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UTILIZING DATE PITS IN MICROENCAPSULATION: EFFECT OF DIFFERENT VARIATIONS ON PROBIOTIC SURVIVABILITY UNDER IN VITRO DIGESTION

Asmaa Samir Al Hamayda

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering

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Cover: Symbolized Figure about the Objective of the Study

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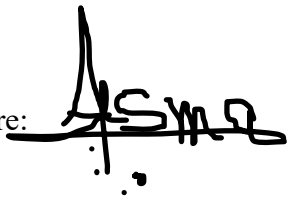
I, Asmaa Samir Al Hamayda, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Utilizing Date Pits in Microencapsulation: Effect of Different Variations on Probiotic Survivability Under in Vitro Digestion*", hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Basim Abu-Jdayil in the College of Chemical and Petroleum Engineering at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: 

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Abstract

This work deals with the medicinal use of date pit powder, which turns it from a waste product to a useful stabilizer for the release of beneficial probiotics in the human intestine. The main objective of this work is to encapsulate probiotics with date pit beads and their subsequent release in the human intestine. The beads were formed using gravitational dripping technique, while INFOGEST protocol was used to test the digested beads and the release of viable probiotic cells. For the three date varieties Raziz, Naghal and Khadrawy, it was found that 0.1 g of date pit powder per 0.2 g of sodium alginate in the solution mixture, i.e., a ratio of (0.5:1), is the best scenario for the highest desired viable probiotic cells, Log_{10} CFU/ml 5.8, 5.3 and 4.7, respectively. On the other hand, Lulu date pit showed that the ratio of 1:1 date pit powder and sodium alginate colonized the highest viable probiotic cells of 10^6 CFU/ml, while Naptit Saif required the least amount of date pit powder in the mixture to obtain the highest viable probiotic cells of Log_{10} 6.2 CFU/ml. Thus, the use of date pit powder, which was considered as waste, showed great results in terms of encapsulating beneficial probiotics and delivering them to the target organ of the body. Moreover, the encapsulation technique is approved as a protective procedure for the survival of probiotics in the intestine, since the resulting number of bacteria in free cells was zero, which means that there were no viable probiotic cells after the simulated digestive system was performed with free cells. Another finding worth mentioning is that treatment of date pit powder with water before performing the INFOGEST protocol resulted in a lower number of viable probiotic cells, ranging from Log_{10} 3.9 to 4.7 CFU/ml, compared to the number of viable probiotic cells when using only washed, dried and ground raw date pits, ranging from Log_{10} 5.1 to 6 CFU/ml. In general, this study showed that the viable probiotic cells in the intestinal phase were increased by the presence of date pits in the beads.

Keywords: Date Pit Powder, Medicinal Use, Human Intestine, In Vitro Digestion, Probiotic Cells, Microencapsulation, Gravitational Dripping, INFOGEST Protocol.

Title and Abstract (in Arabic)

تغليف البكتيريا النافعة في حبيبات الدواء بوجود بذور نواة التمر المطحون

الملخص

تعني هذه الرسالة باستخدام بذور نواة التمر المطحون بطريقة طبية بحيث تحوله من مخلفات محلية طبيعية إلى عامل مفيد لوجود البكتيريا النافعة في أمعاء جسم الإنسان. الهدف الرئيسي من هذه الأطروحة هو توصيل البكتيريا النافعة باستخدام الحبيبات التي تحتوي على بذور نواة التمر المطحون وإطلاقها في أمعاء جسم الإنسان. تم استخدام تقنية التنقيط التجاذبي لتشكيل الحبيبات ، كما تم استخدام بروتوكول INFOGEST لاختبار الحبيبات المهضومة ودراسة عدد المستعمرات البكتيرية النافعة التي يمكن ان يحصل عليها الانسان بعد عملية الهضم. وأظهرت الدراسة زيادة عدد المستعمرات البكتيرية في الطور المعوي في وجود بذور نواة التمر المطحون في الحبيبات. وقد أظهر استخدام بذور نواة التمر المطحون، والذي كان يعتبر من المخلفات الشائعة في دولة الامارات العربية المتحدة، نتائج عظيمة في الحفاظ على قوة الحبيبات و نتائج عظيمة من حيث وصول البكتيريا النافعة إلى العضو المستهدف من الجسم. تم استخدام خمس انواع مختلفة من نواة التمر و يمكن القول ان نمط انتاج حبيبات ألجينات الصوديوم مع نواة التمر للرزاز و النغال و الخضراوي متشابهة من حيث كمية المواد المستخدمة. حيث أثبتت تجاربهم ان استخدام 0.1 جرام من بذور نواة التمر المطحون مع 0.2 جرام من ألجينات الصوديوم في الخليط المستخدم لانتاج حبيبات ألجينات الصوديوم يؤدي إلى الحصول على أعلى قراءات لعدد المستعمرات البكتيرية النافعة بعد عملية ال INFOGEST و كانت النتائج كالتالي :لوغاريثم 5.8 لرزاز , 5.3 للنغال و 4.7 للخضراوي. بينما نواة التمر لنبتة سيف و اللولو أضهروا نتائج مغايرة, حيث أظهرت نتائج اللولو ان نسبة (1:1) هي افضل ما يمكن استخدامه مما يعني استخدام 0.1جرام من ألجينات الصوديوم و نواة التمر بينما نبتة سيف أظهرت نتائج ايجابية باستخدام كمية اقل من نواة التمر المطحون، حيث أن 0.05جرام من نواة التمر كافية لكل 0.2 جرام من ألجينات الصوديوم لانتاج اوغاريثم 6.2 من المستعمرات البكتيرية النافعة بعد عملية ال INFOGEST .

