Formulation & stability of *Lactobacillus acidophilus* microcapsules with a clinical trial for treatment and eradication of *Helicobacter pylori* infections

التصيغ و الدراسة الثباتية للكبسولات المجهرية للـ *Lactobacillus acidophilus* مع دراسة سريرية في علاج اصابات الـ *Helicobacter pylori*

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Abstract

Probiotics are live microorganisms that administered through the digestive tract, have a positive impact on the hosts health. In this study research has shown that extraction method (encapsulation in alginate systems) can be used as an effective method for preparation of immobilized *Lactobacillus acidophilus.* All formulas were studied, from characterization, encapsulation yield, particle size measurement and mechanical stability of microcapsules in simulated gastric conditions which resulted that alginate – starch microcapsules gave the best results. based on this finding that starch – alginate can be used to protect living microbes, alginate starch microcapsules has been further studied for: efficacy of cell release, thermal stability (refrigeration and shelf life) which achieved good results. Finally the selected formula was added to standard anti- *Helicobacter pylori* therapy (triple therapy) in a parallel clinical study (42 patients) which showed that the addition of *Lactobacillus acidophilus* has a significant effect in increasing the eradication rate of *Helicobacter pylori*.

المستخلص

الأحياء العلاجية هي كائنات مجهرية حية عندما تعطى عن طريق الجهاز الهضمي لها تأثير ايجابي على صحة الحاضن . أظهرت النتائج أن طريقة الأنبثاق (التي تمثل بالكبسولات المجهرية في نظام الالجينات) ممكن استخدامها كطريقة فعالة لتحضير تكتلات بكتريا الـ *Lactobacillus acidophilus* . تم دراسة جميع الصيغ الركيبية المحضرة والتي تشمل: المواصفات ، نسبة انتاج الكبسولات المجهرية ، قياس حجم الجزيئات و الأستقرار الميكانيكي للكبسولات المجهرية في ظروف معدية خارج الجسم و اظهرت النتائج ان تركيبة الكبسولات المجهرية المكونة من الالجينيت و النشأ أعطت افضل النتائج . من ثم تم دراسة كفاءة تحرر الخلايا البكتيرية والاستقرارية الحراريه (عمر الخزن و الرف) التي أعطت نتائج مقبولة للصيغة التركيبية المفضلة . أخيرا الصيغة المختارة أضيفت الى علاج مضادات بكتريا Helicobacter Pylori القياسي (العلاج الثلاثي) في دراسة سريرية متوازية (42 مريض) والتي أظهرت أن أضافة الـ*Lactobacillus acidophilus* له تأثير أيجابي في زيادة سرعة ابادة بكتريا الـ *Helicobacter pylori* .

Introduction

Probiotics are live microorganisms that administered in adequate amounts confer a health benefit on the host [1]. Due to their perceived health benefit probiotics have been increasingly included in yoghurts and fermented milk during the past two decades. Most commonly they have been *Lactobacilli* such as *Lactobacillus acidophilus*, and *Bifidobacteria* often referred to as *Bifidus* [2].

Key words: probiotics, *Lactobacillus acidophilus*, microencapsulation, *Helicoba*

Before a probiotic can benefit human health it must fulfill several criteria: it must have good technological properties so that it can be manufactured and incorporated into food products and pharmaceutics without losing viability and functionality or cresting unpleasant flavors or textures; it must survive passage through the upper gastrointestinal tract and arrive alive at its site of action, and it must be able to function in the gut environment. To study the probiotic strain in the gastrointestinal tract, molecular technique must be established for distinguishing the ingested probiotic strain from the potentially thousands of other bacterial strains that makeup gastrointestinal ecosystem. Addionaly, techniques are required to establish the effect of the probiotic strain on other members of the intestinal microbia and importantly on the host. This includes not only positive health benefits, but also demonstration that probiotic strains do not have any deleterious effect. Armed with this knowledge, the probiotic can enter human pilot studies that attempt to assess their health benefits to consumers [3, 4, 5].

