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Immunomodulatory and Anticancer Activities of Enterocin Oe-342 Produced by Enterococcus Feacalis Isolated from Stool

Omnia Momtaz Al-Fakharany*, Ahmed A. Abdel Aziz, Tarek El-Said El-Banna and Fatma Ibrahim Sonbol

Department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University, Tanta 31111, Egypt

***Corresponding author:** Omnia Momtaz Al-Fakharany, Department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University, Tanta 31111, Egypt, Tel: +20 1282065777; E-mail: momtaz_omnia@yahoo.com

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Abstract

Objectives: This study evaluated the anticancer activity of *Entr. faecalis* enterocin OE-342 by testing its direct antitumor effects on colorectal cancer cell line, as well as, its immunomodulatory effects.

Methods: The anticancer potentiality of this enterocin against human colorectal adenocarcinoma (HCT-116) cells was evaluated using cytotoxicity, cell cycle analysis, apoptosis and monitoring morphological changes of the treated cells. Also the immunomodulatory effects of the tested enterocin were quantified on LPS-induced PBMCs cell model with monitoring TNF-α and IFN- γ levels using flowcytometry.

Results: The obtained data showed that enterocin OE- 342 inhibited HCT-116 cellular viability in a concentration dependant manner with somewhat high IC_{50} value recording 49.920 mg/ml. Upon HCT-116 treatment with this enterocin the appearance of undergoing apoptotic cells characterized by cellular rounding up, shrinkage and membrane blebbing was observed. Cytotoxicity of enterocin OE-342 was accompanied by cell cycle arrest in the G2/M phase (24.66%) accompanied with an increase in the ratio of the apoptotic cells in the pre- G1cell cycle phase. Also, this enterocin affected the generation of TNF-α and IFN- γ from LPS-induced PBMCs cell resulting in inhibition of the first by 27.3% and induction of the second by 69.5% which suggest the immunomodulatory effects of the tested enterocin.

Conclusion: Taken together, our results suggest the anticancer potentialities of enterocin OE-342 against human colon cancer cells represented through its effects on both apoptotic and inflammatory pathways.

Keywords: *Enterococcus faecalis;* Enterocin; Cytotoxicity; Apoptosis; TNF-α; Immunomodulatory; Colon cancer

Introduction

The gastrointestinal tract (GIT) microflora plays a crucial role in maintaining the health status of humans and animals [1]. Humans are colonized by various commensal microorganisms, which form the microbiome. The human gut microbiome modulates many host processes, including metabolism, inflammation, and immune and cellular responses. They can influence human health by preventing the growth of pathogens, producing beneficial microbial products and metabolizing nutrients and toxins. It is becoming increasingly apparent that the microbiome can influence the development of cancer. In addition, cancer treatment with microbial agents or their products has the potential to shrink tumours [2].

Cancer is one of the major causes of morbidity and mortality worldwide [3]. Cancer is the result of uncontrolled growth of a given cell type that occurs together with the invasion of surrounding tissue and the spread of malignant cells [4]. Cancer can only develop and progress in the context of failed immunosurveillance and is often associated with systemic immunosuppressive effects, which can alter the microbiota [2]. Cancer cells are characterized by the presence of multiple cell physiology alterations including; resistance to growth-inhibitory signals and programmed cell death, limitless replicative potential and metastasis [5]. The available approaches for cancer treatment include chemotherapy, surgery and radiation. Cancer cells might develop resistance to conventional chemotherapeutic agents through various mechanisms [6]. Therefore, there is a pressing demand for targeted therapies that are capable of killing cancer cells selectively without affecting normal cells or at least to act as adjuvants to lower the therapeutic doses and increase efficiency of conventional anticancer drugs. Previous studies have reported that some lactic acid bacterial (LAB) strains could inhibit colon cancer progression [7]. This activity has been related to the immunomodulatory activity they possess, and likely on the basis of the changes in the cytokine profiles they induce. Among LABs, there is a group of genera that are phylogenetically related by their biochemical and ecological characteristics (*Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Carnobacterium, Pediococcus*, etc.). As immunomodulators, the genera Enterococcus and Streptococcus have been the least studied [8-10].