ما تم أيضاً تأكيده في هذا العمل هو أهمية عملية التنقيط التجاذبي حيث أن البكتيريا النافعة ماتت تماماً خلال عملة الهضم و كانت نتائجها صفر و التي تعني عدم تكون اي مستعمرات بكتيرية في نهاية التجربة. ما تم استنتاجه أيضا هو ان استخدام بذور نواة التمر كما هي سيؤدي الى حصولنا على نتائج أعلى في نمو البكتريا في نهاية عملية الهضم.

ستساعد إمكانية استخدام بذور نواة التمر المطحون في تصنيع الأدوية على بدء سوق جديد لما يُعرف بأنه يهدر بكميات ضخمة و له اسعار منخفضة القيمة في بلداننا. لذلك ، من منظور اقتصادي ، فإن الوصول إلى مسحوق نواة التمر، بتكلفة منخفضة للغاية، سيجعل إنتاج هذه الحبيبات التي تحتوي على بكتيريا نافعة و نواة التمر عمليًا نجاح اقتصادي لدولة الامارات العربية المتحدة.

مفاهيم البحث الرئيسية: بذور نواة التمر المطحون ، بكتيريا نافعة ، عملية التغليف، نواة التمر، المستعمرات البكتيرية.

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The first thank will always go to ALLAH, who guided me through my whole life and gave me this opportunity to complete my master's degree.

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Special thanks go to my dearest friend Fatima Al Mheiri, for her support and encouragement during the years of tough times and happy ones and I am so grateful for her presence in my life.

Dedication

To my beloved parents, family, and friends

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List of Abbreviations

CFU	Colony Forming Unit
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared
GCC	Gulf Cooperation Council
IDF	International Dairy Federation
LAB	Lactic Acid Bacteria
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestine Fluid
SSF	Simulated Salivary Fluid

Chapter 1: Introduction

1.1 Overview

Due to its effects on the environment, society, and the economy, the extraordinary level of food waste in the world's food supply chains is receiving more attention. Food waste is a waste stream that is receiving more and more attention in the developing field of waste management. Food waste is increasingly acknowledged as being essential to a more sustainable solution to the world's waste problem as the scope of its detrimental environmental, social, and economic impacts on ensuring global food security becomes clearer [1]. According to the Food and Agricultural Organization (FAO), 1.3 billion tons of various foods are wasted annually along the supply chain, which could feed up to two billion people without having any further negative effects on the environment. Food waste was “one of the great paradoxes of our times” [2]. What was observed lately is that there is a research trend that could be advantageous to farmers, the food industry, and food security, which is the use of food industry waste. Date pits as shown below in Figure 1 are frequently used as supplemental feed for livestock and poultry or as a traditional soil fertilizer. The extracted oil from the date pits can be used for the purposes of production of cosmetics or pharmaceuticals. Utilizing waste and unwanted date-pits in food applications and developing valuable products has been conducted but with limited research [3]. In accordance with what was discussed earlier, the encapsulation process can be considered as a solution to the utilization of date pit into valuable products. For more than fifty years, encapsulation has been used in biological fields and food science disciplines, as well as it, was used in pharmaceutical and nutritional areas [4].