There are a variety of techniques available for the production of encapsulated materials few are co-extrusion and spray drying to prepare capsules, spray chilling, matrix entrapment, gel formation and fluid bed processing. All of these methods are applicable to food ingredients as well as to other materials, a wide range of materials are available, by which it's possible to form capsules, which will release under a variety of conditions [6,7].

Stability of commercial probiotic strains is important to assure that stated levels of viable cells are delivered in probiotics, which is dependent on many factors including growth conditions and storage conditions (relative humidity, oxygen content, stabilizers and temperature). The importance of growth conditions including the presence of calcium, in the production of stable cultures has not been established. [8].

Considerable research interest has been dedicated to the encapsulation of bacterial cells for the growing and promising potential in therapeutic applications such as in kidney failure, uremia, cancer therapy, diarrhea, cholesteremia and other diseases [9].

In this paper we will study the bio-encapsulation method by gel particle technique with different materials to produce stable microcapsules of *Lactobacillus acidophilus*. Then to study its clinical application in the eradication of *Helicobacter pylori*.

Materials and methods

Isolation

The isolation of *Lactobacillus acidophilus* from Supermulti-duphilus® capsule (West Coast Naturals) was performed by MRS (peptone, meat extract, yeast extract glucose, tween 80) medium. Briefly one capsule was mixed and votrexed into MRS broth medium, inculcated at 37 ° c for 24 hr.

Growth from MRS broth cultures was used to streak on MRS agar plate.

Lactobacillus acidophilus isolates were identified by comparing their sugar fermentation patterns with the scheme described in Bergeys manual of systematic bacteriology [10, 11].

Cellular viability technique

To determine the viable counts of *Lactobacillus acidophilus*, 1 ml of broth culture was taken and it was diluted with 9 ml sterile saline solution. From this solution several dilutions were made and 0.1 ml of each one was dispersed in Petri plates containing MRS agar. The plates were incubated at 38° C for 48 hr. at the end of the incubation the *Lactobacillus acidophilus* colonies were counted and the results were reported [12].

Preparation of the bio-microcapsules

The gel-particle technique was used to prepare alginate, alginate-gelatin and alginate-starch microcapsules [13].

A sterile 2% (w/w) sodium alginate solution was prepared, 0.2 mol^{-1} Ca Cl_2 sterile solution at room temperature, 2% starch solution and 24% sterile gelatin solution to prepare different formulas:

Formula 1: was prepared by mixing 5ml cell suspension (concentration 5.7$\pm$ 0.6 x 10^{10} CFU) and 30 ml sterile solution of 2% sodium alginate solution at 37° C.

Formula 2: was prepared by mixing 5 ml cell suspension (concentration 5.7$\pm$ 0.6 x 10^{10} CFU) and 30 ml sterile mixture of 2% sodium alginate -2% gelatin in a ratio of (2:1) at 37°C.

Formula 3: was prepared by mixing 5 ml cell suspension (concentration 5.7$\pm$ 0.6 x 10^{10} CFU) and 30 ml sterile solution of 2% sodium alginate - 24% maize starch in a ratio (1:1) at 37°C.

Each mixture (formula 1,2,3) was dropped (using a 10 ml syringe needle into a gently agitated 0.2 mol^{-1} $CaCl_2$ solution at room temperature 25°C , the distance between the syringe and the $CaCl_2$ collecting solution was 10 cm.

The beads were allowed to stand for 1 hr for hardening before aseptically transferred to a sterile flask for storage.

The encapsulation yield (EY)

Which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure was calculated as:

$EY = N / N_o * 100$

Where N is the number of viable entrapped cells released from the microspheres and N_o is the number of free cells added to the biopolymer mix during the production of microspheres [14].

Particle size measurement of microspheres

The particle sizes of microspheres were determined by measuring diameters of 300 microspheres using an optical microscope (Olympus) at X 100 magnification fitted with a micrometer scale. The mean diameters of microspheres from all the experimental treatments were calculated and presented with standard deviations (n-1) [15].

Mechanical stability in simulated gastro -intestinal fluids [16]

To evaluate the stability of microcapsules for oral therapy, knowledge of encapsulent dynamics is required under relevant physiological conditions the following was used

to represent the gastric juice (2gm NaCl, 3.2 gm pepsin, 7ml HCl, complete volume with water to 1000ml, pH 1.6).

