

Combining Prebiotic and Mixture of Freeze Dried Probiotics to Develop Synbiotics that Induce Apoptosis in Human Colon Tumor Cells

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IN THE PRESENT study, we first aimed to select some potential anti-cancer probiotics *in vitro*. Using the inulin fermentation product by the selected five probiotic bacteria (*Lactobacillus paraplantarum* strain S143, *L. pentosus* strain P2-3, *L. plantarum* strain J12, *L. gasseri* strain F. and *L. reuteri* strain IRT), Caco-2 cells were exposed to 50% filtrate supernatant of each probiotic bacteria after 24 h of incubation. Results showed that inulin fermentation product of the selected probiotics cause apoptosis in the Caco-2 cell line. Also, the viability of the freeze-dried powders of the five probiotics using skimmed milk as cryoprotectant was followed up for 12 weeks of storage at refrigerated conditions and also at room temperature. Results proved that lyophilized powders showed slight decrease in their viable count during storage both at room and refrigerated temperatures. This would ensure a good cell concentration for at least 3 months to be administered as a probiotic and would guarantee the long-term delivery of sufficient active, viable, and functional cultures.

Keywords: Probiotic, Mixture, Freeze dried, Synbiotic, Apoptosis and Tumor.

Introduction

Probiotics are live microorganism which, when consumed in sufficient amounts, provide a health benefits to the host (FAO/WHO, 2006). Generally regarded as safe (GRAS) organisms, lactic acid bacteria (LAB) have become the most widely used probiotics for medicine and food production. As probiotics, firstly, the microorganisms must be non-pathogenic and non-toxicogenic; secondly, they should withstand external and internal environments, including gastric acid and bile salts. In addition, the adhesive ability of LAB is a primary consideration in the selection of probiotics (Saad et al., 2013).

Lactic acid bacteria together with bifidobacteria are most investigated probiotics since decades. The uniqueness of LAB is that they provide several health benefits as well as they are involved in food fermentations (Abushelaibi et al., 2017). Several criteria have been used to consider new LAB isolates as probiotic including tolerance to acid and bile conditions, cholesterol

lowering potential, ability to hydrolyze bile salt, ability to possess antimicrobial properties, and able to survive during the fermentation process (Vijaya Kumar et al., 2015).

Based on the aforementioned, administration of beneficial microorganisms (called probiotics) or prebiotics (non-digestible ingredients of food that, when administered in sufficient amounts, selectively stimulate the growth and/or activity of one or a few number of health promoting bacteria in the colon) represents a promising approach for treating several gastrointestinal alterations and can have many beneficial effects on the body (Buigues et al., 2016).

Many recent publications have reviewed the role of probiotics, prebiotic and Synbiotics in Colorectal cancer (Orlando & Russo, 2013). Colon cancer is the third most common cancer worldwide, with high morbidity and mortality (Hagggar & Boushey, 2009). world wide deaths from colon cancer have increased from 608,000 in 2008 to 694,000 in 2012 (WHO, 2014).

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Colorectal cancer (CRC) is a well-defined series of sequences caused by mutations, activations, and deletions of oncogenes and tumor suppressor genes leading to adenoma/carcinoma (Commane et al., 2005). Epidemiological, experimental, case, studies have shown a strong ability of mutagens to induce cancer (Biondi et al., 2012). CRC is influenced by environmental factors such as, diet and dietary habits, physical inactivity, consumption of tobacco and other occupational hazards (Stewart & Kleihues, 2003). Food carcinogens produced during cooking at high temperatures and air pollution include, polycyclic aromatic hydrocarbons (PAH), heterocyclic amines (HCA), N-nitroso compounds (NOC), mycotoxins (aflatoxins) and acrylamide that represents a potential risk factors for CRC and other cancer types (Cross et al., 2010).

