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Original article

Encapsulation of *Lactobacillus rhamnosus* in alginate, pectin and whey protein targeting colon cancer

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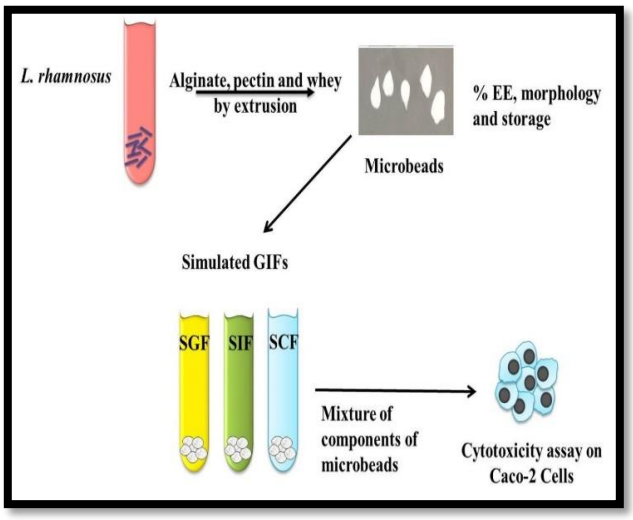
Abstract:



The encapsulation of probiotic cells in prebiotic polymers is one of the most effective techniques to maintain probiotic viability during gastrointestinal transit and storage. The purpose of this study was to fabricate a suitable colonic delivery system for *Lactobacillus rhamnosus* using alginate, pectin, and whey protein for oral administration. The effects of biopolymers at different concentrations and ratios under various sterilization temperatures on *Lactobacillus rhamnosus* viability were evaluated. The microbeads were evaluated for entrapment efficiency, shape, and morphology by SEM. The viability of free and encapsulated *Lactobacillus rhamnosus* in simulated gastric fluids was also evaluated. Moreover, MTT assay was implemented to determine the cytotoxic effect of *Lactobacillus rhamnosus* microbeads using the colon cancer cell line Caco-2. Different signaling pathways in the Caco-2 cell line were also studied. The results showed that the formulae produced from polymers sterilized at low temperature had the highest entrapment efficiency and viability for *L. rhamnosus*. The symbiotic effect of probiotics and prebiotics was observed mainly after incubation in simulated colon fluid for 4 h and 24 h. The mixture of components of the microbeads M12 showed an anticancer effect on Caco-2 by upregulation of *Bax* and caspase-3 and downregulation of *Bcl-2.*

Keywords: Probiotics, prebiotics, cytotoxicity, Caco 2, caspase-3, *Bax* and *Bcl-2*.

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1. Introduction

Colon cancer has been increasing gradually throughout the last century in many areas of the world mainly in developing countries. In 2018, the number of new patients diagnosed with colon cancer was 18.1 million and may reach 29.4 million in 2040 [(1)]. The main causes of colon cancer are environmental and lifestyle factors such as exposure to toxic substances, UV radiation, diet alteration, increase in alcohol consumption, and lack of physical activity. These factors lead to pathological disturbance in the colonic environment, including the composition of the gut microbiota [(2)]. Nutraceuticals such as probiotics and prebiotics show a vital role in the inhibition and treatment of colon cancer. Probiotics are live, non-pathogenic bacteria that, when added to food or taken as dietary supplements guarantee the health of the host. The effective dose should contain $>10^6$–10^8 CFU/g (colony-forming units) of probiotic bacteria. The anticancer effects depend on strain type, metabolic properties, and components secreted. They modify the progress of tumors through different pathways including encouraging apoptosis and inducing cell cycle arrest as well as via anti-mutagenic, anti-oxidative, anti-inflammatory, and anti-angiogenic effects [(3)]. *Lactobacillus rhamnosus* is one of the probiotics that documented to have colon anticancer effect. Sharma, M. and his colleagues [(4)] prepared cell-free supernatants from different *Lactobacillus* strains to determine their cytotoxic effect using two human colon cancer cells HT-29 and Caco-2. Among many tested strains, *L. rhamnosus* revealed a high anti-proliferative effect due to metabolites produced by *L. rhamnosus*.

Also, Di, W *et al*. [(5)] reported the effect of exopolysaccharides (EPS) secreted by *L. rhamnosus* on HT-29 cell line. The anticancer effect of EPS varied depending on the dose applied to the cancer cell line; the anti-proliferative effect of EPS improved as the concentration of EPS increased.

Orlando *et al*. [(6)] studied the anticancer effect of live and heat-killed *L. paracasei* and *L. rhamnosus* on colon cell line DLD-1. They observed that both live and dead strains were shown to be capable of inhibiting the viability of DLD-1 cells.

Prebiotics are defined as food ingredients like carbohydrates, resistant starch, non-starch polysaccharides, and non-digestible oligosaccharides, which are not digested or absorbed in the small intestine with partial or whole fermentation in the large intestine. They affect the host by motivating the growth and activity of one or more health-promoting bacteria. Probiotics are more effective when administrated with prebiotics than probiotics alone in the inhibition and management of colon cancer [(7)].

The main factor that has been found to stimulate the functionality and survivability of probiotics is a transition through the gastrointestinal tract (GIT) mainly, in the stomach, and small intestine. The high acidic (pH 1 to 3), high ionic strength, and enzyme activity (pepsin) environments in the gastric fluids cause a reduction in the viability of bacteria. The presence of bile acids in the small intestine decreases the viability of many probiotics. Probiotics can persist under the pH environments prevailing within the human colon, that is between pH 6 to 7 [(8)].

The microencapsulation method can retain the microbial cells from adverse GI conditions through cell entrapment inside a biopolymeric matrix. Probiotics have been encapsulated using a variety of techniques e.g. low-temperature drying process, such as ultrasonic vacuum spray drying [(9)], spray chilling [(10)], electrospinning [(11)], and supercritical technology [(12)]. These techniques present extremely low or high temperature and osmotic stress, which are destructive to the survival of probiotic cells [(13)]. In the meantime, high-temperature drying techniques, for instance, spray drying [(14)] and fluid bed drying [(15)] have also gained adequate attention. Nevertheless, proper protective wall materials are still needed to overcome the heat, shear stress, and long

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period of oxygen exposure during high-temperature processing [16]. On the other hand, due to the sensitivity of probiotic bacteria to gastric acid and bile conditions during gastrointestinal digestion, the digestion properties of these encapsulating wall materials used in low or high temperature drying technologies also need to be pre-considered [17, 18]

The extrusion technique is the mildest one as it does not require high temperature or surfactants or any solvents to confirm great cell viability. The extrusion method depends on adding the probiotic cells to the polymeric solution and then extruding into a crosslinking solution like calcium chloride by a syringe needle or nozzle [19].

Several polymers have been studied for the microencapsulation procedure of probiotics, including polysaccharides [20], proteins [21], and lipids [22]. Sodium alginate is the common encapsulating polymer used for this purpose. Alginate microcapsules are prepared by ionotropic gelation in the presence of divalent cations [23].

