Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Prebiotic and Probiotic: A Promising Tool in Human Colon Cancer Prevention

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THIS study examines the action of probiotics (*Enterococcus faecium*) and prebiotics (glucan and mannan of *Saccharomyces cerevisiae* cell wall) in the prevention of human colon cancer using the Caco-2 cell line; by measuring the adhesion between probiotics, prebiotics, and Caco-2 cell by plate count agar technique and confocal microscope, estimation of mucin expression by measuring (MUC-2gene), estimation of cytokine expression by measuring interleukin 6, 8, 10 (IL6, IL8, IL10), CXCL1 and tumor necrosis factor (TNF) genes. The confocal microscope indicated the valuable of probiotics and prebiotics in modifying the propagation and apoptosis of cancer cells. The data indicated that the Enterococcus faecium probiotic strain induces elevation on the expression of cxcl1, and IL10 genes and decrease the expression of IL6, IL8, MUC, and TNF genes. While the glucan and mannan of Saccharomyces cerevisiae cell wall induce elevation in the expression of IL6, and MUC genes and decrease the expression of cxcl1, IL8, IL10, and TNF genes. Synbiotic (combination of the *Enterococcus faecium* and glucan and mannan of *Saccharomyces cerevisiae* cell wall) induced elevation in the expression of cxcl1, and MUC genes and decrease the expression of IL6, IL8, IL10, and TNF genes. Many research proofs that *Enterococcus faecium* can be employed as supportive anti-cancer therapies therefore it can protect from cancer.

Keywords: Caco-2 cells, Interleukin (IL), Mucin gene, Prebiotics, Probiotics, Synbiotics, Tumor necrosis factor (TNF).

Introduction

Recently, probiotics use globally for the prevention and treatment of human diseases [1]. Cancer increases the human death rate in the world and the risk of developing cancer depends on the body's immune situation and genetic factors, [2]. Probiotics have different effects on cancer patients; the effects are different on healthy people and enhance several critical concerns. In medicine, several researches proved that probiotics support the immune system and improve the intestinal health, they are generally unharmed, and in extraordinary cases, they may give rise to trouble [3]. Goldin and Gorbach [4] were among the first to demonstrate the association between a diet reinforced with Lactobacillus and a reduced incidence of colon cancer (40% vs. 77% in controls). Several studies prove the potential enforcement of probiotics in the prevention and treatment of cancer [5,6,7]. Kotzampassi *et al.* [8] demonstrated the beneficial effects of probiotics (*Saccharomyces boulardii*, Lactobacillus: plantarum, acidophilus; and *Bifidobacterium lactis*) in patients who were exposed to colorectal surgery for cancer. Probiotic bacteria can raise and reduce the anti-inflammatory cytokines production that show a serious function in protection from carcinogenesis [9].

According to the adjustment of bile acid profile and pH, probiotics have been demonstrated to be one of the most important agents in cancer prevention [10] and it has a role in improving the intestinal barrier through the production of an excessive amount of mucus, and in the propagation of healthy cells [11]. Probiotics may have the ability to decrease and detoxify carcinogenic materials [12]. Bacteriocins play a greater role in the modification of host immunity [13]. Fiber-enriched diet has a prebiotic activity, which increases the level of beneficial bacteria, which make favorable result to prevent colorectal cancer [9].

This research aimed to investigate the effects of probiotics (*Enterococcus faecium*) and prebiotics

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(Received 03/10/2023, accepted 08/12/2023)
DOI: 10.21608/EJVS.2023.238590.1626

(glucan and mannan of *Saccharomyces cerevisiae* cell wall) on the proliferation and immune response genes of human colon cancer Caco-2 cell.

Material and Methods

Probiotic Strain

Enterococcus faecium was kindly obtained from Food Safety Laboratory, Regional Center for Food and Feed, A.R.C., Giza, Egypt. The culture was adjusted at a concentration of 108 (5x108) colony-forming units /ml, by pour plate assay using VRP agar and standard serial dilution techniques [14]. The bacterial strain was washed twice with phosphate buffered saline (PBS) and re-suspended in Roswell Park Memorial Institute Medium (RPMI medium) [15].