Treatments include either single or combination therapies such as chemotherapy, radiation, surgery, or biologically targeted management (Jemal et al., 2006). However, these therapies are less effective during metastasis, therefore prevention and early detection are key factors for reducing the risk of coloncancer (WHO, 2014). Recent attention has been focused on increasing dietary intake of probiotic and fibers (Globocan, 2012). LAB and their products of fermentation are claimed to be antimutagenic and Anticarcinogenic (Ljungh & Wadström, 2006). Fermentation supernatant fractions of inulin showed a significant growth-inhibition and apoptosis induction in the human colon cancer cells (Munjaj et al., 2009). The consumption of synbiotics, a mixture of probiotics and non digestible constituents such as dietary fiber, has been reported to reduce the risk of colon cancer (Steina et al, 2012). It is generally believed that probiotics act via alteration of the intestinal microflora, enhancement of the host's immune response, cell cycle arrest, promotion of apoptosis and induction of short-chain fatty acid (SCFA) production (Faghfoori et al., 2015). In addition, the ability of probiotic cells to adhere to the intestinal mucosa is a critical parameter to probiotic action. It has been shown that specific probiotic strains exert anti-proliferative effects via synergic actions between adhesion to colon cancer cells and production of short-chain fatty acids, mainly butyric and propionic acids (Thirabunyanon & Hongwittayakorn, 2013).

The present study was aimed for the production

of gelatin capsules containing the prebiotic inulin and freeze-dried mixture of synergistic probiotic bacteria capable of induction of apoptosis of colorectal cancer cell line and studying the survival of the freeze-dried powders of the probiotics during a 3 months storage period.

Materials and Methods

Selection of Inulin fermenting LAB

In a previous study 42 isolates of LAB bacteria have been tested for their probiotic potential, including; tolerance to simulated gastric and intestinal juices, antibiotic resistance, antagonistic activity toward some pathogenic bacteria and adhesion to Caco-2 cell line. The isolates with desirable characters have been selected for this study.

The obtained isolates were tested for their ability to utilize inulin as follows: MRS broth without any sugar (basal MRS) containing 40 mg/L of bromocresol purple was prepared and autoclaved. The inulin sugar solution was prepared, sterilized through a syringe filter (0.22 µm pore size) and added to the sterile medium to obtain the concentration of 10 g/liter. Isolates were activated in 10 ml MRS medium and incubated at 37 °C for 24 h. Then, they were centrifuged for 10 min at 10000 rpm. Pellets were washed twice and resuspended in MRS without glucose and containing pH indicator bromecresol purple. Then inoculated (2%, vol/vol) in 10 ml inulin medium, glucose solution was used as positive control while suspended cells in basal medium containing pH indicator bromecresol purple was used as a negative one. After 24 h incubation at 37°C under anaerobic conditions, the color change from purple to yellow was recorded as positive fermentation results compared with the positive and negative controls (Hedberg et al., 2008).

The anticancer effect of inulin fermentation product of probiotic bacteria on Caco-2 cells

Probiotic isolates were selected for further investigation to evaluate their ability to induce apoptosis in Caco-2 cells. Probiotic isolates were grown in MRS basal medium containing 10 gm/L filter sterilized inulin solution at 37 °C for 24 h anaerobically (Barry et al., 1995). For *in vitro* preparation of the cell-free filtrate, cultured bacterial cells were centrifuged at 11,000 × g for 30 min. then supernatant was filtered using a 0.22-µm syringe filter and was stored at 4 °C until use.

Human Colorectal Carcinoma cell line, Caco-2, was purchased from VACSERA (Egyptian company for production of vaccines and sera - tissue culture unit). The cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum and incubated at 37°C in a CO₂ incubator. The Caco-2 monolayers were at 90 % confluence prepared in 6-well plates, then washed twice with phosphate buffered saline (PBS) (pH 7.4). Each supernatant was mixed with the RPMI-1640 medium in concentration 50% (1 ml supernatants: 1 ml RPMI-1640 medium). MRS containing RPMI1640 medium was used as negative controls and doxorubicin HCL (0.4µg/ml RPMI1640 medium) as positive control. The treatments were added to each well of the plates and incubated for 24 h under aseptic conditions in humidified atmosphere and 5 % CO₂ at 37°C. DNA-binding dyes Acridine orange (AO) and ethidium bromide (EB) double staining were carried out to detect the apoptosis in Caco-2 cells.