Alginate has several benefits, such as being naturally sourced, biocompatible, non-toxic, and easily applied during the encapsulation process. However, microcapsules fabricated by sodium alginate have a porous nature that does not provide integrity to the capsule wall leading to the low efficiency of encapsulation. They are less stable in stomach juice which makes their capsule sensitive leading to the premature release of the entrapped probiotic which is invented to be released in the intestine [24]. Mixing alginate with other biopolymers could act as a useful method in supporting the structure of microcapsules.

Prebiotic dietary fiber like pectin meets many regulations, pectin is a heteropolysaccharide, generally extracted from fruits and resistant to low pH [25]. In the pharmaceutical industry, pectin is used as a polymer to prevent cancer and has many health benefits. It may offer various forms of protection to cells against mutagenic events. Primary, pectin is fermented by bacteria in the colon, and one of the metabolites generated by this process is butyrate, which reduces colon inflammation and prevents carcinogenesis. Moreover, pectin can prevent cell metastasis, and initiate apoptosis in cancer. It is resistant to proteases and amylases, which are active in the gastrointestinal tract, and being susceptible to degradation by the colon microflora makes it appropriate for colon treatments [26]. Low methoxy pectin especially citrus pectin with desterification of less than 50% forms rigid gels through the action of multivalent cations, which cross-link the galacturonic acid chains [27].

It is composed of a mixture of globular proteins mostly of β-lactoglobulin and α-lactoalbumin. The effects of whey protein on human health are studied in many areas to decrease disease risk or as a supplementary therapy [28]. Whey protein can interact with polysaccharides to produce soluble or insoluble complexes. The encapsulation of probiotics by whey protein protects them from the stomach acidic environment. Moreover, it has been stated that whey protein can decrease losses of probiotic cells during the drying process [29].

It has been assumed that whey protein protects the hydrophobic bacteria via initial attachment to the unfolded whey protein. The hydrophobic interactions between hydrophobic bacteria and whey protein give rise to cells being implanted within the walls of the capsules [30]. Coating of alginate beads by whey protein has also been considered as another probiotic carrier. Gbassi *et al.* [31] stated that whey protein coating considerably improved the survival of *L. plantarum* in alginate beads.

This study aims to increase the survivability of *Lactobacillus rhamnosus* after oral ingestion by using a simple encapsulation technique that could be scaled up easily. The

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commercially available cheap sodium alginate, amidated low-esterified pectin, and whey protein are chosen as encapsulating polymers.

To the best of our knowledge, this study is the first to assess the impact of the prebiotic polymer ratio on the effectiveness of *L. rhamnosus* encapsulation as well as the impact of prebiotic polymers sterilization temperature on the stabilization of probiotics in gastrointestinal fluids. Antiprofilative, different signaling pathways in Caco-2 cell line and cytotoxic effects of *L. rhamnosus* microbeads were also evaluated.

2. Materials and methods

2.1. Materials

GRINDSTED Pectin SF 580 (batch number 49626779) of low esterification less than 30% ester and sodium alginate (batch number H501598331) were purchased from DANSCO Laboratories, (New Zealand). Whey protein was bought from the local market, in Egypt. Peptone and bile extract powder was purchased from Loba Chemi Laboratories (India). Agar and broth for De Man, Rogosa, and Sharpe (MRS) were purchased from LABM Laboratories (UK). Pepsin in powder form (1:3000), Potassium monophosphate, and Calcium chloride anhydrous were purchased from Oxford Laboratories (India) and Alpha Chemika (Egypt) respectively. Pancreatic extract powder and MTT (3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Bio Basic (Canada) and Serva (Germany). *Lactobacillus rhamnosus* was isolated in our lab. Dulbecco's Modified Eagle Medium (DMEM) and Fetal bovine serum (FBS) were purchased from Lonza (Switzerland). High-Capacity cDNA Synthesis Kit, SYBR GREEN no ROX Master Mix kits, and TRIzol reagent were purchased from Thermo Fisher Scientific (USA). The Center of Excellence for Research in Regenerative Medicine and its Applications provided the human colorectal adenocarcinoma Caco-2 cell line (CERMA, EGYPT).

2.2. Preparation of *Lactobacillus rhamnosus* cells suspension

Lactobacillus rhamnosus cells in freeze-dried form were used to cultivate the cells in MRS broth media with 1.0% (w/v) inoculation. Cells at the stationary stage were collected after 17 hours of incubation at 37ºC and re-inoculated in MRS broth repeatedly three times. Cells were harvested at the stationary growth phase by centrifugation at 4000 xg for 20 min. The concentrated cells obtained after centrifugation were resuspended into sterile 0.1% peptone solution and centrifuged under the same conditions, followed by resuspension into peptone solution to obtain a final cell concentration of 10 to 12 log CFU/mL [32].

2.3. Preparation of prebiotic polymer-based solutions

Alginate and pectin aqueous solutions were separately prepared in two different concentrations (2g/100 mL and 4g/100 mL). Each solution was autoclaved at 121°C for 20 min. or 110 °C for 10 min. On the other hand; whey protein was prepared at a concentration of 4g/100 mL and sterilized at 110 °C for 10 min.

2.4. Microencapsulation of *Lactobacillus rhamnosus*

Microencapsulation of *L. rhamnosus* with polymers was performed by extrusion/ionotropic gelation method [33]. The *Lactobacillus* cells were mixed with 10 mL of sterile prebiotics polymeric solution for five minutes to get a homogenous suspension under the conditions shown in **Table 1**.

The final bacterial cell count was from 10^{10} to 10^{12} CFU/mL of suspension. The homogeneous mixtures were taken in a 26-gauge nozzle syringe and extruded into sterile aqueous 5% $CaCl_2$ solution at a flow rate of 2.2 mL/min with low-speed stirring and left for 30 min at room temperature. After 30 min, the beads were collected and then

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washed with sterile distilled water. All beads were kept in separate vials for further analysis [34].

Table 1: Microbeads encapsulated *Lactobacillus rhamnosus*

Ratio of polymers				
Microbeads code	**Alginate 2%**	**Pectin 2%**	**Whey 4%**	**Sterilization temp.**
M1	1	1		121 ^{0}C for 20 min
M2	2	1		
M3	1	1	1	
M4	1	1		110 ^{0}C for 10 min
M5	2	1		
M6	1	1	1	
	4%Alginate	**4%Pectin**	**4%Whey**	
M7	1	1		121 ^{0}C for 20 min
M8	2	1		
M9	1	1	1	
M10	1	1		110 ^{0}C for 10 min
M11	2	1		
M12	1	1	1	

2.5. Physicochemical characterization of microbeads encapsulated *Lactobacillus rhamnosus*

2.5.1. Determination of encapsulation efficiency

The number of viable cells in the prepared microbeads was evaluated after the encapsulation process. One gram of beads was mixed with 9 mL of 1% sodium citrate and shaken for 30 min at 180 rpm in an orbital shaker until the complete release of the lactic acid bacteria. The resulting bacterial suspension was serially diluted in 0.1% peptone water (w/v), and aliquots of these dilutions were plated on MRS Agar. The dishes were incubated at 37 °C for 48 h. In addition, before the encapsulation process, the bacterial count in one mL of feed solutions was assessed using the same dilution, seeding, and incubation conditions. The survival is expressed as log CFU/ mL (log of colony-forming units per millilitre). The encapsulation efficiency was calculated by dividing of viable bacteria remaining after encapsulation by the initial viable count of bacteria used for the encapsulation, **Eq. 1**.

$$\text{Encapsulation efficiency} = \frac{Log_{10}N}{Log_{10}N_0} \times 100 \quad (1)$$

N represents the number of viable bacterial cells after encapsulation and N_0 represents the number of viable bacterial cells before encapsulation [35].