Enterococcus faecalis is a Gram-positive, commensal bacterium belonging to LABs and inhabiting the gastrointestinal tracts of humans and other mammals [11]. Bacteriocin production by Enterococci was recognized many years ago [12,13]. Bacteriocins are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms [14]. Bacteriocins have been investigated as a therapeutic agent, because of their ability to perform many biological functions, notably among them are inhibition

of membrane protein synthesis, DNA synthesis, antiviral properties, and apoptosis or cytotoxicity of tumor cells [15]. The human gastrointestinal tract appears to be an excellent environment for bacteriocin-producing enterococci. We and others have shown that bacteriocin-producing enterococci are found in most stool samples of healthy babies at relatively high frequencies, suggesting that bacteriocin-producing enterococci are important in the development of the gut flora in infants. Although enterocins have been extensively studied, it was generally in regard to their application in the food industry as preservative. Some of the immunomodulatory factors produced by *E. faecalis* have been identified [16-18] however the extent of immunomodulatory capacities amongst non-pathogenic gut strains, and the identity of the bioactives that underpin them, remains largely unknown. In addition, there is a scarcity of reports about the efficacy of enterocins in immunity enhancement and cancer treatment. Therefore, in this study we aimed to evaluate the immunomodulatory effects as well as the anticancer potential of enterocin OE-342 produced by *Enterococcus faecalis* strain that was isolated from stool of apparently healthy neonate using human PBMCs cells and human colorectal cancer cell line (HCT-116) respectively.

Methods

Mammalian cell lines

Human colorectal carcinoma (HCT-116) cells were kindly provided by tissue culture department of the Holding Company for Production of Vaccines, Sera and Drugs (VACSERA-EgyVac). Cells were cultured in RPMI 1640 medium and DMEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin– streptomycin (Invitrogen, USA) and then incubated (Jouan-France) at 37°C in a humidified atmosphere containing 5% CO_2.

Peripheral blood mononuclear cells (PBMC) are the primary source of lymphoid cells for investigation of the human immune system. PBMCs were obtained from healthy donor. Its use was facilitated by Ficoll-Hypaque density gradient centrifugation [19]. Cells were cultured in RPMI medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS) and 1% penicillin-streptomycin solution in flat bottom 6-12-24 or 96 well plates, incubated at 37°C in a humidified atmosphere of 5%CO_2, 95% air for 24 h.

Bacterial strains and culture conditions

Previously isolated *Enterococcus faecalis* (*Ent. faecalis*) OE-342 from the faeces of a healthy neonate in the Delta region, Egypt was stored under 60% glycerol at -80°C and resuscitated on De Man-Rogosa-Sharpe (MRS) agar. Several sub-culturing steps were then carried out to obtain a pure culture of the isolate [20]. For inoculation, aliquot of 1 ml (109CFU/ml) of Ent. faecalis OE-342 overnight culture, was inoculated in MRS broth (Oxoid, UK) and then incubated at 37°C, under aerobic conditions for 24 h. The crude bacteriocin from OE-342 could be obtained by centrifugation at 10,000 rpm at 4°C for 30 min and culture supernatant was separated carefully and was filter-sterilized by passage through a 0.45-µm pore-size Whatman membrane filter (Sigma, USA) and stored frozen at -80°C till used [21]. Staphylococcus aureus (ATCC 25923) was used as indicator strain and was grown at 37°C in nutrient broth for 18-24 h.

Production and purification of enterocin

The cell free supernatant (CFS) produced by Ent. faecalis OE-342 was obtained as described above, and the bacteriocin activity in it was tested by agar well diffusion assay against the tested indicator strain before and after treatment with proteolytic enzyme proteinase K (to ensure the proteinaceous nature of the tested enterocin). Bacteriocins were precipitated from CFS using ammonium sulphate (40% saturation) with 4 h of stirring at 4°C. Precipitated proteins were collected by centrifuging for 30 min at 10.000 rpm at 4°C, resuspended in phosphate-buffered saline (PBS), pH7 and the residual enterocin activity was determined according to [22] by the agar well diffusion method using S. aureus ATCC 25923 as indicator strain and the recoverd highly active bacteriocin was stored at -80°C till use. 16% Tricin SDS- PAGE was performed to confirm the molecular mass of the partially purified bacteriocin [23].

Targetting bacteriocin genes

To detect genes encoding the known enterocins, genomic DNA of strainOE-342 was used as template. PCR reactions were performed in a Thermal cycler (Techne,UK) in a 25 µl volume containing up to 25 µl PCR-grade water, 12.5 µl of 2X FastGene® Optima HotStart ReadyMix with dye (Genetics, Germany), 1.25 µl of each primer (0.5 µM), and 2 µl DNA template (100 ng/ml). The PCR conditions consisted of an initial denaturing step of 3 min at 95°C, followed by 40 cycles of 15 sec denaturing at 95°C, 15 sec annealing at a temperature specific for the primers for each of the known enterocin genes, and 1 min extension at 72°C. A final extension was carried out for 1 min at 72°C. The primer sequences for PCR amplification and PCR conditions for enterocin genes in *Enterococcus faecalis* OE- are illustrated in Table 1 and Table 2. The PCR products were visualized by electrophoresis in 1.5% agarose gels.