Acridine orange/ethidium bromide (AO-EB) stock solution (100x): acridine orange, 15 mg; ethidium bromide, 50 mg dissolved in 1 ml 95% ethanol and QS to a total volume of 45 ml in purified water. A working solution of (AO-EB) was prepared by diluting a 1-ml aliquot of the stock solution into 100 ml PBS. After 24 h incubation of cells with the supernatant, the media were discarded and 100 µl of the dye mix working solution was added to each well. The dye was immediately (fast uptake) examined by fluorescence inverted microscope (LEICA DMI3000 B) filter I3 at 200x magnification recording changes in morphology and color changes of the nucleus because of each supernatant, each assay was carried out in triplicate. The cells were divided into three categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin) and late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) (Attari et al., 2009).

Preparation of freeze-dried probiotic powders

This was carried out according to Zárate & Nader-Macias (2006) with slight modifications, each probiotic bacterium was cultivated in MRS broth; then, 10 ml of each culture was inoculated into 250 ml of sterile MRS-broth and incubated at 37°C. Cells were harvested by centrifugation (×7000 g, 10 min, 4°C), washed twice with sterile PBS and then resuspended in 4 ml of 10 % sterile reconstituted skimmed milk. This suspension

was fractioned into 2 bottles (50 ml) and frozen at -20°C. Frozen media were lyophilized under vacuum for 8 h (ilShinBioBase freeze dryer) and freeze-dried powders were placed aseptically into empty gelatin capsules (50 mg/capsule approximately). Samples were obtained from each culture before and after freeze-drying and the numbers of viable cells were enumerated.

Storage stability of the freeze-dried powders

Gelatin capsules containing 20 mg of lyophilized powder in each capsule were stored at two temperatures: room temperature and 4°C, inside sterile containers containing silica-gel desiccants and stored under darkness. Duplicate samples were taken from storage at 0, 1, 2, 4, 10 and 12 weeks for testing of probiotic viability by plate counting. The lyophilized powders were rehydrated in 1 ml of saline solution (0.9% NaCl) and these suspensions were serially 10-fold diluted in sterile peptone-water, all colonies were enumerated after incubation of plates at 37°C for 48 h. Results were expressed as log of CFU/capsule of lyophilized powder. The weight of freeze dried microorganisms was determined by the difference between full and empty capsules (Jalali et al., 2012).

Results

Depending on a previous study for selection of the LAB isolates with probiotic properties and inulin fermentation ability, of all isolates five strains (*Lactobacillus paraplantarum* strain S143, *L. pentosus* strain P2-3, *L. plantarum* strain J12, *L. gasseri* strain F., and *L. Retire* strain IRT) showed remarkable probiotic properties, thus they were selected for continuing anticancer examinations and with details presenting their characteristics.

The anticancer effect of inulin fermentation product of probiotic bacteria on Caco-2 cells

Since one of the most desired strategies in cancer therapy is induction of apoptosis in tumor cells, to analyze the incidence of apoptosis in Caco-2 cancer cells and prove the anticancer effect of inulin fermentation product by the selected five probiotic bacteria, the Caco-2 were exposed to 50% filtrate supernatant of inulin fermentation product by the tested probiotic bacteria after 24 h of incubation to visualize apoptosis using the Acridine orange / Ethidium bromide double staining and observation by fluorescent microscopy.

DNA-binding dyes AO and EB were utilized for the detection of apoptotic cells morphology. Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. The cells were divided into three categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin) and late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation).

Results showed that none of the apoptosis related signs were observed in the negative control cells, they appear as viable cells with uniform bright green nuclei with organized structure. While positive control treated cells are orange-stained nuclei with chromatin condensation or fragmentation.

The Morphological features of apoptosis can be seen in Fig. 1 cells treated by inulin fermentation product of the probiotic (*L. paraplantarum* strain S143, *L. pentosus* strain P2-3 and *L. plantarum* strain J12) there were shrunken cells having yellow nuclei with condensed or fragmented chromatin; these apoptosis characteristics belonged to late apoptosis stage.