Prebiotic

Using yeast cell (*Saccharomyces cerevisiae*) as prebiotic (240g / kg glucan, 200 g / kg mannan) was estimated and diluted in buffered saline 0.327 g of sample in 100 ml buffer as described by Shoaf *et al.* [16].

Human Colonic Carcinoma (Caco-2) cells

The cell line was obtained from VACSERA (Egyptian Organization for Biological Products and Vaccines), and grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (Difco), 2 mM l-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Lonza, Belgium). Cells were incubated in a 5% CO2 humidified atmosphere at a constant temperature of 37 ◦C. Cells were grown to 70% confluence and harvested following trypsinization with trypsin-EDTA (Gibco), washed, resuspended in RPMI, and seeded at a density of 1 ×105. The cells were grown for 14 days to allow them to reach confluence and fully differentiation. Media were changed every other day.

Adhesion Assay [15]

Caco-2 cells were seeded in a 24-well plate (2 cm2/well; BD Falcon, Franklin Lakes, NJ). Adhesion experiments were performed 15d after confluence, a time when morphological and functional differentiation is complete the viable cell number, counted in a Neubauer chamber, was about 6 × 105 cells per well. Cell monolayers were carefully washed twice with sterile phosphate-buffered saline (PBS; pH 7.3) before bacterial cells were added. *Enterococcus faecium* 48h-old culture was collected by centrifugation (5000 rpm for 5min), washed, and resuspended in RPMI for assays with Caco-2 cells for reference purposes (100% values), 1 ml aliquots of the original bacterial cell from Probiotic and prebiotic suspensions (125 µl per well) were then added directly on washed intestinal epithelial cell layer and incubated CO_2 incubator) at 37 °C for 2hr. After incubation, non-adhered bacteria were removed by washing the cell cultures twice with PBS. Cells with adhered bacteria were treated with 250 µl of trypsin-EDTA (Invitrogen; Life Technologies Europe) per well for 10 min at 37 °C followed by the addition of 250 µl culture medium containing FBS to stop the trypsin reaction. All incubations were performed in biologically independent triplicates. Plates were then incubated at 37°C, 5% CO_2 for 2h, after which all monolayers were washed gently three times with PBS to release unbound bacteria.

Confocal Microscope

Cells were cultured on glass-based Petri dishes and stained with 10 µm acridine orange, the signal was collected by exciting samples with 405 nm and 488 nm lasers, and emission was collected at 542 nm (represented by red) and 494 nm (represented by green), respectively and imaged with confocal scanning microscope (CLSM, LSM 710, Carl Zeiss, Germany)

Quantitative Real-Time PCR:

According to Pfaffl [17] the messenger RNA (mRNA) of MUC-2, TNF, IL6, IL8, CXCL1, and IL10, was detected and assayed in Caco-2 cells using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) following the manufacturer's protocol by reverse-transcription polymerase chain reaction (RT-PCR) using specific primers as shown in Table 1. Assessment of both RNA concentration and purity in extracted samples can be carried out using a (Nano Drop 1000, USA) spectrophotometer. Absorbance at 260 Nanometer (nm) gives a specific measurement of RNA concentration and the absorbance at 280 nm and 230 nm.

Quantitative Reverse Transcription PCR (RT-qPCR) complementary DNA (cDNA) was synthesized by using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific / Ferments, Vilnius, Lithuania), following the manufacturer's recommendations. The reverse transcriptase enzyme uses the RNA template and short-sequence primers to direct the synthesis of the first strand cDNA, which is then used as a template for the qPCR reaction. Amplification curves and CT values were determined to estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with the control group, according to method stated by Yuan *et al.* [18].