Genes	Primer	Sequence (5' to 3')	Amplicon size (bps)	Reference
L50AB	EntL50AB-F	5-ATGGGAGCAATCGAAAATTAGTAG-3	130	23
	EntL50AB-R	5-TTAATGTCTTTTTAGCCATTTTTCAATTTG-3		
AS48	EntAS48-F	5/-GAGGAGTITCATGITTAAAGA-3/	150	23
	EntAS48-R	5/-CATATTGTTAAATTACCAAGCAA-3/		
31	Ent31-F	f:5-TATTACGGAAATGGTTTATATTGT-3	130	24
	Ent31-R	r:5-TCTAGGAGCCCAAGGGCC-3		

Table 1: Primer sequences for PCR amplification of enterocin genes in *Enterococcus faecalis* OE-342. F: Forward; R: Reverse

Cell proliferation inhibition assay

For the assessment of the dose dependent cell proliferation inhibition effect of *Ent. faecalis* enterocin OE-342, HCT-116 human colorectal adenocarcinoma cells were propagated in 75 cm^2 cell culture flasks using RPMI-1640 medium (Gibco-USA) supplemented with

10% (v/v) fetal bovine serum (Gibco-USA) and incubated in 5% (v/v) CO2 incubator at a temperature of 37°C. Confluent cells were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylenediaminetetraacetic acid (EDTA) (Gibco-USA) for 5 min. Cells were plated at a concentration of 2×10^5 cells/ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 h to achieve confluency. The medium was decanted and fresh medium containing various concentrations of enterocin OE-342 was added for cytotoxicity determination using colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) reduction assay (25). Dead cells were washed out using PBS, and 50 µl of MTT stock solution (5 mg/ml) were added to each well. After4 h incubation period, the supernatants were discarded and the formazan precipitates were solubilized by addition of 50 µl / well of dimethyl sulfoxide (DMSO). Plates were incubated in the dark for 30 min at a temperature of 37°C, and absorbance was determined at a wavelength of 570 nm using microplate reader (Biotek ELX -800,USA). The cell viability percentage was calculated using the following formula.

Genes	Initial denaturation		Denaturation		Annealing		Extension		No. of cycles	Final extension	
	Temp. (°C)	Time (min)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	
L50AB	95	3	95	15	58	15	72	1	40	72	1
AS48	95	3	95	15	52	15	72	1	40	72	1
31	95	3	95	15	52	15	72	1	40	72	1

Table 2: PCR conditions for the selected genes

The MTT assay was performed three times with three replicates conducted for each experiment. The cell viability (%) was blotted against the tested enterocin concentrations. Statistical significance between treated and untreated cells was determined using Student's unpaired t-test. Differences at P values less than 0.05 were considered significant. The IC50 value of the tested enterocin was determined using Masterplex-2010 software program.

Anticancer activities of enterocin OE-342

The anticancer activities of enterocin OE-342 on HCT-116 colon cancer cells were determined through monitoring the morphological alterations of the treated cells using an inverted microscope (Nikon-Japanred).

Cell cycle analysis

The alterations in cell cycle pattern were determined by using flow cytometry and according to [26]. In this assay, propidium iodide (PI) can be employed to discriminate living cells from dead cells, or for cell cycle analysis; cell cycle analysis is based on the stoichiometric binding of PI to intracellular DNA. HCT-116 colorectal carcinoma cells pre-cultured in 25 cm^2 cell culture flasks were treated with an IC10 (10 mg/ml) of tested enterocin dissolved in RPMI-1640 medium, for 24h. The cells were then washed with warm PBS and collected by trypsinization. For cell cycle analyses, the collected cells (about 2×10^5 cells/ml) were then resuspended in warm PBS, fixed gently with about 4 ml ice cold 70% (v/v) ethanol, maintained at a temperature of 4°C overnight and then stained with 0.5 mL of warm PI solution (7 ml of PI solution consists of 0.35 ml of PI solution (1 mg/ml), 0.7 ml RNase A solution (1 mg/ml), 6 ml of PBS and 0.1% (v/v) Triton X-100) in a dark room. After 30 min at 37˚C, the cells were then analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The alterations in cell cycle pattern were determined by using flow cytometry and according to [26]. In this assay, propidium iodide (PI) can be employed to discriminate living cells from dead cells, or for cell cycle analysis; cell cycle analysis is based on the stoichiometric binding of PI to intracellular DNA. HCT-116 colorectal carcinoma cells pre-cultured in 25 cm^2 cell culture flasks were treated with an IC10 (10 mg/ml) of tested enterocin dissolved in RPMI-1640 medium, for 24 h. The cells were then washed with warm PBS and collected by trypsinization. For cell cycle analyses, the collected cells (about 2×10^5 cells/ml) were then resuspended in warm PBS, fixed gently with about 4 ml ice cold 70% (v/v) ethanol ,maintained at a temperature of4°C overnight and then stained with 0.5 mL of warm PI solution (7 ml of PI solution consists of 0.35 ml of PI solution (1 mg/ml), 0.7 ml RNase A solution (1 mg/ml), 6 ml of PBS and 0.1% (v/v) Triton X-100) in a dark room. After 30 min at 37˚C, the cells were then analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The obtained cell cycle profile and sub-G1 group were analyzed using CellQuest version 3.2 and Win MDI version 2.8 softwares [27]. The flow cytometric analysis was performed at the Center of Excellence and Cancer Research (CECR), Tanta University, Eygpt.