In this study, a shake method was used and percentage of microcapsules undamaged was observed by microscope to provide information on the mechanical resistance of the microcapsules.

Efficacy of cell release: [17]

To determine the viable counts of the entrapped bacteria, 0.1 gm of microcapsules were resuspended for (F3) in 10 ml of phosphate buffer (pH7) followed by gentle shaking at room temperature. Samples were taken at different time intervals to determine the complete release of encapsulated bacteria by plating on MRS ager.

Storage viability study (Stability):

Lactobacillus acidophilus microcapsules (F3) were placed in 100 ml polyethylene bottles and subjected to controlled temperatures (4, 30)^{0}C.

The product was subjected to microbiological stability testing at the following time intervals (1, 2, 3) months for refrigerated samples. While for shelf life determination (30)^{o}C enumeration was performed at (1,2,3,6) months using numeration method (12) as follows:

The immobilized *Lactobacilli* was released from the polymer wall by digestion using trypsase solution (1% w/v) at 37^{o}C for 60 min; two successive dilutions at a 1:10 ratio in sterile PBS (pH 7) was carried out; 100ml of these diluted aqueous phase was spread on MRS ager Petri dishes, and the Petri dishes were placed in an incubator at 37^{o}C for 48 hr for numeration [18].

Clinical study

Forty two patients suffering of *Helicobacter pylori* with an age range (23-45) years of both sexes were conducted for the clinical study, all patients were diagnosed for *Helicobacter pylori* infection from the signs and symptoms in addition to *Helicobacter pylori* Ab combo rapid test -(serum/ plasma / whole blood) kit(CTK Biotech. Inc.), an antibody response as a marker for the disease and the patient with bleeding undergone endoscopy.

All tests and the endoscopy was done in saint Raphael hospital while the clinical investigation and follow up was done in a private clinic.

The patients were divided into 2 groups for a parallel study.

1st group (18 patients) was put on:

Nexium® 20 mg once a day
Amoxillin® 500mg three times a day
Clathramycin® 500mg twice a day

For 2 weeks.

While the 2nd group (24 patients) took:

The above remedy in addition to

4.5 billion *lactobacillus acidophilus* three times daily for 2 weeks (Formula 3).

Results and Discussion

Characterization of bio-microcapsules

The *Lactobacillus acidophilus* loaded microcapsules were prepared by extrusion method with alginate, alginate/ gelatin and alginate/ starch and dried at 4°C. The shapes of the micro particles were investigated using light microscope and shown in the figures (1, 2, 3).