TABLE 1. Primers used in the RT-PCR

Primer	Sequence	References
MUC-2	PF: CTC CAA GCC ACA CTG CCC PR: TGC TCC CCA AAC TAT CTG	[19]
TNF	PF: AGG CGC TCC CCA AGA AGA CA PR: TCC TTG GCA AAA CTG CAC CT	[20]
IL-6	PF: AATTCGGTACATCCTCGACGG PR: GGTTGTTTTCTGCCAGTGCC	[21]
IL-8	PF: GCCAGTGCTTGCAGACCCT PR: CTTCTCCACAACCCTCTGCAC	
IL-10	PF: GGTTGCCAAGCCTTGTCTGA PR: AGGGAGTTCACATGCGCCT	[22]
CXCL1	PF: GCCAGTGCTTGCAGACCCT PR: GGCTATGACTTCGGTTTGGG	[23]
GAPDH	PF:GTCGCTGTTGAAGTCAGAGG PR:GAAACTGTGGCGTGATGG	[21]

Statistical Analysis

Duncan's multiple range test was used to compare the means of cell growth and adhesion, and gene expression levels. Comparisons were statistically significant at $P < 0.05$. The resulting values were analyzed. All data were analyzed using the software SAS, version 9.4 (SAS Institute, Cary, NC, USA). Differences between means were tested by Duncan [24].

Results

The data presented in Figure 1 indicate the valuable properties of probiotics and prebiotics in modifying the propagation and cancer cells apoptosis. In the Caco-2 culture, large bacterial inoculum size decreased the adhesion yields. The Count of *Enterococcus faecium* on Caco-2 cells by plate count agar technique using VRB agar was 7x105. While it was 2x105 using synbiotic-treated cells (*Saccharomyces cerevisiae* cell wall and *Enterococcus faecium*).

The data presented in Table 2 and Figure 2 indicated that the CXCL1 gene was elevated among probiotic and synbiotic treated cells (1.88 and 3.19 respectively) while it decreased in prebiotic treated cells (0.97) compared with non-treated cells (1.78). Proinflammatory cytokine IL6 was decreased in probiotic and synbiotic treated cells (0.68 and 0.74 respectively) while it was slightly elevated in prebiotic treated cells (1.26) compared with non-treated cells (1.01). Proinflammatory cytokine TNF was significantly decreased in the treated groups (probiotic: 0.53, prebiotic: 0.33, and synbiotic treated cells: 0.02 respectively) compared with non-treated cells (1.0). IL8decreased in probiotic, prebiotic, and synbiotic treated cells (0.94, 1.76, and 1.6 respectively) compared with non-treated cells (2.05). IL10 decreased in prebiotic and synbiotic-treated cells (0.12 and 0.28 respectively) while it significantly increased in probiotic-treated cells (2.04) compared with non-treated cells (1.07). The Mucin gene shows a significant increase in prebiotic (3.25) and synbiotic-treated cells (5.03) while it significantly decreases in probiotic-treated cells (0.54) compared with non-treated cells (1.09).

Figure 1: Analysis of Caco-2 cells among the treated groups examined by using confocal microscope.

Caco-2 cell line (Negative control): normal viable cells with intact nuclei and nuclear membrane (arrow). B- Caco-2 cells treated with probiotic (*Enterococcus faecium*): normal viable cells with intact nuclei, nuclear membrane (arrow), and chromatin condensation (2 arrows). C- Caco-2 cells treated with prebiotic (glucan and mannan of *Saccharomyces cerevisiae* cell wall) normal viable cells with intact nuclei and nuclear membrane (arrow), chromatin condensation (2 arrows), and membrane blabbing (3 arrows).

Figure 2: Level of immune response genes among the treated groups

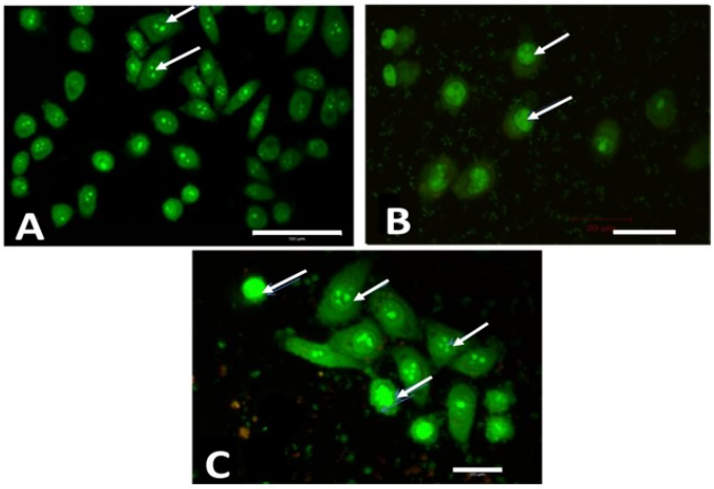

Figure 1. Caco2 cells among the treated groups examined by confocal microscope.