Detection of apoptosis using annexin V staining and propidium iodide (PI)

The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is an early event in apoptosis, and can be used to detect and measure apoptosis. During apoptosis, PS is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure upon the cell membrane. Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. Therefore the measurement of Annexin V binding to the cell surface as indicative for apoptosis has to be performed in conjunction with a dye exclusion test to establish integrity of the cell membrane. Annexin V binding was assessed using bivariate FCM according to [28], and cell staining was evaluated with fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclusion of propidium iodide (PI) (negative for red fluorescence). HCT-116 cancer cells were placed and seeded into a 6-well culture plate to detect apoptotic cells quantitatively. Quadrant statistic calculations were done

using CELLQUEST PRO software (BD Bioscience, San Jose, CA). The experiment was repeated twice with triplicate samples for each experiment. Analyses were conducted on 5000 cells, which were obtained at a rate of 300 cells s-1. FL-1 and FL-3 were represented in dot plots illustrating viable, apoptotic and necrotic cells [29].

Immunomodulatory activities of enterocin OE-342

Quantification of the tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) in LPS-induced PBMC cells: The immunomodulatory role of enterocin OE-342 on human macrophages was investigated in vitro. The effect of the tested enterocin on the expression of tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) in LPSinduced PBMC model was studied using flowcytometric analysis (intracellular staining protocol).

About 2×10^6 cells/ml of PBMCs were suspended in RPMI medium and seeded into a rounded bottom, 6-well plate for 24 h. At the end of incubation, the inflammatory model was induced by stimulating PBMC with 100 µl of E. coli LPS (20 ng/ml) for 24 h in the presence or absence of the treatment (10 mg/ml of enterocin OE-342). The intracellularly induced TNF-α and IFN-γ were assessed by flow cytometric analysis using FITC – labeled TNF alpha and FITC-labelled IFN Gamma respectively. Analyses were conducted on 10.000 cells and bivariate dot plots or probability contour plots could be generated upon data analysis to display the frequencies of and patterns by which individual cells express certain levels of intracellular cytokine proteins. The flow cytometric analysis was performed at the Center of Excellence and Cancer Research (CECR), Tanta University, Eygpt.

Statistical analysis

Data were statistically analyzed using Statistical Package for Social Science version 20 (SPSS) (SPSS Armonk, NY: IBM Corp.). The Student's unpaired t-test was used for pairwise comparisons analysis whereas the one-way analysis of variance (ANOVA) test was used for comparison among different groups. Each experiment was performed at least 3 times and data are shown as mean ± SE and and $p<0.05^*$ is considered statistically significant while $p<0.001^{**}$ highly significant.

Results

Ent. faecalis OE-342 used in this study was isolated from the stool of a neonate in the Delta region, Egypt. MALDI TOF MS analyses identified *Ent. Faecalis* OE-342 isolate to the species level with a correct identification score of 2.46. It had been described as a strain that produced an enterocin with a narrow inhibitory spectrum only on Gram-positive bacteria after exclusion of the effect of lactic acid. The substance responsible for antimicrobial activity was found to be protein in nature as indicated by complete loss of activity after protease treatment. The partially purified bacteriocin OE- 342 produced a single band with a molecular weight ranging about 5 kDa as determined by Tricine SDS-PAGE analysis((data are currently under review for publication).

PCR analysis of bacteriocin genes

PCR amplification using specific primers as shown in Table 1 was used to screen DNA of E. faecalis OE-342 for the existence of the known enterocin genes. This study focused on enterocin genes including 31, L50A andAS-48. *E. faecalis* OE-342 showed three bands: 200,250 and 150 bp corresponding to the specific amplification of enterocins L50A, AS-48 and 31 respectively as shown in Figure 1.

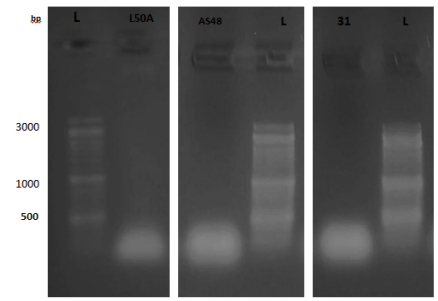

Figure 1: PCR amplification of genes encoding bacteriocin of E. faecalis OE342. L, molecular weight ladder (3000bp); Lanes 1: PCR product of bacteriocin gene. L50,enterocin L50A; AS48: enterocin AS48 and 31:enterocin31.