2.5.2. Morphological characterization

The size and shape of the freshly prepared microbeads were obtained using a digital camera. The cross-sectional areas of microbeads were tested under the scanning electron microscope (Quanta 250, Eindhoven, Netherlands) at a low vacuum. The particles were sliced with a blade without any preliminary sample treatment to examine the internal structure of the particles [36].

2.6. Viability of free and encapsulated *Lactobacillus rhamnosus* in simulated gastrointestinal fluids

Simulated gastric fluid (SGF) was prepared with 0.3% (w/v) sodium chloride and 0.32% (w/v) pepsin, the pH was adjusted to 2.0 with hydrochloric acid. Simulated intestinal fluid (SIF) was prepared by dissolving 0.1% w/v pancreatin and 0.3% w/v bile salts in a sterile saline solution and adjusted to pH 7.0. Simulated colon fluid (SCF) was prepared with 0.1M monopotassium phosphate the pH was adjusted to 8.0. All fluids were sterilized by filtration over a 0.22µm pore filter [37].

One gram of beads was added to test tubes enclosing 9 ml of pre-warmed (37°C) SGF and incubated in a water bath kept at 37°C under agitation at 100 rpm for two hours. One millilitre of free *Lactobacillus* cells was incubated in SGF under the same conditions. Aliquots (100 µL) of each suspension were serially diluted and seeded on MRS plates. The acid-treated free cells were centrifuged at 4000xg for 10 minutes. Acid-treated beads of each formula were collected and drained

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dry. The dried beads and treated free cells pellet were then transferred to 10 mL SIF and incubated in a water bath maintained at 37°C under the agitation of 100 rpm for three hours. Aliquots of 100-µL of each suspension were serially diluted and seeded on MRS plates. After SIF treatment, the free cells and beads were treated as previously described and then transferred to 10 mL of SCF with shaking at 100 rpm for four hours and twenty-four hours at 37°C. Finally, the beads were depolymerized, and the encapsulated cells were released. Both the free cells and the beads were centrifuged, and re-suspended in 1 ml of aseptic peptone water, and the number of viable cells was evaluated [38].

2.7. Viability of encapsulated *Lactobacillus rhamnosus* after storage

The microbeads and free cells were kept at 4°C for 180 days. The samples were taken, and the viability of *Lactobacillus* entrapped microbeads was calculated as described in section 2.5.1.

2.8. Anti-proliferation and cytotoxicity studies of selected microbeads

The cytotoxicity of prepared M12 microbeads which were composed of alginate, pectin, whey protein, and *L. rhamnosus*, was added and evaluated on human colorectal adenocarcinoma Caco-2 by MTT assay. Microbeads are compared with polymers mixture without *L. rhamnosus* and *L. rhamnosus* cell pellets. The concentrations added were 0.002 (mg/µL) from each polymer (alginate, pectin, and whey) and 0.011 log CFU/µL of *lactobacillus rhamnosus*.

The assay measured the formation of blue formazan product as a result of the reduction of MTT by mitochondrial dehydrogenase, which indicates the normal function of mitochondria and cell viability. The polymer mixture, cell pellet of *L. rhamnosus*, and the mixture of components of the selected microbeads M12 were neutralized to pH 7 to be compatible with the pH of DMEM. All treatments were added to the Caco-2 cell line for 48 h, individually, and then cell viability was evaluated using the colorimetric MTT assay. MTT was prepared at concentration (5mg/mL) and 10 µL of MTT was added to each well. After 4 h of incubation at 37°C, the media was discarded and 100 µL of DMSO was added to dissolve the purple crystals. The absorbance was recorded using a microplate reader at 570 nm.

DMEM medium without treatment was used as a negative control. The viability of untreated controls was normalized to 100%, **Eq. 2** [39].

$$\%\ \text{Cell viability} = \frac{\text{absorbance of control} - \text{absorbance of treatment}}{\text{absorbance of control}} \times 100 \quad (2)$$

2.9. Studying the different signaling pathways in Caco-2 cell line (Expression analysis of treated Caco-2 cells)

For expression analysis, Caco-2 cells were seeded in 6-well plates at a density of $3x10^5$ cells/ well for 24 h. The mixture of components of the selected microbeads M12 was incubated for 48 h at 37˚C. Cells were detached and collected by centrifugation for 5 min at 250 x g. The harvested cells were stored at -80˚C until used for further analyses including total RNA isolation and synthesis of complementary DNA.

Real-time qPCR reaction mixture was prepared using Maxima SYBR Green according to the manufacturer's recommendations. Exon-spanning primers (Caspase-3, *Bax*, and *Bcl*-2) were designed to produce an amplicon size varying from 100-200 bp. *HPRT* was used as the housekeeping gene, **Table 2.**

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Table 2: Primers sequences used in qPCR.

Gene	Primers' sequences (From 5' to 3')	Annealing Temperature (°C)
HPRT	FWD: TGACACTGGCAAAACAAT REV: GGTCCTTTTCACCAGCAA	57
CASP3	FWD: TTTTTCAGAGGGGATCGTTG REV: CGGCCTCCACTGGTATTTTA	57
BCL2	FWD: CACCCTGTGGTCCACCTGAC REV: ACGCTCTCCACACACATGAC	57
BAX	FWD: TTCATCCAGGATCGAGCAG REV: TGAGACACTCGCTCAGCTTC	57

2.9.1. Relative quantitation using fold change

The expression of the four mentioned genes was determined using the delta C_T (ΔCT) method, **Eq. 3**.

$$\Delta C_T = (\text{Average } C_T \text{ of gene of interest} - \text{Average } C_T \text{ of } \textit{HPRT}) \quad (3)$$

Required calculations include calculating the ΔC_T for the test sample relative to the target of interest and the total *HPRT* calibrator; (calculation of $2^{-\Delta\Delta CT}$ to obtain relative quantitative value; fold change) [40]. The values of gene expression obtained by drug treatment were plotted as (fold change) [41].

2.10. Statistical data analysis

The statistical analysis was accomplished by one-way analysis of variance (ANOVA) followed when needed by Tukey's. Statistical significance was determined at $p \leq 0.05$. Assessment of the effect of the different conditions on viable counts for each formula was performed by Student's t-test with a 95% confidence interval.