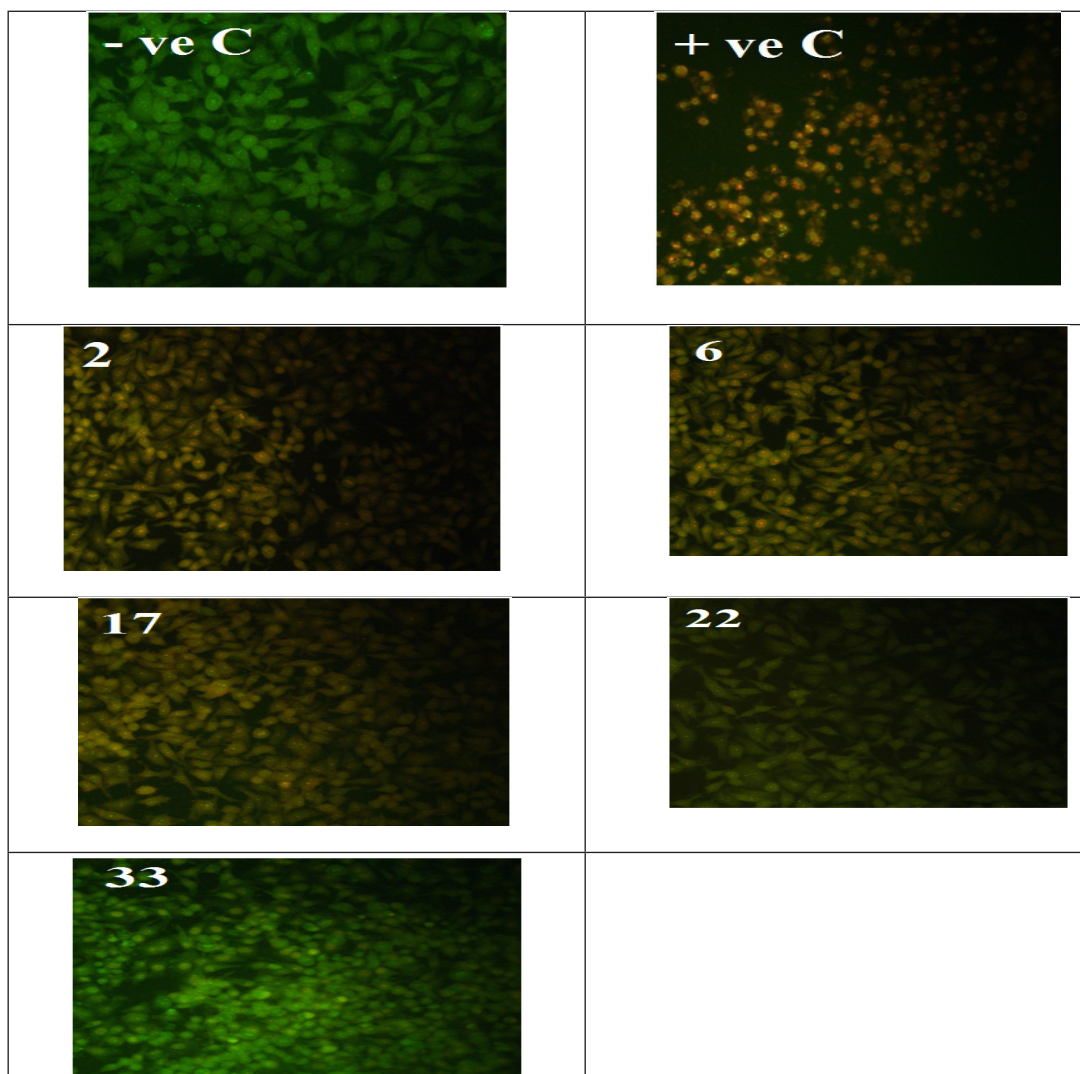


Fig. 1. Fluorescent microscope pic. of Caco-2 cell line treated with filtrate supernatant of inulin fermentation product by the tested probiotic bacteria after 24 h of incubation to visualize apoptosis using the Acridine orange / Ethidium bromide double staining (-ve C: negative control, +ve C: positive control, 2: *L. paraplantarum* strain S143, 6: *L. pentosus* strain P2-3, 17: *L. plantarum* strain J12, 22: *L. gasseri* strain F and 33: *L. reuteri* strain IRT).