Figure 1: Date-pits and its powder [5]

The encapsulation process includes protecting a certain item, which could be a functional ingredient or a microorganism, by shielding it. The products formed are considered carriers, and they will have different sizes [6]. Due to the constrained use of food-grade materials as carriers, creating new formulations of the encapsulating materials continues to be a significant challenge in the food industry. Polysaccharides, proteins, and lipids have all been used as encapsulates thus far. Probiotics, which are living microorganisms and are considered beneficial bacteria for humans, are one of the active ingredients that are used in the encapsulation process. The substance that must be enclosed or kept inside is referred to as the active agent or core ingredient, and the probiotics are shielded by the coating wall. In the food sector, encapsulation typically serves a variety of purposes. The main goal, however, is to protect the encapsulated active core from external factors that might damage them until they reach its final target in the human intestine [7]. In previous studies, it has been mentioned that cellulose acetate phthalate gelatin, vegetable gum, fats, or κ — carrageenan were utilized as material for the encapsulating agents in the encapsulation process, but alginate is the most common bio polymer agent used[8]. Normal alginates that are available in the markets are obtained from Phaeophyta, which are known as brown algae such as *Ascophyllum nodosum* and *Macrocystis pyrifera*. Alginates are mainly polysaccharides characterized as un-branched anions [9].

Alginate is mostly useful in entrapment active core and forming small beads that cover the microorganisms inside as shown below in Figure 2 in the encapsulation process due to its gelation capability in simple conditions, as well as its great mechanical properties, its biodegradability, and good biocompatibility [10]. Although alginate, after forming in beads, have very attractive features, they possess some drawbacks as they are sensitive to acidic conditions, which will show less resistance in the stomach if used as a carrier in the digestion experiment. The concentration of alginate and the probiotics, the viscosity of the alginate solution, the length of time beads take to harden, the diameter of the beads, and the distance between the coagulation solution and the outlet are some of the factors that affect how well encapsulating material functions during the whole process. The survival rate of the active core, such as the probiotics, increases proportionally as alginate concentration increases. As the size of the beads increases, the probiotics' effectiveness in the simulated gastric fluid increases [11].

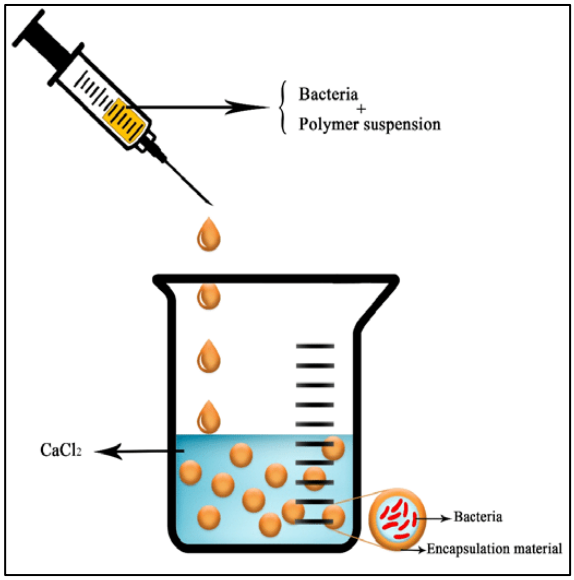

Figure 2: Schematic illustration of the dripping technique [12]

1.2 Statement of the Problem

In many nations around the world, the buildup of agricultural industry wastes is regarded as a serious environmental issue. This is especially true for plants where there are no waste recycling facilities or where the staff lacks the skills necessary to complete the recycling process. Date pits are one of these forms of plant waste. Date seeds are considered a type of dry waste because they are large and lightweight (i.e., low density and quality), and when they accumulate in large quantities, they become a serious problem because they are thought to be an environmental hazard when burned, in addition to providing a shelter for pests and rodents. Therefore, it's crucial to figure out how to incorporate these wastes into green and sustainable engineering applications as a competitive, economical, and environmentally friendly alternative to synthesis materials currently in use and limited to the feed industry. Although date seed pits are typically observed as wastes, there are many uses for them today, some of which are distinctly unexpected. Date pits were traditionally either thrown away or used as animal feed, a practice that is still widely used today.

1.3 Research Objectives

In the past, millions of people in dry and semi-arid regions all over the world relied on dates as a staple food source. Dates are a common fruit in the Middle East. The date fruit consists of a fleshy pericarp that makes up a sizeable portion of the weight, and it contains date seeds, which are also referred to as kernels or pits. Depending on maturity and grade, dates vary. The objective of this study is to encapsulate probiotics using date pit-based beads to enhance the release of the beneficial bacteria (probiotics) in the targeted area of the large intestine in the human body. Different types of date-pit mixed with biopolymer of sodium alginate will be used in the formulation of these beads. The encapsulation process using the gravitational dripping will be optimized in terms of degradation in the human body, as well as the resistance of encapsulated probiotics to the in-vitro gastrointestinal tract conditions. Beads should exhibit an optimum diameter size, great shape, longer shelf-life and a release of probiotics in a certain place (-organ) in the human colon. Several tests will be conducted to evaluate the performance of the prepared encapsulations. The potential for date pits to be used in the production of medicines will help to open up a new market for what is currently regarded as waste or low-value products in our countries. As a result, from an economic standpoint, the availability of this date pit powder at a very low price makes the production of these beads feasible.