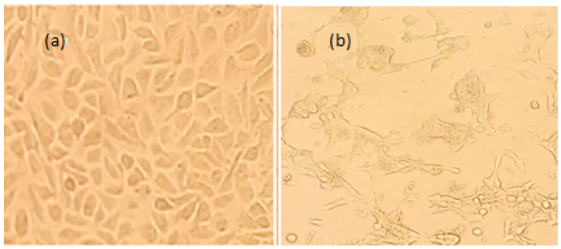

Figure 2: Cytotoxic effect of the enterocin OE-342 showing the morphological changes of HCT-116 cells 24 hr post treatment using inverted microscope: (a) Untreated (control) cells showing polygonal shape with homogenous cellular contents. (b) Treated cells showing membrane blebing, cellular deformation with severe shrinkage and condensation of cell contents.

Cytotoxicity assay of enterocin OE-342

The cytotoxic effect of tested enterocin on HCT-116 colon cancer cells was assessed by recording the various morphological changes of the cells before and after treatment. The untreated culture fields showed a polygonal shape with distinct boundaries and homogenous cellular contents. On the other hand, Induced cell toxicity was accompanied with morphological abnormalities of cells 24 h post treatment with the enterocin OE-342. Figure 2 shows the appearance of undergoing apoptotic cells that characterized by cellular rounding up, deformation with severe shrinkage and condensation of their cellular contents, membrane blebbing, cellular detaching out of the culture surface and loss of cell adhesion. Regarding the antiproliferative potential of the tested enterocin, it was noticed that cell viability was concentration dependent as the cytotoxicity was significantly increased at higher concentrations of the tested enterocin (Figure 3) and the IC50 value was somewhat high recording 49.920 mg/ml determined using Masterplex 2010 Software.

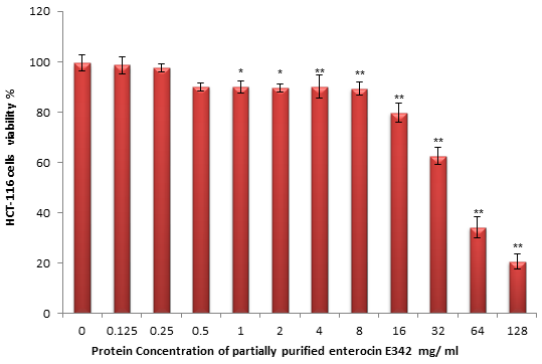

Figure 3: Enterocin OE-342 reduced the proliferation of HCT-116 human colorectal cancer cells. HCT-116 cells were cultured in 0, 0.125, 0.25, 0.5, 1,2,4,8,16,32, 64, 128 mg enterocin/ml for 24 h and then the cell viability was assessed using MTT asaay. Values are means ± SD (n=3), where p<0.05* is considered statistically significant while p<0.001** indicate highly significant decreases from untreated cells .

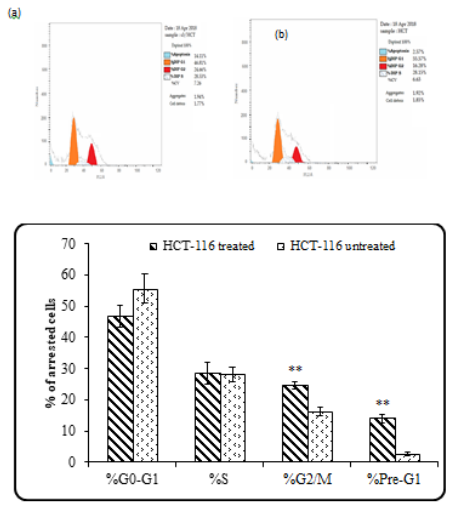

Figure 4: The effect of enterocin OE-342 treatment on the regulation of the cell cycle of colon cancer cells. The percentage and distribution of arrested cells in pre-phase, G1, S and G2/M phase of the cell cycle are shown in the graph presented (**P<0.001, Student's unpaired t-test).

Cell Cycle analysis and apoptosis

The flow cytometric analysis for the cell cycle pattern of the treated cells is shown in Figure 4. After treatment, the population percentages in the pre-G1 that represented the apoptotic cells were increased significantly (p<0.001) in HCT-116 treated cells to reach 14.11%, in comparison to the untreated control cells. Cell cycle analysis have demonstrated that toxicity of enterocin OE-342 was accompanied by cell cycle arrest in the G2/M phase, there was a significant (p<0.001**) elevated arrested cells in the G2/M phase 24.66% ± 1.08 after cellular treatment as compared with control cells. In addition, there was a non-significant (p>0.05) arrest observed in the S phase and also during the G0-G1 phase.

It was noticed that cell arrest was accompanied with apoptotic profile in the pre-G1 phase recording a total 14.11% ± 1.61 (p<0.001**) apoptotic cells. The highest % was in the late apoptosis phase (8.3 ± 1.62, p<0.001**) followed by early apoptosis (3.12% ± 1.15, p=0.056) as shown in Figures 5 and 6). Necrosis was also observed (2.69% ± 0.76, p=0.013*).

