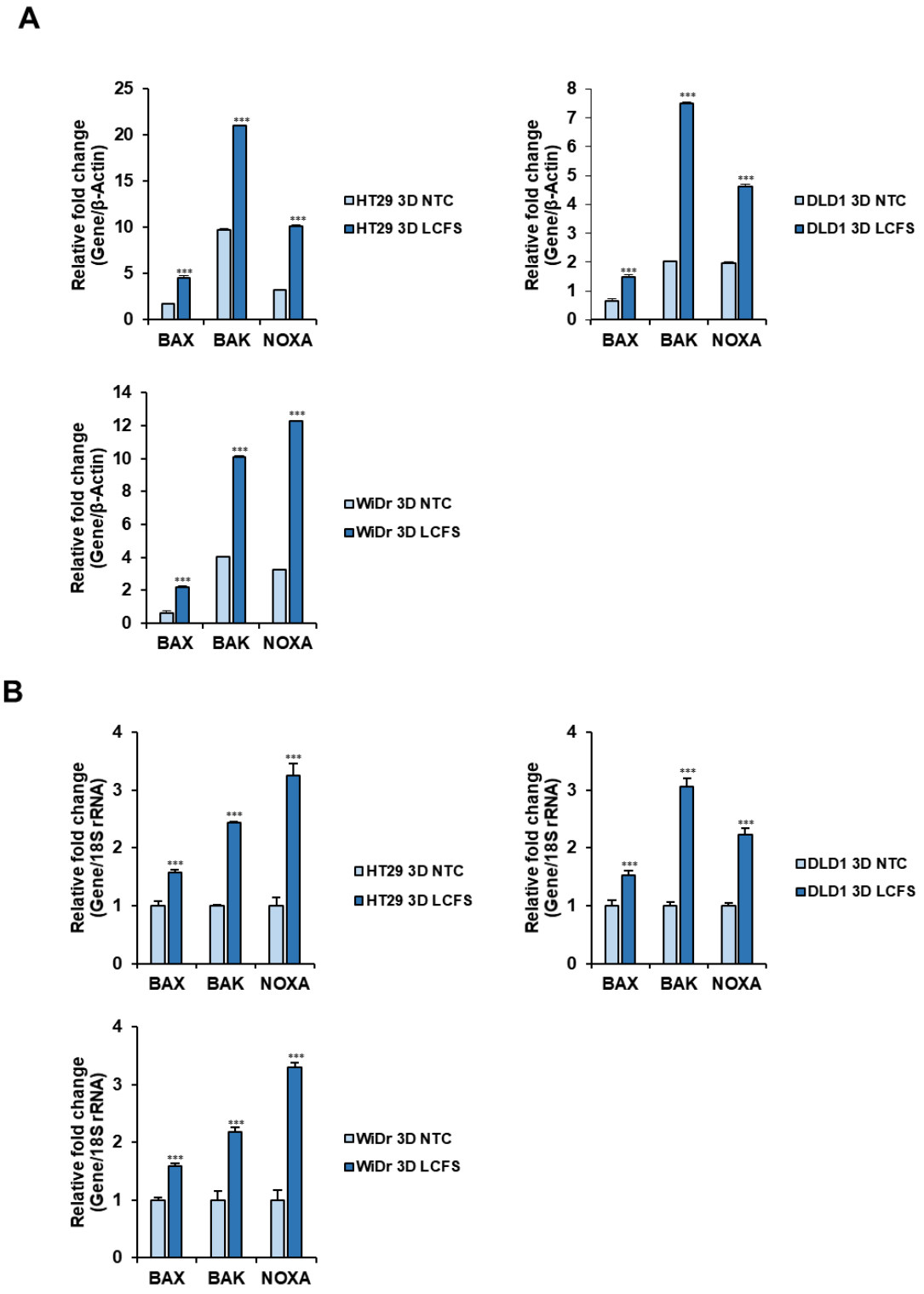

Figure 5: Apoptosis markers were identified using qRT-PCR. Apoptosis markers, such as BAX, BAK and NOXA, were quantified. mRNA is presented as a relative expression normalized to (A) β-actin and (B) 18s rRNA. The mean ± SEM is shown. ***, P < 0.05 (n=3 for each

experiment).

Additions

- Description of the results in the text: line 250-251
- Data: Figure 5A, B

4. Please provide a non-stained sample plot from each condition depicted in the FACS dot plot (Fig. 4F).

We sincerely appreciate the reviewer's comments. As pointed out by the reviewer, we added a non-stained sample plot from each condition.