1.4 Relevant Literature

1.4.1 Pollution

In the last few years, there has been a lot of research done on the impacts of pollution on health. Increases in hospital admissions and mortality have been linked to pollution exposure in all its forms. Short-term studies that link daily fluctuations in pollution and health have discovered these impacts [13]. One of the major environmental risks that pose an economic challenge is the generation of new solid wastes and careless treatment of these unwanted items, which results in high costs for collection, transfer, and disposal. As a result, it's essential to increase awareness of pollution and to find an effective way that is environmentally responsible by cutting down on waste generation and optimizing the recycling of used goods [6]. There are many hierarchy diagrams that were used all over the world to represent solutions for the treatment of waste, but all of them show the same steps as shown below in Figure 3 [14].

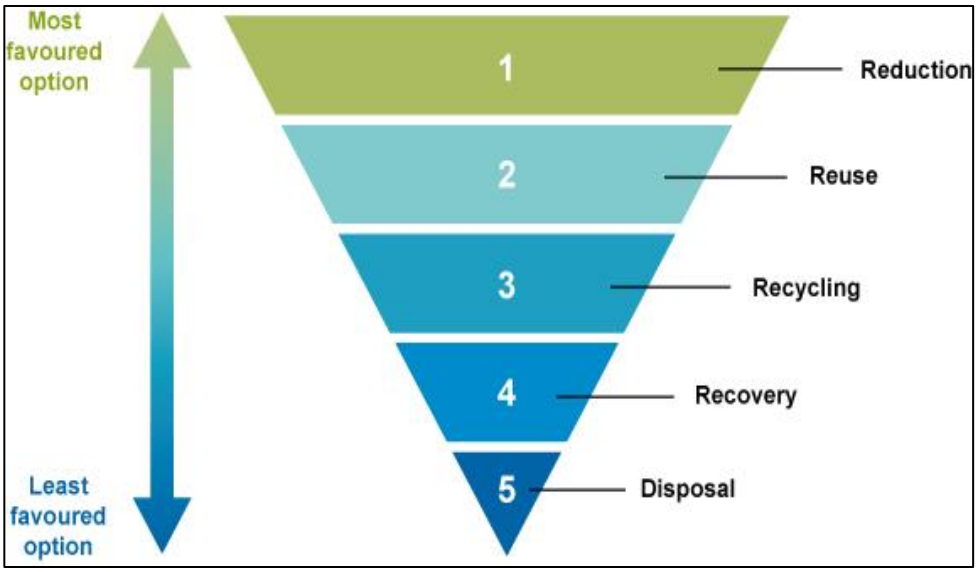

Figure 3: Hierarchy of treating waste [14]

The best way and most effective one to limit the environmental impacts of waste are to not create them. So, waste reduction is at the top of the hierarchy. The idea of reusing something, is putting the item in use without any additional modification, altering its form, or even changing its original essence. Reusing solid wastes is considered the second choice in the hierarchy. Reusing several kinds of solid trash is possible, including bottles, worn-out clothes, or anything

else that can be used for a certain purpose. Reuse results in the production of less solid waste. Transferring usable goods from individuals, whom no longer desire to those who do, offers additional benefits. Recycling is the third option after the reduction and reuse steps. Recycling waste is defined as re-producing new products from certain items that have been used earlier. The environment and human health may be impacted by the reprocessing processes, but these effects are often less severe than if the product had been made from brand-new raw materials. Option four in the waste hierarchy is recovery, which entails finding alternative applications for wastes that allow them to be used after extraction or repossession [14].

Removal and suitable disposal of by-products and bio-waste processing products can actually provide the opportunity to decrease serious environmental concerns. 1.3 billion tons of various sorts of food are wasted annually [1]. FAO estimates that, across the food supply chain, they can feed two billion people without negatively affecting the environment further. Lately, researchers are exploring more on the use of waste from the food industry, and this idea of waste utilization could offer economic gain to different sectors in the country, such as food security, the farmers, and environmental safety and sustainability.

What has been done and discussed in this thesis is choice number 2 in the hierarchy shown in fig.3 above, which is the idea of reusing unwanted products. What has been done is reusing fruit parts as useful products, which gave promising results in the following study field. One of the most famous solid wastes in the Middle East is the parts of fruit. Seed, pumice, rind, and skin of fruits are the primary parts that are produced in the industry of fruit processing. By employing alternative processes, it is possible to decrease the quantity of plant waste. The byproducts of fruits are abundant sources of many kinds of chemicals with medicinal benefits. Waste produced by the processing of fruits can be recycled to create innovative medications, dietary supplements, or functional meals.