Also in Fig. 1 cells treated with inulin fermentation product of the probiotic (*L. gasseri* strain F and *L. reuteri* strain IRT) show green shrinking cells which still have intact membranes but have started to undergo DNA cleavage with perinuclear chromatin condensation visible as bright green patches. These distinctive signs of apoptosis belonged to early apoptosis stage.

Storage of capsules

According to the data presented in the Tables 1 and 2, when the probiotic cultures were freeze-dried in skimmed milk as a cryoprotectant, their

viable counts decreased about 0 – 0.2 log CFU when compared to the viable counts before freeze drying. After 12 weeks storage of the freeze dried probiotic bacteria at room temperature their viable counts showed slight decrease about 1 – 1.4 log CFU/ capsule when compared to their viable counts immediately after freeze drying.

Finally, the lyophilized probiotic bacteria conserved higher viability after 12 weeks of storage at 4°C as their viable count decreased about 0.5 – 1.1 log CFU/ capsule.

TABLE 1. Survival of the probiotic cultures after freeze-drying and 12 weeks storage at room temperature.

| Isolate No. | Counts before freeze drying log CFU/ml | Viable cell counts (log CFU/ capsule) after freeze drying during storage at room temperature | | | | | |
|-------------------------------------|--|--|------|------|------|------|------|
| | | 0 w | 1 w | 2 w | 4 w | 10 w | 12 w |
| <i>L. paraplantarum</i> strain S143 | 12.4 | 12.3 | 11.9 | 11.9 | 11.6 | 11.4 | 11.1 |
| <i>L. pentosus</i> strain P2-3 | 12.3 | 12.3 | 12.2 | 12.2 | 12.1 | 11.3 | 11.3 |
| <i>L. plantarum</i> strain J12 | 12.5 | 12.4 | 12.3 | 12 | 11.7 | 11.6 | 11.4 |
| <i>L. gasseri</i> strain F | 12.6 | 12.4 | 12.3 | 12.2 | 12.1 | 11.9 | 11.3 |
| <i>L. reuteri</i> strain IRT | 12.4 | 12.4 | 12.3 | 12 | 11.9 | 11.7 | 11 |

TABLE 2. Survival of the probiotic cultures after freeze-drying and 12 weeks storage at 4°C.

| Probiotics | Counts before freeze drying log CFU/ml | Viable cell counts (log CFU/ capsule) after freeze drying during storage at 4°C | | | | | |
|-------------------------------------|--|---|------|------|------|------|------|
| | | 0 w | 1 w | 2 w | 4 w | 10 w | 12 w |
| <i>L. paraplantarum</i> strain S143 | 12.4 | 12.3 | 12.4 | 12.1 | 11.9 | 11.9 | 11.6 |
| <i>L. pentosus</i> strain P2-3 | 12.3 | 12.3 | 12.3 | 12.2 | 12.1 | 12 | 11.3 |
| <i>L. plantarum</i> strain J12 | 12.5 | 12.4 | 12.3 | 12.2 | 12.2 | 11.7 | 11.7 |
| <i>L. gasseri</i> strain F | 12.6 | 12.4 | 12.5 | 12.4 | 12.3 | 12.1 | 11.9 |
| <i>L. reuteri</i> strain IRT | 12.4 | 12.4 | 12.3 | 12.1 | 12 | 11.7 | 11.3 |

Discussion

In each year, about 1.2 million new cases of colorectal cancer are diagnosed all over the world (Jemal et al., 2010). At present, the therapy for colon cancer includes surgery, radiation therapy, chemotherapy, as well as immunotherapy and gene therapy. Surgical resection is generally applied to early stage of cancer, which likely leads to bowel dysfunction. Quite a few patients suffered the postoperative recurrence and metastasis and were required to undergo chemotherapy (Lim et al., 2013). At present, the drugs used for chemotherapy not only inhibit the cancer cell growth, but also

destruct the normal cells. Therefore, exploring effective therapies that are specifically sensitive to cancer cells not to normal cells is particularly important. Probiotic microbes have been associated with a variety of health benefits including enhanced immune performance, suppression of tumor cells and protection against colon cancer (Suzuki et al., 2008). Health promoting effects of probiotic bacteria is found to be strain dependent and their positive effect in colon cancer may also vary from one strain to another (Sadeghi-Aliabadi et al., 2014).