3. Results and discussion

3.1. Physicochemical characterization of encapsulated *Lactobacillus rhamnosus*

3.1.1. Encapsulation efficiency (EE %)

The encapsulation efficiency is an important parameter that affects the efficiency of drug delivery systems. The encapsulation efficiency of *L. rhamnosus* was found to be highly varied according to polymers ratio and concentrations. In addition, the temperature at which polymers are sterilized affects the capacity of encapsulation. The EE% of the first group of formulations (M1 to M3) ranged from 49 to 64% as shown in **Fig. 1**

The second group (M4 to M6) showed efficiency between 63 to 75%. The third group (M7 to M9) demonstrated encapsulation between 82-85%. The last group (M10 to M12) had EE% ranging between 91 to 97%. The ability of the fourth group to entrap *L. rhamnosus* possesses the highest EE%. The EE % increased by increasing the concentration of polymers [42] and decreasing the temperature of sterilization. This means that sterilization temperature affected the encapsulation behaviour of the polymers.

Concerning sodium alginate, according to the European Pharmacopoeia, autoclave sterilization for 15 minutes at 121 °C for solutions and powders of sodium alginate is acceptable. However, the gel strength of the calcium alginate beads and the viscosity of sodium alginate solutions have both been shown to decrease with rising sterilization temperatures. These effects can be attributable to a reduction in the degree of polymerization of the alginate molecules as a result of the heat treatments [43].

Fortunately, pectin was reported to be autoclavable and stable when heated at 121 for 60 min [44].

However, whey protein, which is mainly composed of globulin, will inevitably form aggregation. Due to denaturation and aggregation at a sufficiently high temperature via disulfide bond and/or hydrophobic interaction [45].

Consequently, lowering the sterilizing temperature and duration would better protect the structure and integrity of polymers. The integrity of the polymer

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during sterilization will eventually affect the entrapment efficiency.

It became evident that utilizing a mixture of 4% alginate, 4% pectin, and 4% whey protein sterilized at 110 ºC for 10 minutes was the optimum condition to achieve the highest encapsulation efficiency. The combination between polysaccharides and protein polymers was reported to be better than using one polymer. This is explained by the attraction between the negative-carboxyl group of alginate and the positive-amino acid group of whey protein, these electrostatic and hydrogen bonds formed lead to enhance the stability of biopolymer. Furthermore, upon the increasing concentration of polysaccharide polymers and protein polymer, encapsulation efficiency increased due to the elevation of the density of functional groups in the biopolymer matrix and prevented leakage of cells during the gelation process [46].

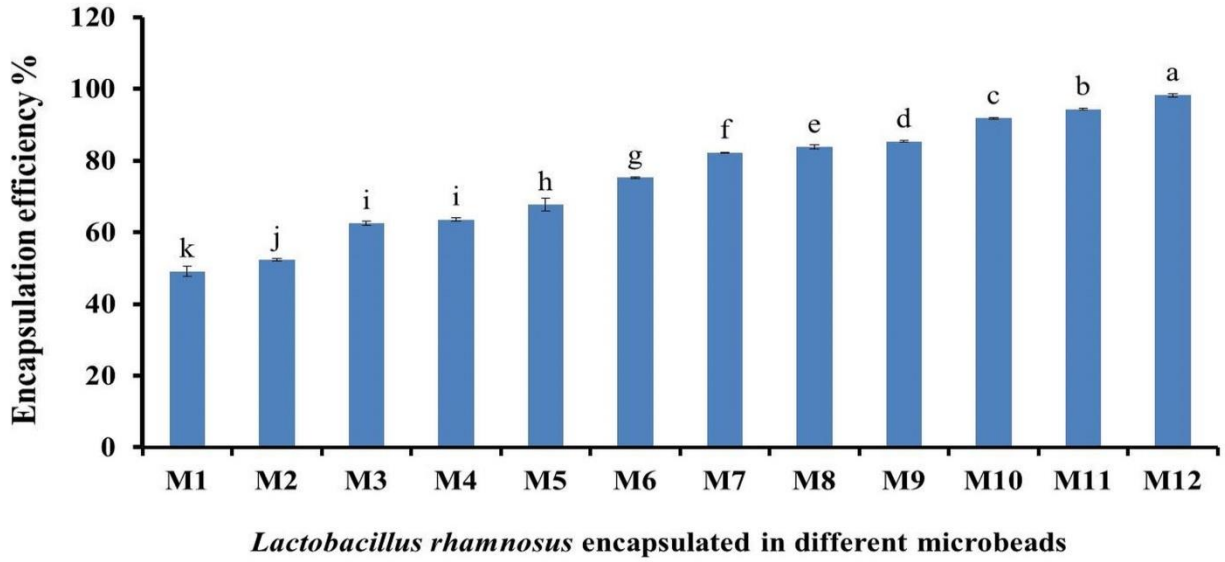

Fig. 1: Encapsulation efficiency of *Lactobacillus rhamnosus* entrapped in microbeads prepared with different concentrations of alginate, pectin, and whey. Different letters indicate that the means differ significantly ($p \leq 0.05$). N.B. Means with different letters are statistically significant a > b > c > d > e > f > g > h > I > j> k

3.1.2. Morphological characterization

The beads composed of alginate and pectin showed a quite transparent appearance as shown in **Fig. 2 (A and B)** while beads composed of alginate, pectin, and whey protein became milky and opaque. The beads composed of alginate and pectin showed a quite transparent appearance as shown in **Fig. 2C**. All the beads revealed a homogeneous size distribution. The microbeads composed of a mixture of 4% alginate, 4% pectin, and 4% whey protein have an average diameter of 2.1 mm ± 0.3. The microbeads composed of 4% alginate and 4% pectin at a ratio of 1:1 showed an average diameter of 2.3 mm ± 0.4, while microbeads formulated by a mixture of 4% alginate and 4% pectin at a ratio 2:1 showed an average diameter of 2.4 mm ± 0.4. Alginate, pectin, and whey protein beads displayed a tailing phenomenon as compared to alginate and pectin beads due to the viscosity shift in the preparation process as water is replaced by whey protein. The internal cross-sectional areas of beads composed of alginate and pectin showed a compact and dense network probably due to a total collapse of the fibers of the gel as shown in **Fig. 2 (A and B)** [47]. It could be

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observed that the internal structure of the beads fabricated by alginate, pectin, and whey were porous and the pores were interconnected as shown in **Fig. 2 C.**

It has been reported that the existence of protein altered the internal morphology of the microspheres with lamella structure [47, 48].