The production of various fruits worldwide, which topped one billion tons in 2017, according to the Organization of Food and Agriculture in the United Nations, obviously causes the formation of significant volumes of trash and byproducts [15]. Approximately 100 Mt of waste is produced annually in only Europe as a result of the beverage and fruit companies. Treating the by-products of fruits and vegetables is one of the main problems that face the industries of agri-food. Large amounts of waste with a high concentration of biological compounds can be dangerous because they can lead to phytotoxicity phenomena, which can affect the growth of certain plants, contaminate aqueous media, negatively affect the quality of the drinking water,

kill marine organisms, and might cause intestinal problems in animals fed by those by-products plants [16]. With proper treatment, even though these vast volumes of waste might cause environmental problems, they can also serve as a useful low-cost raw material that is rich in important components for other sectors of industry.

Gulf Cooperation Council (GCC) countries are famous in the date palm production sector. This particular sector is considered an economic activity in the Arabian Peninsula. Furthermore, date palm production plays an important role among the population of the Peninsula. Domestic consumption of date has its own importance in the GCC countries, as well as it has other important roles such as considering employment opportunities and money earnings, and mostly they use it as trade in many countries. Worldwide, date fruits are marketed under various names due to their nutritional and spiritual value. Local communities in arid areas still rely heavily on date fruit for resilience subsistence. Date palm exportation from GCC countries has been significantly impacted by the globalization of markets, raising a whole new set of important competitive factors [17].

About 125 thousand metric tons of palm dates were consumed in the United Arab Emirates in the twenty-first century. Comparing this number to the prior years, a considerable rise has occurred. Figure 4 below represents that year-over-year; there is a major increase in date consumption.

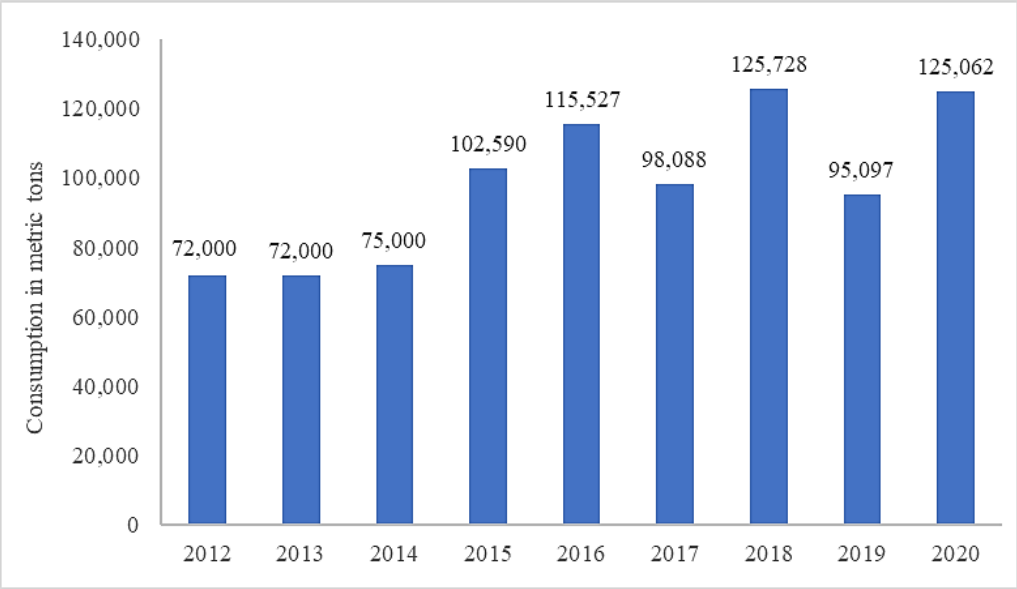

Figure 4: Analysis of date palm consumption in the U.A.E in metric tons in the period of 2012 – 2020 [18]

Because the Middle Eastern nations are the world's largest date producers, finding ways to use the pits of the dates will give viable chances to many applications in different sectors. Furthermore, it could decrease environmental pollution by decreasing the reduction of agricultural waste [19].

1.4.2 Date Seeds

Almost 2000 distinct date types have been found globally, each with unique physical and chemical properties [20]. Dates have a special shape of an ellipsoid, they don’t have a fixed color, but they vary from red to light yellow depending on the stage of the date. A date pit, or as called seed or kernel, is surrounded by an endocarp, which is a very thin layer and then this layer is covered by a mesocarp, which is a pulp of the fruit and then these parts are covered by an Epicarp, which is a thin layer of flesh [21].