a. Caco2 cell line (Negative control): normal viable cells with intact nuclei and nuclear membrane (arrow). B- Caco2 cells treated with probiotic (*Enterococcus faecium*): normal viable cells with intact nuclei, nuclear membrane (arrow), and chromatin condensation (2 arrows). C- Caco2 cells treated with prebiotic (glucan & mannan of *Saccharomyces cerevisiae* cell wall) normal viable cells with intact nuclei and nuclear membrane (arrow), chromatin condensation (2 arrows), and membrane blebbing (3 arrows).

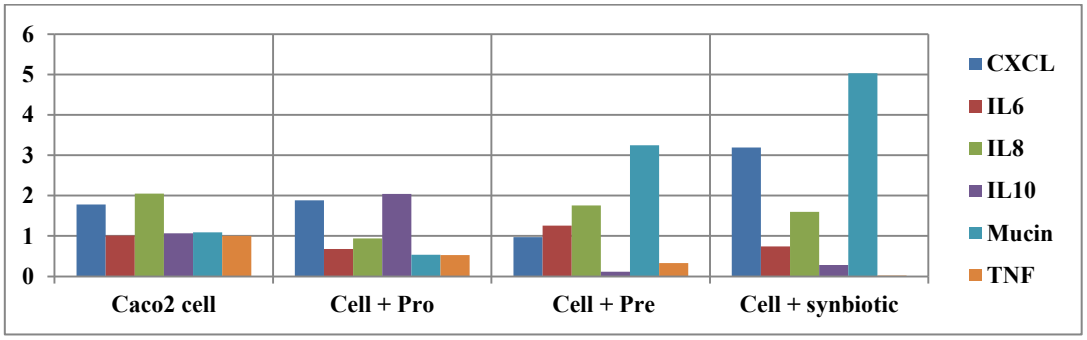

Figure 2. immune response genes among the treated groups

TABLE 2. immune response genes among the treated groups

Groups	*CXCL*	*IL-6*	*IL-8*	*IL-10*	Mucin	*TNF*
Caco2 cell (-ve control)	1.78^{C}	1.01^{bc}	2.05^{a}	1.07^{cd}	1.09^{b}	1.00^{b}
Caco2 cell treated with *Enterococcus faecium*	1.88^{c}	0.68^{c}	0.94^{a}	2.04^{b}	0.54^{b}	0.53^{b}
Caco2 cell treated with *Saccharomyces cerevisiae* cell wall (glucan & mannan)	0.97^{c}	1.26^{bc}	1.76^{a}	0.12^{d}	3.25^{a}	0.33^{b}
Caco2 cell treated with synbiotic (*Saccharomyces cerevisiae* cell wall and *Enterococcus faecium*)	3.19^{bc}	0.74^{c}	1.60^{a}	0.28^{d}	5.03^{a}	0.02^{b}

a-d Means with the same letters within each column of the trait are non-significantly different (P≥0.05)

Discussion

The reproduction of cancer cell and apoptosis define the evolution of cancer. Probiotics decrease inflammation and prevent damage to the neighbor cells as it modulates cellular proliferation and apoptosis [25].

A lot of researchers indicate that probiotic has a significant antiproliferative role and/or induction of apoptosis in cancer cells [26, 27]. Śliżewska *et al.* [2] showed that probiotic has profitable properties in modulating the proliferation and apoptosis of cancer cells.

In the present study, the confocal microscope indicated beneficial properties of probiotics (*Enterococcus faecium*) and prebiotics (glucan and mannan of *Saccharomyces cerevisiae* cell wall) in modulating the proliferation and apoptosis of cancer cells.

Enterococcus faecium RM11 decreased cell proliferation by 21% and activation of apoptosis of Caco-2 after being treated with Enterococcus faecium FP51. They suggested that both strains could be used as potential probiotics in functional food or for colon cancer biological products [28].

The anticancer activity of probiotics includes changes in metabolic mechanisms, the declination, and binding of carcinogenic compounds, improving chronic inflammation by immunomodulation, lowering pH, and the inhibition of enzymes that produce potential carcinogenic compounds [29].