A prebiotic is a selectively fermented ingredient that permits specific changes, in the

composition and/or activity in the gastrointestinal microbiota that confers benefits upon the host well-being and health (Vandenplas, 2016). Prebiotics are non-digestible carbohydrates acting as a nutritive substrate to stimulate the growth and metabolism of probiotic bacteria (Ghoury et al., 2014). Whereas probiotics introduce exogenous bacteria in the colonic microbiota, prebiotics aim to modulate the endogenous luminal microflora by selectively stimulating the growth of health-promoting bacteria already present in the colon. For example, different non-digestible carbohydrates, such as galacto-oligosaccharides, showed the potential to increase the number of bifidobacteria and subsequently the production of short chain fatty acids (SCFAs) in healthy volunteers (Bouhnik et al., 2004).

Butyrate has received much attention as a potential chemopreventive agent (Abrahamse et al., 1999). Butyrate act as an energy source for non-transformed cells, and also reduce the survival of tumor cells by inducing apoptosis and differentiation as well as inhibiting proliferation. These mechanisms may play an important role in the reduction and / or inhibition of promotion and progression of cancer development (Sengupta et al., 2006).

Apoptosis is programmed cell death that plays an important role in controlling cell numbers. Therefore, the regulation of cell proliferation and death can be one of cancer treatment strategies (Zhong et al., 2014). Probiotic bacteria can regulate cell apoptosis and proliferation. LAB isolated from infant faeces inhibited colon cancer cell proliferation through direct adhesion to cancer cells and production of SCFA (Thirabunyanon & Hongwittayakorn, 2013). The anti-proliferative effect of *L. acidophilus* and *L. plantarum* on colon cancer cells via induction of apoptosis has been previously reported by several researchers (Nami et al., 2014).

In this study, using the EB/AO stain results showed that after 24 h of Caco-2 cell line exposure to filter sterilized inulin fermentation supernatant of the probiotics (*L. paraplantarum* strain S143, *L. pentosus* strain P2-3 and *L. plantarum* strain J12) displayed morphological feature of late apoptosis; while those of (*L. gasseri* strain F and *L. reuteri* strain IRT) showed early apoptosis stage.

These results are similar to previous results obtained by Haghshenas et al. (2014), they

investigated acceptable cytotoxic effect of the secreted metabolites of *Lactococcus lactis* subsp. *Lactis* 44Lac against all of the treated HT29 cancer cell lines. Cytotoxic assessments through flow cytometry and fluorescent microscope proved that apoptosis is the main cytotoxic mechanism for the secreted metabolites.

Also, Haghshenas et al. (2015) studied the effect of *L. plantarum* 17C on HT-29 cell viability, HT-29 cells were exposed to the supernatant produced during the late stationary growth phase of *L. plantarum* 17C and analyzed by fluorescent microscope and found a significant number of apoptotic cells after 24 h of incubation with 50 µg/ml of *L. plantarum* 17C supernatant.

Soltan et al. (2015) reported that the supernatants of *L. acidophilus* ATCC 4356 and *L. casei* ATCC 39392 decreased CaCo-2 cell proliferation and increased cell apoptosis.

The utilization of prebiotic substances in combination with probiotic strains (synbiotics) is an important topic for industrial purposes. In functional foods, prebiotic substances have a double role: they improve probiotic survival during storage in foods, and when reconstituted in the bowel, they act to enhance the digestive process (Roberfroid, 2007). Therefore, the study of new synbiotic formulations is particularly anticipated (Casiraghi et al., 2007). One of the main benefits of synbiotics is to increase the persistence of the probiotic bacteria in the gastrointestinal tract (Rastall & Maitin, 2002).