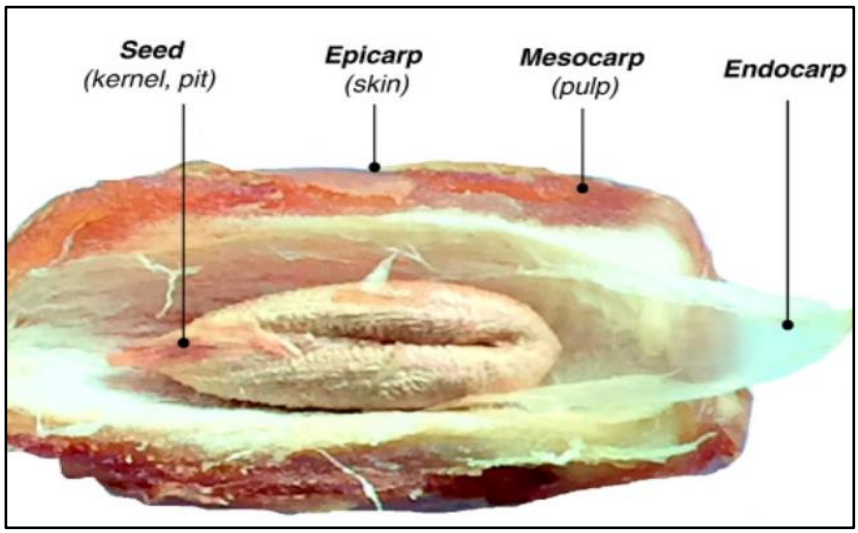

Figure 5: The anatomy of the date fruit representing the endocarb, mesocarp, epicarp, and seed [21]

Dates pits do not have any toxic effects, unlike many other fruit seeds. They can substitute cocoa, as well as they are considered a great source of functional food ingredients. Date pits contain great number of essential minerals for health which includes magnesium, phosphorus, iron, calcium, potassium, and zinc, as well as vitamins and polyphenols. Date pits are a great source of phenolic compounds and antioxidants, which are important in reducing oxidative stress and improving the human immunity system [22].

1.4.2.1 Date–pits as a source of antioxidants

Plants need to protect themselves from harsh conditions of the environment and from infections, so different types of metabolites are naturally produced by plants, such as polyphenols, examples are shown below in Figure 6. Plants' polyphenols are very important compounds in plants that help to maintain proper functions, as well as homeostasis, and they help plants and animals to prevent diseases because of the bioactive properties that polyphenols give to them [23]. It is now well established that eating green vegetables regularly, as well as eating fruits and fiber on a regular basis, can help to prevent or delay the onset of cardiovascular diseases, aging, cancer, and inflammation. Antioxidant supplements can be produced in the pharmaceutical and nutritional fields by using the date pit as one of the components formula [24].

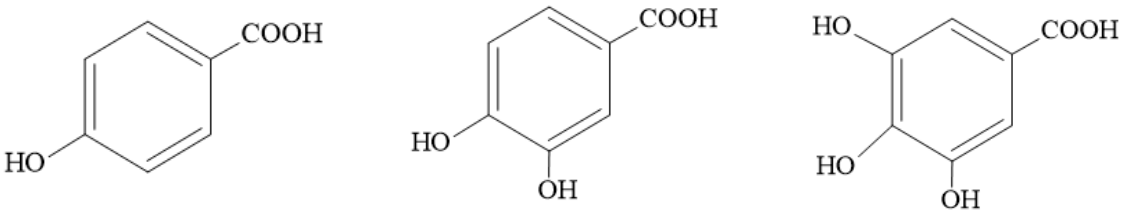

Figure 6: Examples of polyphenols present in the date seeds. I: p-Hydroxybenzoic acid, II: Protocatechuic acid, III: Gallic acid [25]

In the past, date seed powder was utilized as a herbal remedy to cure conditions like anemia, impotence, and progeria [26,27]. As a result, middle-aged women can frequently utilize it to preserve their health, strengthen their immune systems, and guarding against chronic illnesses [27]. Date seeds interact with the intestinal microbiota, particularly probiotic bacteria, and can be utilized as a probiotic, according to studies [28]. Furthermore, it can be decided that the consumption of date seeds has a function as an anti-inflammatory and basically can possibly raise the body's immune status.

1.4.3 Alginate

D-mannuronic acid and l-guluronic acid are the main units of alginic acid, which is found in naturally occurring alginates. It can be extracted from brown seaweeds. Alginates have very

attractive and unique features including antibacterial activity, biodegradability, biocompatibility, and nontoxicity, which make their uses exceptional in many sectors such as the food sector and pharmaceuticals.