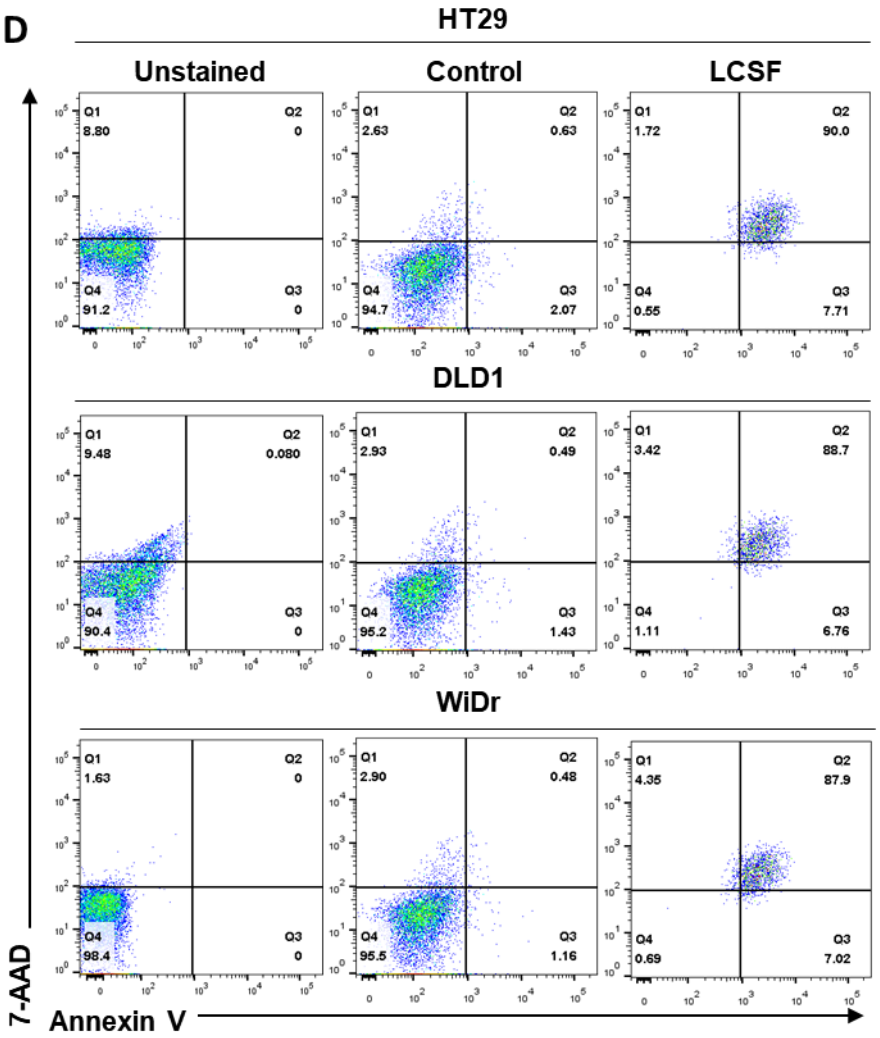

Figure 6. Apoptosis markers were determined via Western blotting and FACS analysis of the spheroids. Shown in the figure are Western blots of (A) HT-29, (B) DLD1, and (C) WiDr cells after LCFS treatment. The antibodies that were detected were PARP1, Bcl-xL, and p-IκBα. β-actin expression was used as an internal control. (D) FACS analysis of apoptosis in HT-29, DLD1, and WiDr spheroids incubated with LCFS. Apoptotic cells were detected by the increase in the fluorescence intensity of Annexin V-FITC.

Additions

- Description of the results in the text: line 251-252
- Data: Figure 6D

Minor Concerns:
1. I would rewrite the sentence "Probiotics are the most advantageous microorganisms in the gut that improve the host". Although the authors describe "what" probiotics "improve the host" in the next sentences, it does not sound appropriated to end this sentence with a unclear and general idea that probiotics "improve the host". I'd recommended include in the same sentence the benefits of probiotics as described in the lines 53-57.

We truly apologize for the vague expression. "Probiotics are the most advantageous microorganisms in the gut that improve the host" has been changed to "Probiotics are the most advantageous microorganisms in the gut that improves immune homeostasis and host energy metabolism."

Response to Reviewer 4 Comments

We are profoundly grateful for your valuable comments on our manuscript.

Reviewer #4:
Manuscript Summary:
Clear and well drafted

Major Concerns:
None

Minor Concerns:
None

Certificate of Editing

Imbac English Services

The following manuscript has been proofread by Imbac English Services for English grammar, language, and style errors.

Thesis Title: Assessing Cell Death using cell-free supernatant of probiotics in Three-Dimensional Spheroid Cultures of Cancer Cells

Authors: Jina Lee, Joo-Eun Lee, Dukjin Kang, Seil Kim, Hee Min Yoo

Issue Date: March 26th, 2020

Hee On Choi

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Title of Article:	Assessing Cell Death using cell−free supernatant of probiotics in Three−Dimensional Spheroid Cultures of Cancer Cells
Author(s):	Jina Lee, Joo−Eun Lee, Seil Kim, Dukjin Kang and Hee Min Yoo

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