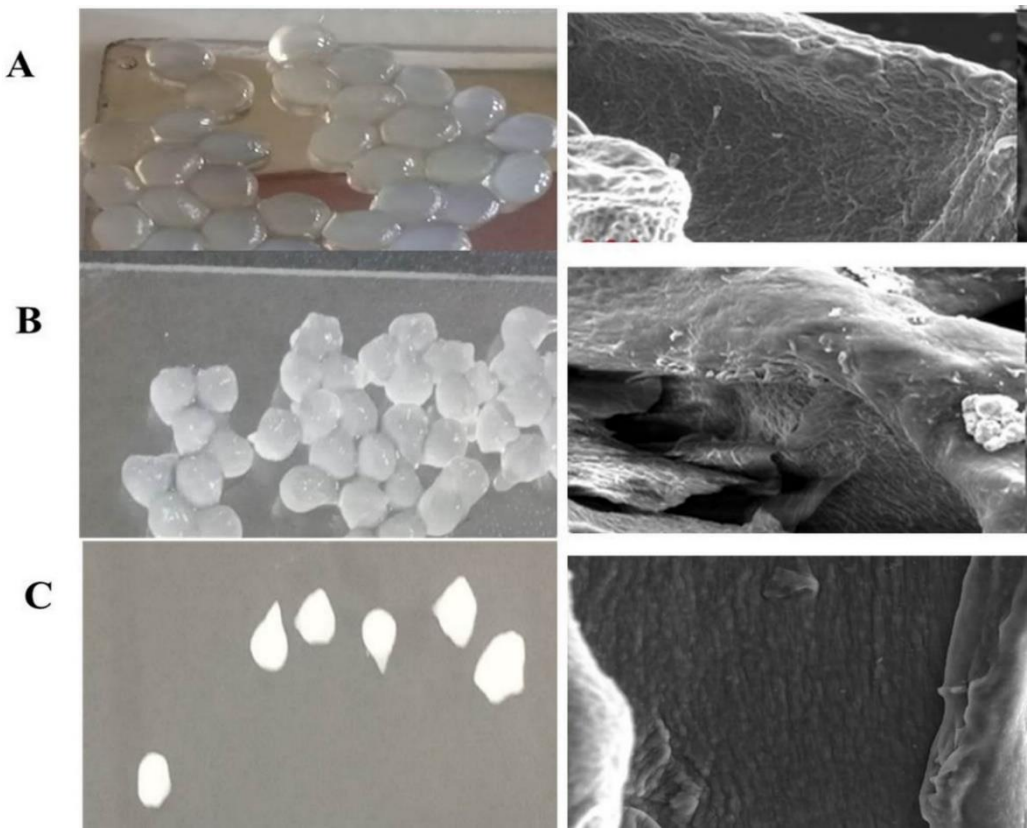

Fig. 2: Shape of the microbeads and internal cross-section at magnification power 800x (A) alginate and pectin beads (1:1) (B) alginate and pectin beads (2:1) (C) alginate, pectin, and whey protein beads (1:1:1).

3.2. Viability of free and encapsulated *Lactobacillus rhamnosus* in simulated gastrointestinal fluids

This section of the work aimed to evaluate the effectiveness of encapsulation systems in protecting viable cells of *L. rhamnosus* during incubation in simulated gastrointestinal fluids. The microbeads (M7 to M12) were selected for evaluation in comparison with free *L. rhamnosus* cells as their entrapment efficiencies were more than 80%. All microbeads in this section were prepared using 4% of polymers. The polymers of microbeads M7 to M9 were sterilized at 121°C for 20 min, while polymers of microbeads M10 to M12 were sterilized at 110°C for 10 min.

Free and entrapped *L. rhamnosus* was subjected to gastrointestinal stress, and their ability to tolerate gastrointestinal stress was presented in **Fig. 3**.

3.2.1. Stability of *L. rhamnosus* in simulated GIT fluids

During incubation of free *L. rhamnosus* in SGF for 120 min, the viable cells decreased from 11 log CFU/mL to approximately 4 log CFU/mL. The recovered pellet of free cells from SGF was then subjected to SIF for 240

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min; the viability further decreased to about 3 log CFU/mL. Then the pellet recovered from SIF was washed and subjected to SCF for 240 min. and 24 hours. The viability of free *L. rhamnosus* remained at 3 log CFU/g and did not decrease by significant value as the colon pH is favourable media for *Lactobacillus* [49].

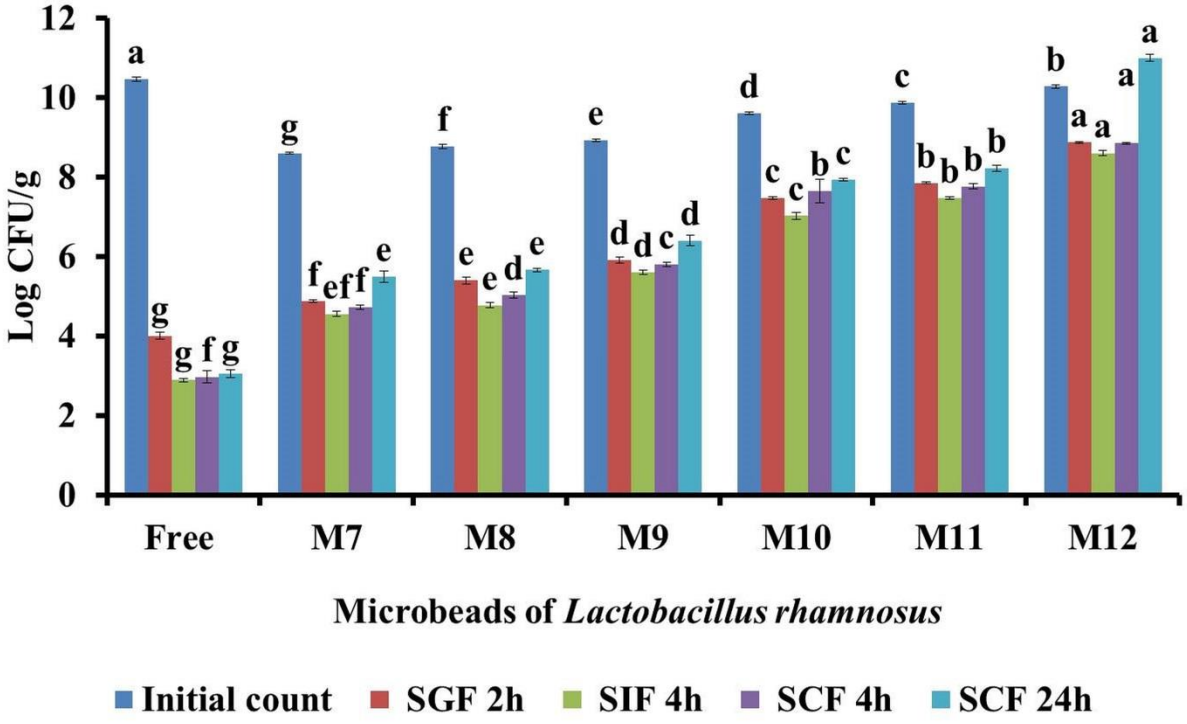

Fig. 3: Viable count of *Lactobacillus rhamnosus* (Log CFU/g) encapsulated in microbeads in comparison with free cells during incubation with simulated gastrointestinal fluids. Different letters indicate that the means differ significantly ($p \leq 0.05$). N.B. Means with different letters are statistically significant a > b >c > d> e> f> g

3.2.2. Effect of sterilization at 121°C for 20 min on GIT stability of *encapsulated L. rhamnosus*

Herein, we evaluated the effect of sterilization temperature 121°C for 20 min on the stability of microbeads in simulated GIT fluids. The viable count encapsulated in microbeads M7 formulated with 4% alginate and 4% pectin at a ratio of 1:1 was 8.6 log CFU/g. After incubation in SGF for 120 min., the viable count decreased to 5 log CFU/g then decreased to approximately 4.5 log CFU/g after exposure to SIF for 240 min. After being exposed to SCF for 240 min and 24 h, the count was around 4.7 log and 5.5 log CFU/g, respectively.