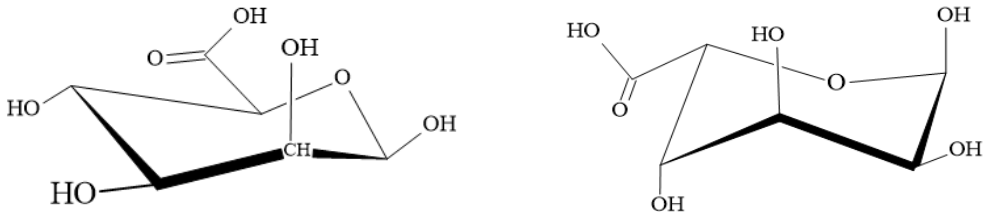

Figure 7: D-mannuronic acid and L-guluronic acid structures.

Sodium Alginate; its structure is shown below in Figure 8; is able to form gels in the existence of bipolar ions because of the carboxyl group in the polymer chain. Because of its porosity and strong water-absorption ability, sodium alginate gel is a strongly advisable material for drug or microorganism delivery. Alginate is used as an emulsifier by food industries [29] because it increases the viscosity of a solution when dissolved in water. Textile industries also can benefit from sodium alginate by using it as a thickener, and because it does not react with dyes, sodium alginate is easily washed out.

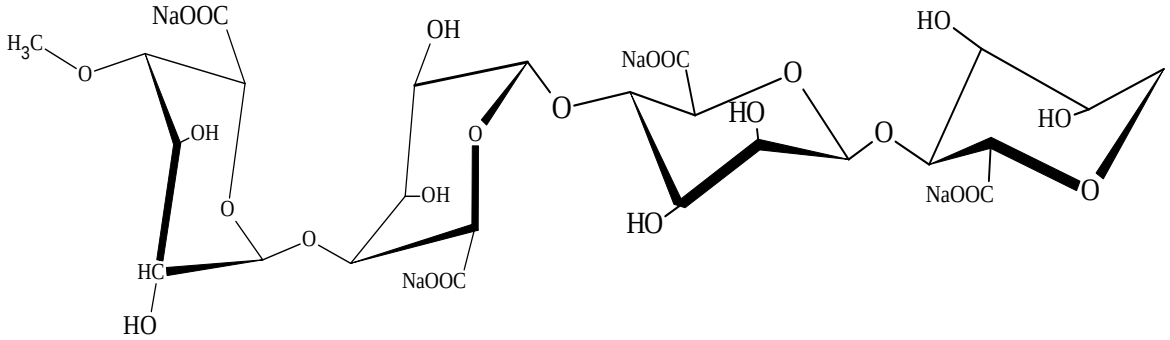

Figure 8: Structure of sodium alginate

Alginate is considered an excellent probiotic carrier, as well as aiding in probiotic survival. Some studies have found specific drawbacks to using alginate alone in the probiotic encapsulation process. They have mentioned that, although the beads, which are made out of

alginate, are good in protecting probiotics during storage, they are not able to do the same in low pH conditions such as in gastrointestinal fluids of the stomach [30]. What was also found in previous research is that the porosity of beads alginate limits the targeted function of being a good carrier, because this high porosity can easily release the loaded molecules, and the encapsulation efficiency will be low. In addition to that, the easy decomposition of beads in acidic conditions will limit the transportation of the probiotics to the human intestine [31]. Therefore, some modifications in the chemical or physical properties of alginate beads are required in order to overcome the above disadvantages.

1.4.4 Encapsulation

Through a variety of methods, such as extrusion, spray freezing, freeze drying, and emulsification, cells can be completely enclosed in the wall material. All of these processes are examples of the encapsulation process. The most typical method for creating hydrocolloid capsules is extrusion and it is considered the oldest method. A hydrocolloid solution is simply made, microorganisms are added to it, and then the cell suspension is extruded through a syringe needle in the form of droplets to fall freely into a hardening solution. The simplicity, ease, low cost, and gentle formulation conditions that guarantee high retention of cell viability when using biopolymers like Sodium Alginate make this method the most popular, see below Figure 9 [32]. Using the encapsulation process in the food industry took place among researchers and scientists. Probiotics are more likely to survive industrial processes and gastrointestinal transit when they are enclosed in appropriate wall materials. Probiotic strains must be encapsulated to be shielded from harsh conditions and to enhance the functional food's final sensory quality [33].