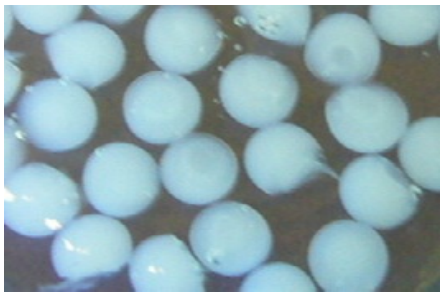


Fig (1): Microcapsules of Formula 1

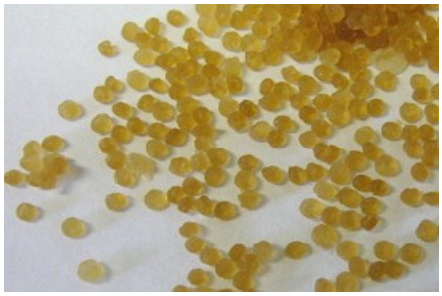


Fig (2): Dried of microcapsules of Formula 2

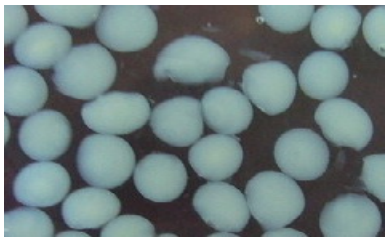


Fig (3): Microcapsules of formula 3

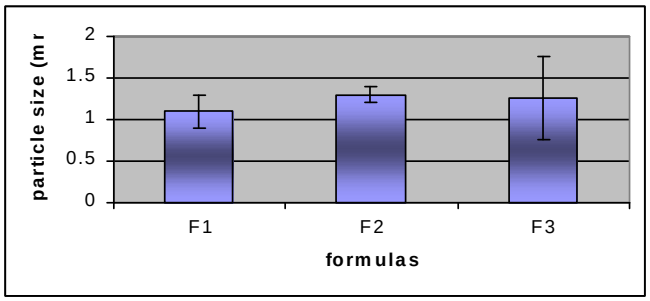
The micro particles homogeneously distributed without evidence of collapsed spheres. The spherical shape of the microcapsules in wet state was lost after drying and the surface of the microcapsules becomes rough.

The encapsulation yields (EY) for viable cells were

Formula 1 45.9%± 3.3
Formula 2 44.1%± 4.3
Formula 3 48.5% ±6.2

Particle size of micro capsules obtained is shown in figure (4) the mean particle diameter was as follows:

Formula 1 1.1 ±0.2 mm
Formula 2 1.3 ± 0.1 mm
Formula 3 1.26 ± 0.5 mm



***(n= 300) standard deviation (n-1) , (p< 0.05)**

Fig (4): Particle size distribution for different formulas of microcapsules.

Mechanical stability

The viability of *Lactobacillus acidophilus* and integrity of microcapsules under high acidic condition (simulated gastric juice, pH 1.6) for 90 min s as shown in figure(5).

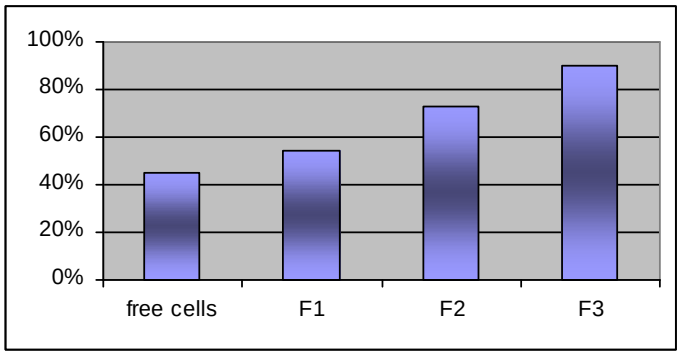


Fig (5): Mechanical stability of microcapsules in simulated gastric juice

After 3 hrs in simulated gastric conditions (pH 1.6) there is a dramatic reduction in free cell plate count while we noticed that F3 had the highest physical resistant to damage (10% damaged) when compared to other mechanical studies of similar quantities of microcapsules of F1 and F2 (using an optical light microscope) and these results were compatible with [19, 20].

Therefore large number of the initial cells must be entrapped in microcapsules to facilitate colonization.

Increase in the number of bacterial survivors at the end of 3 hr s in simulated gastric conditions was shown in Figure (5) which is similar with other studies [21].

F3 was selected for further studies as efficacy of cell release and for thermal stability studies because it had the highest encapsulation yield and the best mechanical stability against simulated gastric juice.

Efficacy of cell release

The pH of phosphate had no significant effect on the release rate of bacterial cells ($p > 0.05$); the microcapsules of F3 ruptured and gave release of the viable cells within (10 min) as shown in Figure (6).

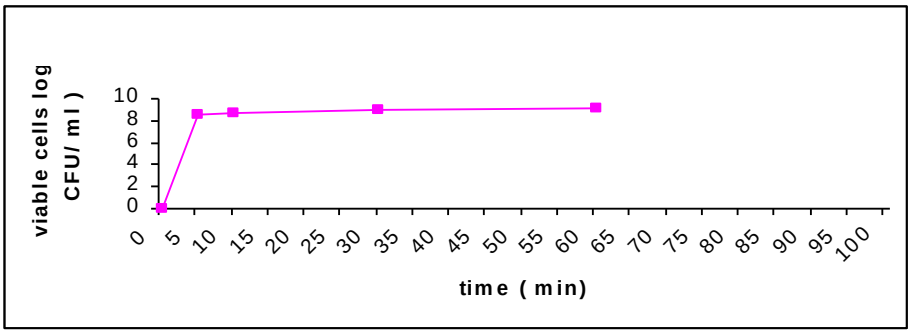


Fig (6): Viable cell release in phosphate buffer

Stability (storage viability study)

The study consisted determining the effect of storage conditions for F3 with a viable count over (2.45×10^9) during refrigerated storage 4°C for 3 months and 30°C with a viable count over (1.08×10^{10}) at room temperature for a period of 6 months each treatment was replicated for three times.