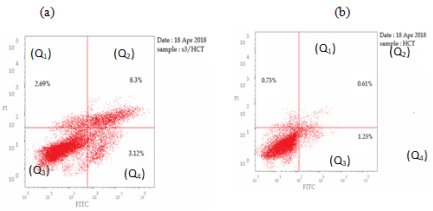

Figure 5: Flow cytometric analysis of HCT-116 colon cancer cells after treatment by enterocin OE-342 for 24 h (a) and without treating by enterocin OE-342 for 24 h (b). Dots with Annexin V-/PI- (Q3), Annexin V+/PI- (Q4), and Annexin V+/PI+ (Q2) Annexin V-/PI+ (Q1) features represent viable intact, early apoptotic, dead late apoptotic cells and necrotic cells respectively.

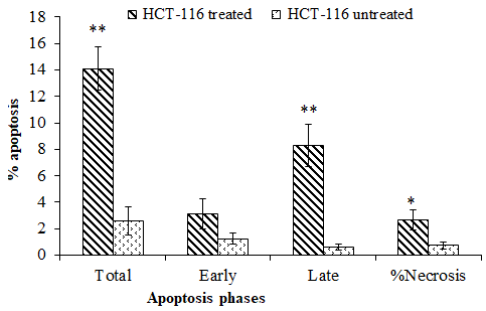

Figure 6: Apoptotic profile of enterocin OE-342 treated HCT-116 colorectal cell line. The percentages of arrested cells in each apoptosis phases are indicated in the graph presented. Necrosis was also observed (*P<0.05, **P<0.001, Student's unpaired t-test).

Immunomodulatory activities of enterocin OE-342

Quantification of the tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) in LPS-induced PBMC cell model: At least 2 × 10^4 cells were analyzed using flow cytometry after treatment with the non-toxic concentration of the tested enterocin for 48 h. The induced intracellular TNF-α and IFN- γ levels in PBMCs cell model after treatments were quantified by measuring the emitted relative fluorescence of the fluorescent probe using flow cytometry. TNF-α was detected in approximately 8.2% of LPS-induced PBMC cells from healthy donors, up to 5.8% of the cells produced TNF-α after treatment with enterocin OE-342. On the other hand, IFN- γ was detected in 3.4% of LPS-induced PBMC cells from healthy donors, up to 10.5 % of the cells produced IFN- γ after treatment with enterocin OE-342. Thus according to the obtained results, it was found that the recovered enterocin OE-342 significantly (p<0.001**) reduced the percentage of

induced TNF-α from and also significantly ($p<0.001^{**}$) increased the percentage of induced IFN- γ in LPS-induced PBMCs cell model after treatment as shown in Figures 7 and 8.

Discussion

Enterococci, which belong to the group of lactic acid bacteria (LAB), have received increased attention in recent years for various reasons [30]. *Enterococcus faecalis* is the dominant Enterocuccus in the gastrointestinal tract and is frequently found in human stool samples. Several studies have shown that there is a high frequency of bacteriocin production among enterococci, and it has also been reported that a major fraction of the isolated enterococci from stool samples do produce bacteriocins [31]. Large populations of bacteriocin-producing enterococci exist in human intestinal flora. The commensal/probiotic role of enterococci in humans and animals has evolved through thousands of years, enterococci have many positive traits that have been appreciated in food fermentation and preservation, and may also serve as probiotics to promote health. Production of bacteriocins (enterocins) is the useful biotechnological trait of Enterococcus bacteria [30].

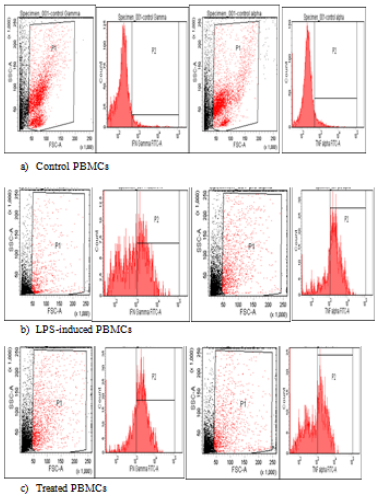

Figure 7: Flow cytometric analysis of PBMCs cells before and after treatment by enterocin OE-342. a) untreated PBMCs (control), b) LPS-induced PBMCs (Positive control), c) Treated PBMCs.

The microbiota can have a major effect on the formation and progression of cancer, and may even influence the outcome of chemotherapies and immunotherapies. Although most of these effects are mediated by indirect effects on immunosurveillance, they also may involve the direct effects of microbial products on cancer cells through various processes [2]. Cancer cells are known to develop chemotherapy resistance throughout the course of treatment. Thus, there is a continuously growing demand for novel anti-cancer and immunomodulator agents. The present study was conducted to evaluate the potential of enterocin OE-342 as an immunomodulator and anticancer agent against one of the most prevalent cancers in Egypt; colon cancer.