The initial number of cells in microbeads M8 prepared at alginate: pectin ratio 2: 1 and sterilized at 121°C for 20 min was 8.7 log CFU/g, then decreased to 5.4 log CFU/g, after 120 min in SGF. The viable count decreased again after incubation in SIF for 240 min to 4.7 log CFU/g. There was a slight increase in the count after 240 min in SCF; the viable count was 5 log CFU/g. After 24 h in SCF, the viable count further increased to 5.6 log CFU/g.

The microbeads M9 were prepared by 4% alginate, 4% pectin, and 4% whey protein at

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a ratio of 1:1:1, where alginate and pectin were sterilized at 121 ºC for 20 min, while 4% whey protein sterilized at 110 ºC for 10 min possessed initial viable count of 8.9 log CFU/g. There was a decline in the viable count after incubation in SGF for 120 min 5.9 log CFU/g. After incubation in SIF for 240 min, a further slight decline in the viable count 5.6 log CFU/g, was observed. After 240 min of exposure to SCF, the viable counts were about 5.8 log CFU/g. The final count after exposure to SCF for 24 h was about 6.4 log CFU/g.

3.2.3. Effect of sterilization at 110°C for 10 min. on GIT stability of *encapsulated L.rhamnosus*

Herein, we evaluated the effect of decreasing the sterilization temperature to 110°C for 10 min on the stability of microbeads in simulated GIT fluids. The microbeads M10 were prepared with 4% alginate and 4% pectin at a ratio (1:1) and sterilized at 110 ºC for 10 min. The viable count was about 9.6 log CFU/g, The count of cells decreased to about 7.5 log CFU/g, after incubation in SGF for 120 min. Another decline in the viable count was observed after exposure to SIF for 240 min, the count was around 7 log CFU/g. After incubation in SCF for 240 min, the viable count was about 7.5 log CFU/g. After the final stage of incubation in SCF for 24 h, the viable count surprisingly increased to 8 log CFU/g.

The viable counts of microbeads M11 prepared as pervious at a ratio of alginate to pectin 2:1 was 9.8 log CFU/g. The counts declined to 7.8 log CFU/g after 120 min in SGF. Then another decline occurred after exposure to SIF for 240 min, the count reached 7.4 log CFU/g. There was a slight increase in the count after 240 min in SCF 7.7 log CFU/g. The final count after exposure to SCF for 24 h increased to 8.2 log CFU/g.

The initial counts were encapsulated in microbeads M12 prepared with 4% alginate, 4% pectin, and 4% whey protein at a ratio of 1:1:1 and sterilized at 110 °C 10.2 log CFU/g. The count decreased to 8.6 log CFU/g after 120 min in SGF. After 240 minutes in SIF, the count reached 8.6 log CFU/g. There was a slight increase after exposure to SCF for 240 min; the count reached 8.8 log CFU/g. The final counts after incubation in SCF for 24 h showed a reasonable increase to 11 log CFU/g. Overall, the previous results showed that free cells had low tolerance to gastrointestinal fluids.

Similarly, In 2019, Liao *et.al* [50] reported that free *Lactobacillus* cells experienced a significant decrease in viability after sequential cultivation in gastrointestinal fluids to approximately 3 log CFU/mL. It has been reported that the optimal pH for *Lactobacillus* growth was pH 7.4 and 8.5, while they were unable to grow at a low pH [49]. The microbeads showed more resistance to gastrointestinal fluids but with different degrees depending on the type of polymers used, the concentration of each polymer, and the sterilization temperature.

The formulations composed of a mixture of 4% pectin and 4% alginate and sterilized at 110 ºC for 10 min M10, and M11 showed significant cell survival in GIT fluids in comparison with the formulations composed of pectin and alginate and sterilized at 121 ºC for 20 min M7, and M8. This could be explained by the fact that sterilizing polymers at low temperatures preserves their structure and integrity.

The protective effect of polymers on encapsulated *Lactobacillus* has been documented in several studies. Ho *et al*. [51] studied the effect of pectin concentration on stimulation of the growth of *Lactobacillus* at the colon site. Higher pectin concentrations

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give some protection to delay the *Lactobacillus* cell death as pectin contains some oligosaccharides which assisted the probiotics in acid tolerance and survival ability.

The main factors affecting the synergistic interaction between the alginate and pectin in the protection of probiotics were studied. They explained this by pointing out that both polymers are polyuronates and have structural similarities. It is worth mentioning that the pectin structure is composed of galacturonic acid chains and alginate is composed of the glucuronic acid blocks. The interaction between pectin and alginate was raised as the amount of these sequences increased. The mechanism of gelation between pectin and alginate includes intermolecular junction zones made by the union of divalent cations (e.g., Ca^{2+}) with glucuronic acid blocks of alginate or glucuronic acid residues in pectin of an adjacent polymer chain. This creates an egg-box structure that can encapsulate and protect the probiotic [52].

The protective effect of whey protein observed after 24 hours of incubation of beads in simulated colon fluids may be explained by the ability of whey protein to compensate for the loss that occurred through the transition in gastrointestinal fluids [53]. In 2019, Yasmin *et al*. [54] reported that probiotics encapsulated in microbeads composed of whey protein and alginate were able to resist SGF and SIF in comparison with free cells. This could be due to the fact that whey protein is one of the essential sources that maintain the growth of *Lactobacillus* [55]. The importance of a combination of protein and polysaccharides polymers was indicated by our findings. This emphasizes the need to select polymers that are both protective and have prebiotic effect. These results showed that microbeads prepared with 4% alginate, 4% pectin, and 4% whey protein at a ratio of 1:1:1 where polymers sterilized at 110ºC for 10 min. (M12) has excellent resistance to GIT fluids, as these microbeads showed the highest viable count at the end of assessment at different pH.

3.3. Survival of free and encapsulated *Lactobacillus rhamnosus* after storage

Free cells and encapsulated *L.rhamnosus* were stored at 4 °C for 180 days. When encapsulated *L. rhamnosus* cells are compared to free cells, the death rates of encapsulated *L. rhamnosus* were much lower displaying the effect of encapsulation in extending the storage life span of *L. rhamnosus.* [56]. The viability of free *L. rhamnosus* showed a dramatic decrease in the count; it is about 2 log CFU/g after three months of storage **Fig. 4**.