Lactic acid bacteria produce short-chain fatty acids (SCFAs) which are known as signaling molecules in the immune system and responsible for cell death and proliferation [9]. SCFAs inhibit carcinogenesis by increasing apoptosis and suppressing the proliferation of tumor cells [2]. According to Ewaschuk *et al.* [30] some species of probiotic bacteria can produce conjugated linoleic acids (CLAs) from linoleic acid. CLAs affect the expression of genes concerned with the apoptosis process and the cellular response to cell growth factors [2]. In another research, the use of Lactococcus lactis articulation catalase which reduce the reactive oxygen species (ROS) production, such as H_2O_2, decreasing colonic damage, and inflammation, consequently disinclined tumor infestation and propagation [31].

Short-chain fatty acids (SCFAs) secret interleukin-18 (IL-18) and mucin (MUC2) and inhibit the production of intestinal macrophage of proinflammatory cytokines e.g., IL-6, IL-8, IL-1β, and TNFα so they regulate the intestinal barrier function [2].

In the present investigation in vitro study was carried out employing Caco-2 cells (human colorectal adenocarcinoma cells) to estimate the effect of probiotic (*Enterococcus faecium*) and prebiotic (glucan and mannan of Saccharomyces cerevisiae cell wall) on the expression of cytokines genes (CXCL1, IL6, IL8, IL10, and TNF), additionally the expression of MUC gene.

The data indicated that the cxcl1 gene was increased in probiotic, and synbiotic-treated cells (1.88 and 3.19 respectively) compared with prebiotic (0.97) and non-treated cells (1.78). Probiotics can motivate pro-inflammatory and anti-inflammatory responses [3]. Probiotics can adjust cytokines, chemokines, and antimicrobial peptides expression by multiple signaling pathways [32]. Ng *et al.* [33] recorded that proinflammatory cytokines are mostly reliable to initiate an effective defense against exogenous pathogens; however, excessive production of these mediators can be harmful and may finally lead to shock, multiple organ failure, and death. In contrast, anti-inflammatory cytokines are critical for down-regulating the trigger inflammatory process and maintaining homeostasis for the suitable functioning of vital organs, but an exaggerated anti-inflammatory response may also result in the repression of the body's immune function.

The expansion of cancer in the case of colorectal cancer related to tumor necrosis factor-α (TNF-α) and the proinflammatory cytokines IL-1β, IL-6, IL-8, IL-12, IL-17 [34].

IL-6 signaling includes a multimeric receptor complex involving a membrane-bound IL-6 receptor (IL-6R), following ligand-induced interaction of this complex, STAT1 and STAT3 are phosphorylated, exciting their translocation to the nucleus, and the subsequent transcription of IL-6 target genes [35]. In the present study, proinflammatory cytokine IL6 was decreased in probiotic and synbiotic-treated cells (0.68 and 0.74 respectively) while it was slightly elevated in prebiotic-treated cells (1.26) compared with non-treated cells (1.01).

Decreasing the level of IL–8 was recorded by Lopez *et al.* [36] by using the Lactobacillus rhamnosus GG strain. In the present study, chemokine IL8 was significantly decreased in probiotic, prebiotic, and synbiotic-treated cells (0.94, 1.76, and 1.6 respectively) compared with non-treated cells (2.05).

In the present study, *E. faecium* can both increase and decrease the production of the anti-inflammatory cytokine IL10. IL10 decreased in prebiotic and synbiotic-treated cells (0.12 and 0.28 respectively) while it was significantly increased in probiotic-treated cells (2.04) compared with non-treated cells (1.07). IL10 can effectively down-regulate the proinflammatory response [33].

Several groups of probiotic express antioxidant enzymes or IL-10 that suppress the inflammatory-related carcinogenesis, these strains are considered agents causing significant changes in the immune response and causing the entire inhibition of tumor development [37].