In this study, inulin was selected as a prebiotic for the symbiotic formulation with the freeze-dried powders of the five selected probiotic for the production of gelatin capsules.

While they are well-known in the food industry, the enthusiasm in probiotics has spiked lately to the pharmaceutical industry, the administration of pharmaceutical formulations containing such viable health promoting bacteria, these pharmabiotics need to provide their beneficial effects until the end of shelf life (Broeckx et al., 2016).

As probiotic utilization shifted from nutritional supplement to medicinal treatment, product stability is a source of concern (Barrons & Tassone, 2008). The quality of probiotic relies

greatly on their viability, which is a fundamental fulfillment to colonize the human large intestine (Lahtinen et al., 2005).

To be effective, at least 10^8 - 10^9 CFU viable probiotics per daily dose should be consumed and therefore maintained alive in commercial products (Desfosses-Foucault et al., 2012). One of the significant issues concerning the formulation of probiotics and pharmabiotics is their loss of viability after processing and during storage. Many factors, such as temperature, oxygen level, moisture level, pressure and dosage formulation have an influence on the viability of probiotics and thus their health-promoting activity. Although some immune-related effects can also be achieved by non-living probiotics or their isolated compounds (Lebeer et al., 2010), the presence of a suitable amount of viable bacteria in the probiotic formulation is an important criterion for health claims (Hill et al., 2014). To enhance long-term stability of probiotics, it is important to preserve them in a dried form, in which the water content is decreased (Vesterlund et al., 2012).

Subsequently, suitable probiotic microbes and fitting vectors must be chosen. Unfortunately, many studies found that the high number of lactobacilli/bifidobacteria in many commercial probiotic products was not accomplished as claimed in the product data, particularly in the dried form of probiotic products: more than 67% of dried powder or granule products distributed in Taiwan markets contained LAB lower than 10^6 CFU/ml, and half of the product brands contained no viable LAB (Lin et al., 2006). In the European countries, 30% of dried probiotic supplement brands available in the market contained no viable bacteria (Temmerman et al., 2002). Similar observations on products in the UK have been made by Hamilton-Miller & Shah (2002).

The industrial application of LAB depends on concentration and conservation technologies that are needed to ensure the long-term stability of cultures in terms of viability and functional activity (Li et al., 2010). It is basic, both technologically and economically to increase the viability of probiotic culture during drying and storage for long periods (Carvalho et al., 2004). There are many mechanisms that allow us to keep the viability of the bacteria over time, cryopreservation and lyophilization being the most usable (Palmfeldt et al., 2003). Moreover, freeze-drying and subsequent storage

decrease microbial viability since the drying expose microbes to an extra stress. Different species show different degrees of survival to freeze-drying (Font et al., 1983). To limit this damage, cryoprotectant substances are generally utilized. Skimmed milk can avoid harm by balancing the cell membrane and providing a protective layer for the cells (Carvalho et al., 2004). For most LAB cultures of commercial interest in the dairy industry, skim milk powder is chosen as drying medium, since it (i) Avoid cellular injury by balancing out the cell membrane constituents (Selmer-Olsen et al., 1999), (ii) Makes a porous structure in the freeze-dried product that makes rehydration easier, and (iii) Contains proteins that provide a protective covering for the cells (Abadias et al., 2001).

In the present study, the effect of storage on the viability of the freeze-dried powder of the five probiotics in gelatin capsules stored in sterile containers containing silica-gel desiccants were studied at room temperature and at 4°C. The viable count of the lyophilized probiotics showed a slight reduction when stored at 4°C. While at room temperature the viable counts of the probiotics showed a higher reduction.