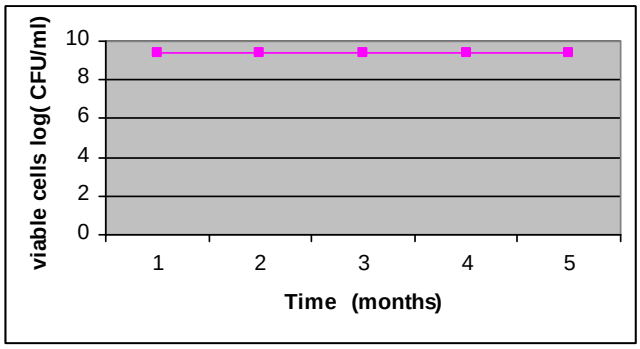


Fig (7): Cell viability at refrigeration temperature

The effect of storage temperatures on storage viability of immobilized *Lactobacillus acidophilus* microcapsules was significantly ($p<0.05$) affected by the storage temperaturesas shown in figure (7, 8). When stored at refrigerated and shelf temperatures.

The population of immobilized bacteria at 4^{o}c indicate that the immobilized bacteria were not devitalized by refrigerated temperature scale when compared to that stored at 30^{0} C (shelf life).

The results were concordant to the reports that enhancement of shelf life of probiotic bacteria could be achieved when storage temperatures were lowered [22, 23].

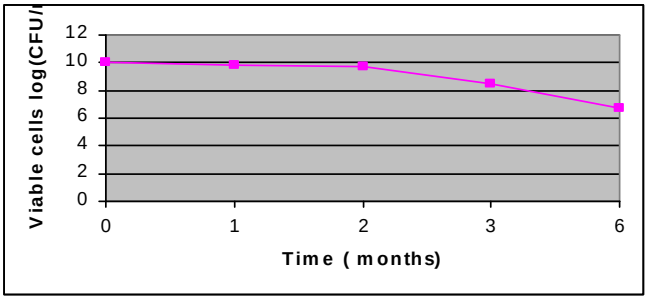


Fig (8): Cell viability at room temperature (shelf life)

Clinical study

Forty two patients with an age range (23-45) of both sexes were diagnosed with *Helicobacter pylori* infection (gastritis, peptic ulcer and duodenal ulcer) according to the sign and symptoms as shown in table (1), in addition to all patient s had positive *Helicobacter pylori* Ab combo rapid test, and 11 patients were subjected to endoscopy (had upper gastric bleeding).

Both groups took their treatment for two weeks after that they were subjected to clinical examination to see the relief of the sign and symptoms.

Therteen patients out of 18 of the 1^{st} group (72.22%) showed full relief and cure while the 2^{nd} group 21 patient out of 24 (87.5 %) showed an increase in the eradication rate. from the above result we observed that the addition of *Lactobacillus acidophilus* gave better results in the eradication of *Helicobacter pylori* which confirms the *in vitro* anti-*Helicobacter pylori* effect of Lactobacillus *acidophilus* could be effective in increasing eradication rates of a standard anti- *Helicobacter pylori* therapy [24, 25].

Table (1): Sign and symptoms of 42 patients with positive *Helicobacter pylori* infection

Symptoms	n	%
Abdominal pain	38	90.48
Pain features		
Burning	25	59.52
Undefined	34	80.95
Night pain	30	71.42
Interference with patients usual activities	25	59.52
Upper gastric bleeding	11	26.19
Other symptoms		
Postprandial fullness	23	54.76
Vomiting	30	71.42
Nausea	29	69.04
Loss of appetite	31	73.8
Anemia	14	33.33
Abdominal sensitivity	20	47.62

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