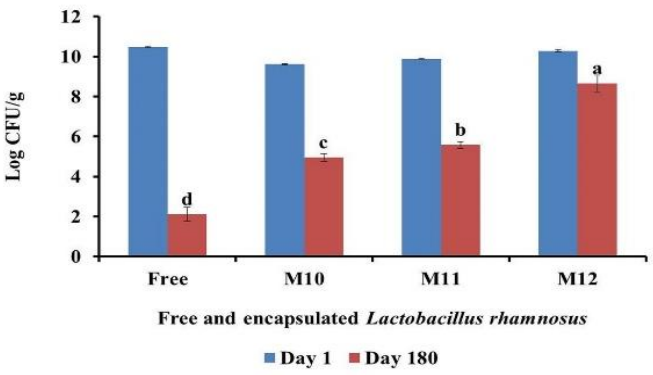

Fig. 4: Viable count of *Lactobacillus rhamnosus* (Log CFU/g)) encapsulated in microbeads in comparison with free cells during storage at 4°C for 180 days. Different letters indicate that the means differ significantly ($p \leq 0.05$). N.B. Means with different letters are statistically significant a >b >c >d.

The encapsulated *L.rhamnosus* viable cell counts of reduced by 0.4 to 1.3 log CFU/g after three months of storage which is an acceptable amount as the total amount after

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the storage is more than 7 log CFU/g. It was obvious that the formulae M11 with alginate to pectin 2:1 showed a significant difference ($p \leq 0.05$) in terms of protection in comparison with the formulae M10 composed of 1:1 alginate and pectin. These findings were accordant with the results obtained by Shahrampour *et al* [57] who documented that the viability count increased with a higher content of alginate. This result was also explained by Soukoulis *et.al* [29] who reported that formulations with a high concentration of pectin were found to have higher moisture content because the structure of pectin has more free hydrophilic groups than alginate. This explains the differences between the water-holding ability of alginate and pectin.

The microbeads M12 prepared by 4% alginate, 4%pectin, and 4% whey protein at a ratio of 1:1:1 has an initial count of 9 log CFU/g, before gradually declining to 8.6 log CFU/g, by the end of storage period. After 180 days of storage, these microbeads lost less than 1 log CFU/g and maintained a viable count of more than 7 log CFU/g, which is required for probiotics therapeutic activity [3]. It is well recognized that proteins can preserve the biological activity of *Lactobacilli* through free radical scavenging which prevents the peroxidation of membrane lipids, and surface adhesion properties that help bacterial cells to overcome physical stresses throughout storage [58]. Furthermore, depending on the solute composition of the embedding substrate, proteins can modulate their molecular mobility and therefore, the occurrence rate of deteriorative enzymatic and chemical reactions taking place during storage. Whey's protective effect may be due to its capacity to decrease osmolytic cell damage that occurs during the dehydration process, as well as its superior cell adhesion properties, as recently verified [29]. Furthermore, whey protein hydrolysis by-products (for example, peptides and amino acids) naturally occurring in whey, as well as those produced by *Lactobacillus* proteolytic action, retain excellent reducing and free radical scavenging properties, inhibiting lipid autoxidation. In addition, the residual lactose may develop stability by improving the membrane through the interaction with the polar head in the cell membrane resulting in maintaining its structural integrity [59, 60]. The previous results revealed that the microbeads M412 prepared by 4% alginate, 4% pectin, and 4% whey protein at a ratio of 1:1:1 have the highest viable count after 180 days of storage at 4ºC.

3.4. Anti-proliferative activity of a mixture of components of the selected microbeads on Caco-2 cells

The microbeads of (M12) which showed the highest viability after incubation in gastrointestinal fluids were chosen for determination of its anticancer effect in comparison with polymers mixture without *L.rhamnosus* and *L.rhamnosus* cell pellet (11 log CFU/ml). The viability of colon cells after the addition of polymers mixture, *L.rhamnosus* cell pellet, and a mixture of components of the M12 for 48 h using MTT assay were 14%, 24%, and 21%, respectively **Fig. 5**. In 2019, Zamorano *et al*. and his team [61] studied the influence of pectin on colon cancer cells. In this study, the viability of HT29 cells was reduced by pectin in a concentration-dependent manner. Cakir and Tunali [62] studied the effect of whey protein on HCT 116 colon cancer cells. They concluded that 10 µg/mL of whey protein has an anticancer effect, that caused 50% apoptotic cell death. They attributed this effect to the upregulation or downregulation

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of certain enzymes, which are responsible for the energy metabolism of HCT 116 cells, specifically linked to the glucose usage of the cells and the adenosine triphosphate (ATP) production. It has been reported that *L.rhamnosus* possesses the ability to inhibit the proliferation of HT-29 cells and the stimulation of the apoptosis process [(63)].

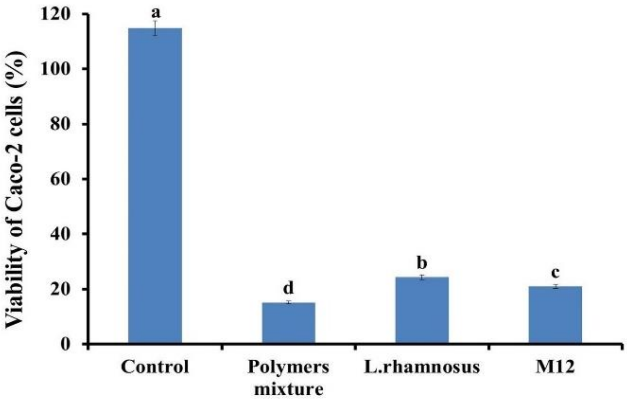

Fig. 5: Viability of Caco-2 cell line after addition of polymers mixture, *L.rhamnosus* (12 log CFU/ml), and mixture of components of the M12 for 48 h using MTT assay. The data shown are the mean ± SD of triplicate experiments. Bars with different letters are significantly different ($p \leq 0.05$) for each sample. a>b>c>d

3.5. Effect of mixtures of components of the selected microbeads on caspase-3 expression

Mammals have a highly specific family of cysteine proteases called caspases, which can activate or deactivate numerous proteins to either trigger or inhibit programmed cell death. The last stage of apoptosis happens when cytotoxic agents are released by cytochrome c, triggering the activity of caspase 9, which in turn activates caspase 3, finally resulting in apoptosis. Caspase-3 (and its cleavage CC3), known as a killer caspase, is an essential executioner molecule in the apoptotic process. It is the major member of the caspase class, in which PARP-1 cleaves into 29- and 85-kDa fragments during the early phases of apoptosis, mediating tumor repopulation in apoptotic tumor cells [(64)]. Caspases were demonstrated to be active during apoptosis in numerous cancer cell lines and played crucial roles in starting apoptosis.

Caspase-3 was markedly upregulated in Caco-2 cells after 48 h treatment with a mixture of components of the M12. The fold change of Caspase-3 in the cells treated with a mixture of components of the M12 demonstrated upregulation in caspase-3 expression on Caco-2 cells was about 12, while the polymers mixture showed minor fold change (0.3) **Fig. 6**. The results suggested that microbeads mixture was inducer of apoptosis, they have the potential to be applied in the treatment of colorectal cancer.