Probiotics can suppress intestinal inflammation through the down regulation of Toll-like receptors (TLRs) expression, secrete metabolites that may inhibit TNF-α from entering blood mononuclear cells, and suppress the NF-κB signaling in enterocytes, furthermore probiotics can adjust the

activity of natural killer (NK) cells [38]. In the present study proinflammatory cytokine TNF was significantly decreased in the treated groups (probiotic: 0.53, prebiotic: 0.33, and synbiotic: 0.02, respectively) compared with non-treated cells (1.0). The use of dextran with *Lactobacillus casei* subsp. Casei boosts the activity of NK cell this property may be implicated to produce cytokine linked to NK cell activity [2]. Moreover, lactic acid bacteria down regulate NF-κB-dependent gene products that regulate cell survival and proliferation. This activity leads to preventing cancer.

The secreted proteins are natural precursors for acquiring mucin function which is rich in proline [39]. In the present study MUC gene shows a significant increase in prebiotic (3.25) and synbiotic treated cells (5.03) while it significantly decreases in probiotic treated cells (0.54) compared with non-treated cells (1.09).

Mucin acts as a protective barrier against environmental injury as it forms mucous layers to lubricate various organs by mediating signaling between epithelial cells [39].

The goblet cell secrets mucin which is gel-forming glycoproteins that act as lubricants and make a protective barrier between the body and the external environment. The process of carcinogenic makes the structure of mucins less glycosylated and decreases the secretion of mucins [2].

Probiotics increase MUC2 gene expression, (up to 5 times) while slightly elevate the expression of MUC1 and MUC3 genes [40]. Lots of proof recorded that the use of *Enterococcus faecium* and glucan and mannan of Saccharomyces cerevisiae cell wall can support anti-cancer therapies.

Conclusions

Enterococcus faecium strain can be employed as adjuvant therapy with anticancer chemotherapy, it decreases the proliferation of cancer cell and has a protective effect against cancer.There is an urgent need to elucidate the effects of synbiotic: *Enterococcus faecium* and glucan and mannan on *Saccharomyces cerevisiae* cell wall in cancer patients.

Acknowledgment:

The authors are indebted to Hisham A. Elshoky, Heba Elsayed Elzorkany researchers at Nanotechnology Lab, Regional Center for Food and Feed, Agricultural Research Center, Egypt for their help.

Funding: Not applicable

Conflicts of interest: Not found

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البريبيوتك والبروبيوتيك: أداة واعدة في الوقاية من سرطان القولون البشري

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تهدف الدراسه الحاليه الي دراسة تأثير البروبيوتيك والبريبايوتك في الوقاية من سرطان القولون البشري باستخدام خلايا سرطان القولون البشريه عن

طريق قياس:

1 -الالتصاق بين البروبيوتيك والبريبيوتك والخلايا السرطان باستخدام المجهر متحد البؤر(Confocal microscope)

2 -قياس تعبير جين الميوسين2 بتقنيه تفاعل البلمرة المتسلسل(Q-PCR)

3-تقدير التعبير السيتوكيني عن طريق قياس جينات الانترلوكين(IL6) ، IL8، IL10، (CXCL1, TNF,)

أشار المجهر متحد البؤر إلى الخصائص المفيدة للبروبيوتيك والبريبيوتك في تعديل تكاثر الخلايا السرطانية وموت الخلايا المبرمج

أشارت البيانات إلى أن سلالة البروبيوتيك *Enterococcus faecium* تحفز الارتفاع في التعبير عن جينات cxcl1 و IL10 وتقليل التعبير عن جينات IL6 و IL8 و MUC وTNF. في حين أن البروبيوتك يحفز الارتفاع في التعبير عن جينات IL6 و MUC ويقلل من التعبير عن جينات cxcl1 و IL8 و IL10 وTNF.

والجمع بين البروبيوتك والبريبيوتك يسبب الارتفاع في التعبير عن جينات cxcl1 و MUC ويقلل من التعبير عن جينات IL6وIL8 و IL10 وTNF . هناك الكثير من الأدلة على أن استخدامEnterococcus faecium يمكن أن يلعب دورًا مهمًا في الوقاية من السرطان ودعم العلاجات المضادة للسرطان.

الكلمات داله : خلايا سرطان القولون البشريه ، الإنترلوكينات ، ميوسين جين ،البروبيونك ،البريبيونك، السمبيوتك، عامل تنخر الورم.