These results agree with the results obtained by Rathnayaka (2013) they studied the effect of lyophilization on viability and probiotic properties of a probiotic microbial mixture containing *Lactobacillus rhamnosus* and *L. plantarum* with UHT milk, sucrose, sorbitol and trehalose during six-month storage at 4°C was also studied. They prepared five types of mixed probiotic cultures: as microbial mixture in PBS, UHT milk, UHT milk added with sucrose, UHT milk added with sorbitol and UHT milk added with trehalose. Better survival of microorganisms after lyophilization was shown in all tried bacterial mixtures except the bacterial mixture in phosphate buffered saline. Highest survival was appeared in the bacterial mixture with UHT milk. According to the results of the Shigella inhibitory assay, probiotic properties of microbes has not been changed by lyophilization or long-term storage.

Also, Gisela et al. (2014) determined the ideal levels of incorporation of different cryoprotectants (skim milk, sucrose and trehalose) in the freeze-drying process of *Lactobacillus plantarum*, and obtained the ideal concentrations of cryoprotective agents: 24.06% milk powder, 6.22% sucrose, 5.63% trehalose.

Moreover, Jalali et al. (2012) analyzed the impact of different formulations of cryoprotectant media containing skim milk, trehalose and sodium ascorbate on the survival rate of probiotic bacteria during lyophilization at different storage temperatures for preparation of a capsule containing the probiotics *Lactobacillus paracaseisub* sp. tolerance and *Lactobacillus delbrueckii* sp. *Bulgaricus*, and found that with no cryoprotectant, small number of bacteria survived, but they maintained higher viability after lyophilization in media with the cryoprotectants. Skim milk in water resulted in an increased viability after freeze-drying. Media with a combination of trehalose and skim milk maintained a higher percentage of viable bacteria, up to 82%. In general, a higher number of viable cells was found in capsules containing freeze-dried bacteria with sodium ascorbate after 3 months of storage. After this period, a marked decline was observed in all samples stored at 23°C compared to those stored at 4°C. The greatest survival rate (about 72-76%) was observed with media containing 6% skim milk, 8% trehalose and 4% sodium ascorbate.

Conclusion

In conclusion, the use of probiotics in enhancing the gut microflora of human shows signs of becoming a success. This study showed promising results to exploit the freeze-dried powders of the strains supplemented with inulin as probiotics mixture with anti-cancer effects for therapeutic purposes. However, more *in vitro/in vivo* investigations should be conducted before this preparation is introduced as potential anti-cancer therapeutics.

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الجمع بين بريبيوتيك وخليط من البروبيوتيك بكتريا المجففة بالتجميد لتطوير سينبيوتيك يحفز الموت المبرمج في خلايا القولون البشري السرطانية

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تهدف هذه الدراسة إلى اختيار بكتريا البروبيوتيك ذات التأثير المضادة للسرطان، باستخدام ناتج تخمير الإينولين من قبل الخمسة بروبيوتيك بكتيريا المختارة (ل. بارابلانتاروم سلالة اس143، ل. بينتوسوس سلالة بي3-2، ل. بلانتاروم سلالة جي 12، ل. غاسيري سلالة اف. و ل. ريوتيري سلالة إرت)، حيث تعرضت مزرعه الخلايا السرطانية للقولون والمستقيم كاكو-2 إلى تركيز 50% من ناتج تخمير الإينولين المرشح من كل بكتيريا بروبيوتيك لمدة 24 ساعة من التحضين. وأظهرت النتائج أن منتج تخمير الإينولين من سلالات البروبيوتيك المختارة يسبب موت الخلايا المبرمج في مزرعه الخلايا السرطانية للقولون والمستقيم كاكو-2. تم أيضا، متابعة حيويه المساحيق المجففة بالتجميد المنتجة من الخمس سلالات البروبيوتيك باستخدام الحليب منزوع الدسم كواقي للخلايا لمدة 12 أسبوعا من التخزين تحت ظروف التبريد وأيضا في درجة حرارة الغرفة. وأثبتت النتائج أن المساحيق المجففة بالتجميد أظهرت انخفاضا طفيفا في اعدادها الحيويه أثناء التخزين سواء في الغرفة أو في درجات الحرارة المبردة. وهذا من شأنه ضمان ما لا يقل عن 3 أشهر من التركيز الجيد للخمائر الحيويه للاستخدام كبروبيوتيك، وضمان على المدى الطويل وصول خمائر وظيفية فعالة حيويه.