In this study, we have succeeded in the extraction and partial purification of enterocin OE-342 from *Enterococcus faecalis* isolated from the stool of apparently healthy neonate. Genes encoding for enterocins including AS-48, 31 and L50A were found in our isolated strain *Enterococcus faecalis* OE-342. According to previous studies, the multiple enterocins- producing isolate could exhibit a wider range of inhibition of the growth of undesirable bacteria than a simple bacteriocin producer [32]. The production of bacteriocins by LAB helps LAB to be colonized in their habitats and to compete with other bacteria [33]. Thus, multiple bacteriocin-producing enterococci and lactobacilli can be useful in controlling the gut microflora.

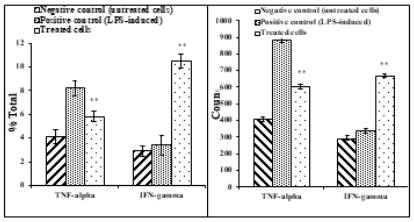

Figure 8: Effects of purified enterocin (OE-342) on the TNF-α and IFN- γ production by LPS- induced PBMC cell model. Cells were incubated with or without LPS (1mg/mL) for 24 h, in the presence or absence of enterocin OE-342 at the indicated concentration. Data are presented as mean± SE where statistical significance was tested using one way ANOVA ($^{**}P<0.001$).

In vitro cell models play an important role in understanding cellular related to up normal human physiological conditions. Investigating the anti-cancer effect of *E. faecalis* was performed with verification of the probable effect of its enterocin OE-342 on the inhibition of colon cancer cell proliferation. The crude bacteriocin from *Entr. faecalis* OE-342 induced a dose-dependent cytotoxicity and apoptosis in HCT-116 colon cancer cell line. This was in agreement with [34] who reported significant cell proliferation inhibition effects of *Entr. faecalis* secreted metabolites on treated cancer cells in a time-dependent and dose-dependent manner. Furthermore, it was observed the anti-proliferative effect of *Ent. faecalis* CECT7121 on a human lymphoblastoid B-cell line (PL104) obtained from bone marrow cells of a patient with an atypical acute myeloid leukaemia [35]. Still to be determined is whether this inhibitory activity differentially affects tumor cells or whether all tissue cultures of human cells would be equally inhibited. However, we report that *Entr. faecalis* OE-342 produces enterocin with apparent inhibitory activity against the proliferation of human colon cancer.

The inhibitory concentration of cell proliferation (IC50) was somewhat high recording 49.920 mg/ml as determined using Masterplex 2010 Software. Despite the anti-bacterial properties of different types of bacteriocins are well described, the anti-proliferative properties of bacteriocins on cancer cell is poorly understood. The sensitivity of tumor cells to bacteriocin may be attributed to the accumulation of glycopeptides and/or glycolipids on cell membranes of these cells, resulting in more affinity for the bacteriocin when compared with normal cells. Therefore, the binding of the bacteriocin to these constituents, create lethal events that could lead to cell death. Furthermore, the cytotoxic activity could be due to the presence in the bacteriocin extract of active products that could probably have anti-growth effects [15]. On the other hand, hypothesized that the ability of a bacteriocins to have a toxic effect on tumor cells will likely depend on the phase of the cell cycle rather than on the presence of precise surface receptors with greater attraction for bacteriocins in tumor cells [36].

Remarkably, the anticancer activity of bacteriocins is facilitated by the presence of high number of microvilli, allowing more binding of antimicrobial peptides to the cancer cells membranes, conversely to the normal cells' [37]. Additional trials are required to discover the exact mechanism of inhibition observed in this study.

Apoptosis is the primary mean of programmed cell death and plays a significant role in the regulation of tissue development and homeostasis [38]. Apoptosis is a process of cell suicide, described by specific morphological changes, such as nuclear fragmentation, condensation of chromatin, presence of apoptotic bodies and blebbing of the plasma membrane [39]. The induction of apoptotic cell death is a favourable emerging scheme for inhibition and treatment of cancer. Morphological changes offer the most direct criteria for distinguishing the apoptotic process [34]. So, inverted microscopy was used to detect apoptosis by monitoring the changes in cellular morphology. In the present study, morphological examination of treated colon cancer HCT-116 cells revealed various features associated with apoptosis, such as cytoplasmic blebbing and chromatin condensation, as well as cell shrinkag. Flow cytometric analysis showed up to 14.11% of apoptotic cells (8.3% late and 3.1% early apoptosis) and 2.69% necrotic cells. Our results were in agreement with [35] who studied induction of apoptosis on LBC cells by *Ente. faecalis* CECT7121 that showed 20 ± 5% apoptosis percentages as determined by morphological examination [34] reported that the supernatant of *Entr. faecalis* isolated from vagina of healthy fertile Iranian women could induce 76% apoptotic (22.64% early and 53.36% late apoptosis) and <1% necrotic cell on HeLa treated cells. The reported values appear to be higher than those obtained in our study presumably due to differences in cell lines and tested enterocin. Considering higher late apoptosis percentage than early apoptosis, it can be speculated that the detected necrosis in treated cells can be attributed to DNA fragmentation in apoptotic cells at 24 h, which is considered as necrosis by flow cytometry. Thus, apoptosis can be considered as the key phenomenon for cell death rather than necrosis.