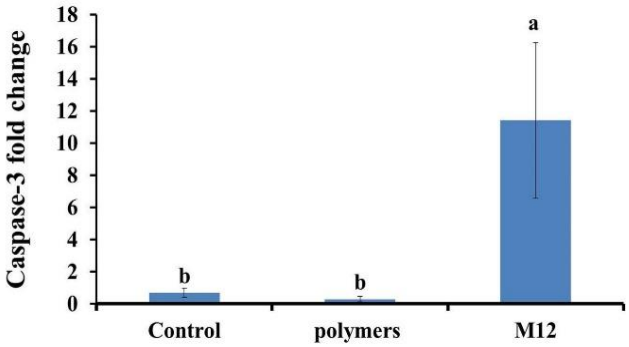

Fig. 6: Expression of caspase-3 gene after addition polymers mixture and mixture of components of M12 on Caco-2 cell line for 48 h using quantitative PCR. Data was normalized to HPRT as a housekeeping gene. The data shown are the mean ± SD of triplicate experiments. Bars with different letters are significantly different ($p \leq 0.05$) for each sample a>b

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3.6. The effect of the polymers mixture and mixtures of components of the selected microbeads on the expression of *Bax* and *Bcl-2*

Proteins of the *Bcl-2* family were the key regulators of apoptosis. *Bax* is proapoptotic, whereas *Bcl-2* is an antiapoptotic gene. The mixture of components of M12 showed high expression of *Bax-2*, the fold change was 3.6, while polymers mixture showed less than 0.4-fold change **Fig. 7.** On the other hand, polymers mixture and M12 showed downregulation of *Bcl-2* gene; the fold change was less than 0.0001 **Fig**. **8.** In 2019, Karimi and his colleague [65] revealed the upregulation of *Bax* gene expression and decrease of *Bcl-2* expression by using heat-killed *L.paracasei* and *L. brevis.* The effect of *L.reuteri* cell wall was found to increase fold change of *Bax* [66].

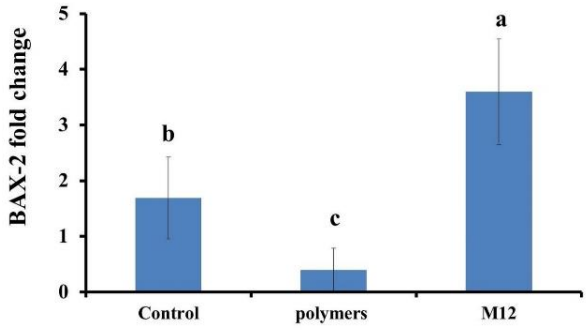

Fig. 7: Expression of *Bax* gene after addition of polymers mixture and mixture of components of M12 on Caco-2 cell line for 48 h using quantitative PCR. Data was normalized to HPRT as a housekeeping gene. The data shown are the mean ± SD of triplicate experiments. Bars with different letters are significantly different ($p \leq 0.05$) for each sample a>b>c

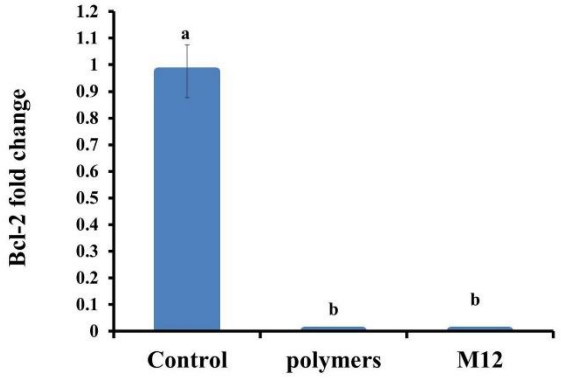

Fig. 8: Expression of *Bcl*-2 gene after addition of polymers mixture and mixture of components of M12 on Caco-2 cell line for 48 h using quantitative PCR. Data was normalized to HPRT as a housekeeping gene. The data shown are the mean ± SD of triplicate experiments. Bars with different letters are significantly different ($p \leq 0.05$) for each sample a>b

Probiotic bacterial supernatant was observed to up-regulate proapoptotic genes involving caspase-3, caspase-9, and *Bax*. Furthermore, they lead to down-regulation of *Bcl-2*. In 2021, Sun, M. *et al*. [67] reported the ability *L. plantarum* exopolysaccharides to activate the *Bax* and caspase-3 while reducing expression of *Bcl-2* in HT-29 cells. These changes lead to apoptosis in HT-29 cells via the mitochondrial pathway. The mixtures of components of the microbeads M12 which contain *L.rhamnosus* extensively stimulated the apoptosis process in Caco-2 colon cell line, due to their ability to induce *Bax* and caspase-3 genes, while reducing the expression of *Bcl-2* gene. Although the results of the MTT test revealed that the polymers mixture without *L. rhamnosus* inhibited Caco-2 cell line growth, it had a minor effect on apoptotic and anti-apoptotic genes. It has been reported that pectin was unable to affect the expression of *Bcl*-2 or caspase-3 activity in HT29 cells [61]. In

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addition, Ramirez-Rico *et al.* documented that the expression of antiapoptotic protein for caspase-3 and *Bcl-2* in intestinal lymphocytes was unaffected upon treatment with whey protein [68]. On the other hand, in 2018, the study of the effect of alginate on pigs' intestines revealed inhibition of *Bax*, caspase-3, and caspase-9 [69].

4. Conclusions

Microbeads encapsulated *Lactobacillus rhamnosus* were successfully prepared using the extrusion method. The following parameters have an obvious effect on the encapsulated *L. rhamnosus*: sterilization temperature of prebiotic polymers (alginate, pectin, and whey), concentration, ratio, and type of prebiotic polymers used.

The microbeads composed of 4% alginate, 4% pectin, and 4% whey protein at a ratio of 1:1:1, where all polymers sterilized at 110 ºC for 10 min showed the highest encapsulation efficiency of *L. rhamnosus* and the highest viable count in simulated GIT fluids. Their stability after storage for 180 days was the highest in comparison with other formulations. These microbeads showed anticancer effect on human colon cell line Caco-2. They were able to inhibit the proliferation of Caco-2 through upregulation of proapoptotic genes caspase-3 and *Bax* genes expression and downregulation of the anti-apoptotic gene *Bcl-2*.

Taken collectively, the selected microbeads encapsulated *L. rhamnosus* significantly inhibited human colon cancer cell growth which makes them a promising candidate for the treatment of colon cancer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Funding

No fund

Highlights

- *Lactobacillus rhamnosus* encapsulated in microbeads prepared by alginate, pectin, and whey had the highest stability in gastrointestinal fluids and during storage.
- Adding whey improved *Lactobacillus rhamnosus* growth at the colon site.
- Microbeads of *Lactobacillus rhamnosus* showed anticancer effect on colon cell line Caco-2.

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