Despite that the tested bacteriocin could induce cell damage, cellular proteins that are associated with controlling the cell cycle and apoptosis are the determinants of cell fate. Cell cycle checkpoints are the pathways that enhance cell death post exposure to toxins and determine the mechanism by which these pathways are regulated [40]. In the present study, it was noticed that the main cell cycle arrest was at G2/M phase in the treated colorectal cancer cell line indicating the growth inhibitory effect of tested enterocin [41]. The increased number of cells in sub-G1 phase points out to the greater incidence of apoptotic cells post treatment [42].

It has long been recognized that infections and inflammation are related to cancer. Moreover, chronic inflammation increases the risk for various cancers. Exemplary studies have indicated that there is an approximately 14% increase in prostate cancer risk due to prostatitis, and a 25% increase in colorectal cancer risk due to ulcerative colitis. Thus, the presence of inflammation appears to induce or facilitate carcinogenesis, indicating that eliminating inflammation may represent a valid strategy for cancer prevention and therapy [43].

Modulation of the immune system involves induction, expression, amplification or inhibition of any phase of the immune response. Different pro-inflammatory mediators and cytokines play critical roles in various inflammatory diseases; therefore, inhibition of those mediators offers a new therapeutic strategy for the treatment of inflammation. Numerous immunomodulators have been identified from different sources including microorganisms, fungi and plants [44]. The positive effects of LABs on cytokine production are complex and inconsistent. Some strains appear to enhance the anti-inflammatory cytokine production, interleukin (IL)-10, while having no effect or slightly decreasing production of the proinflammatory cytokines; chemokine, IFN-γ and TNF-α [45]. In addition, studies on the effect of lactic acid bacteria on cellular immunity are very limited.

Tumor necrosis factor - alpha (TNF- α) is pro-inflammatory cytokine and it has been involved in many inflammatory conditions. It is implicated in the same time in apoptosis and in cell proliferation. TNF-alpha not only acts as pro-inflammatory cytokine conducing to wide spectrum of human diseases including inflammatory diseases, but can also induce tumor development [46]. Interferon-gamma (IFN-γ) is a cytokine that plays an important role in inducing and modulating an array of immune responses [47]. It is primarily secreted by activated T cells and natural killer (NK) cells, and can promote macrophage activation, mediate antiviral and antibacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte-endothelium interaction, regulate Th1/Th2 balance, and control cellular proliferation and apoptosis [48].

Our data demonstrated that the recovered enterocin OE-342 reduced TNF-α production from LPS-stimulated PBMC cells. Moreover, the tested enterocin increased the percentage of induced IFN- γ in LPS-induced PBMC cells from 45.7% to 72.2% after treatment. Similar studies that indicated the same findings have been reported in a few cases. For example [49] demonstrated that peritoneal macrophages from mice intragastrically (ig) pre-treated with *Ent. faecalis* CECT7121 have a higher capacity to respond to certain microorganisms by secreting higher levels of inflammatory cytokines [35], reported the increased IL-10 levels by the administration of *Ent. faecalis* CECT7121 and concluded that this strain can modulate the immune response and influence its polarization from very early stages of the antigen presentation process by dendritic cells [50] indicated the anti-proliferative activity of IFN-gamma that lead to the growth inhibition or cell death, generally induced by apoptosis. In addition, it has been reported that increased levels of the pro-inflammatory cytokine TNF are associated with poor prognosis and disease severity in patients with non-Hodgkin's lymphoma [51]. Thus induction of IFN-γ production by enterocin OE-342 together with its effective ability to inhibit the LPS induced TNF- α may suggest a strong role in cancer immunotherapy.

In conclusion, our study evaluated the anticancer activity of *Entr. faecalis* enterocin OE-342 by testing its direct antitumor effects on colorectal cancer cell line, as well as, its immunomodulatory effects. This study revealed that enterocin OE-342 exerted a direct cytotoxic action on the tumors cells via apoptotic mechanisms in addition to stimulating the immune response providing novel targets to the current therapeutic manipulation of cancer. Finally, based on the current evidences the effects of *Entr. faecalis* enterocin OE-342 on colon cancer are considered to be very promising. Although, further purification, structure analysis and amino acid sequence determination are required to identify the tested enterocin to ensure its efficacy and safety in the prevention and/or treatment of colon cancer.

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Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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