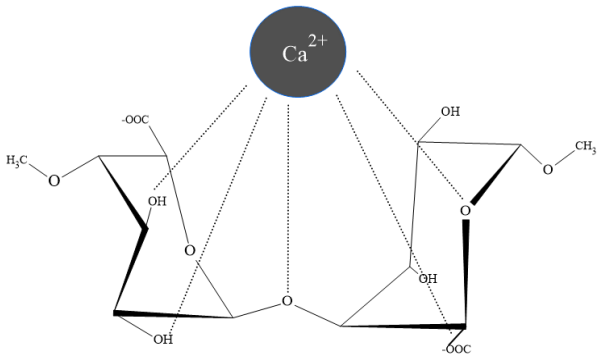

Figure 9: Structure showing the bonds between the cation and the units of alginate in the ‘egg-box’ model.

For probiotics to be released metabolically active in the intestine, they should be protected and shielded from an unwanted or unfavorable environment, so encapsulation is needed. Probiotics need to endure the stomach's acidic environment in order to survive and reach the large intestine. As a recommendation from the International Dairy Federation (IDF), probiotic bacteria will formulate in an efficient therapeutic way by a range of 10^6-10^7 CFU/g in the large intestine [34]. To maintain the structure of the beads throughout the process of food digestion and food matrix, it is important to take the beads' insolubility into account. This should enable the release of cells in the targeted area gradually, the large intestine. The main applications of alginate capsules are biomedical and pharmaceutical: to make a slow and controlled release of drugs and enzymes [35]; in the food industry [36] to microencapsulate probiotics, prebiotics, [37] nutrients, [38] as well as microorganisms that benefit the intestinal flora [39]; and in agriculture, for the treatment of wastewater, adsorption of heavy metals [40], for the encapsulation of bioactive substances [41], eliminate some organic pollutants [42], encapsulation of compounds to remove pathogenic bacteria [43] and to encapsulate bacteria beneficial for plant growth [44]. Probiotic microencapsulation techniques may impart a modified release through the addition of coatings or the blending of polymers.

Picot and Lacroix [45] demonstrated that encapsulation is of a great importance to protect the viable bacterium. They found that encapsulation of *Bifidobacterium* breve with whey protein significantly increased viable cell counts when compared to the non-encapsulated ones. Furthermore, in dairy products, microencapsulation techniques were effectively used to increase the survival of microorganisms [46] or artificial gastrointestinal juice [47].

The wall material of the encapsulated microorganism should be chosen carefully. Alginate is one of the most important encapsulation matrix used by researchers. In addition to alginate, some modifications are needed to help improve the survival of microorganisms especially in harsh conditions. The addition of these excipients should lead to a modification of the rate or circumstances of dissolution of alginate capsules in order to affect the release properties. In pharmaceutical applications, covering alginate microspheres with chitosan results in the "controlled" release of encapsulated medicines in simulated intestinal as opposed to a "burst" release [48]. Lin et al. investigated the stability of alginate microcapsules that encapsulated *E. coli* and then had undergone a chitosan or poly-L-lysine coatings before receiving a second layer of alginate. The capsules were then examined qualitatively using light microscopy. The encapsulated capsules were given to Wistar rats and then collected after 6 hours of digestion. Chitosan-coated particles appeared to be more resistant to digestion than poly-L lysine-coated

particles, but more quantitative data would be required to strengthen conclusions[49]. In another study Chandramouli et al. [50], used the sodium alginate as a polymer for the formation of capsules with no additives or polymer blends, but the optimization was studied regarding the size, the concentration and the hardening time of the gelling process to check the survival of bacteria in simulated gastric conditions. Their results concluded that, encapsulated bacteria outlived non-encapsulated free bacterial cells in simulated gastric conditions, and encapsulated bacteria's viability in simulated gastric conditions increased as alginate gel concentration increased from 0.75% to 1.8% (w/v). Similar results have been reported by Lee and Hoe et al. [51] where they demonstrated that *Bifidobaterium longum* enclosed in alginate spheres outlasted exposure to simulated gastric juice (pH 1.5) substantially better than free cells. Furthermore, Mandal et al. [52] confirmed that *L. casei* microencapsulated in alginate beads survived better than free cells during low pH, high bile salt concentration, and heat treatment. In simulated harsh circumstances of GIT and heat processing, increasing alginate concentrations also improved *L. casei* survivability.

Although alginate has been used extensively to encapsulate probiotic bacteria, the parameters of the encapsulation techniques do not appear to be consistent. Different conclusions have been drawn regarding the usage of alginate as a matrix for bacterial cell encapsulation due to the lack of uniformity or propensity to ignore certain of the conditions of encapsulation. This study is attempted to boost and optimize the encapsulation technique by using the alginate with date pit to increase the survival rate of encapsulated bacteria over free cells when exposed to simulated digestion system conditions. It also aimed to evaluate the performance of this distinctive alginate biopolymer when mixed with different varieties of date pit powder.

1.4.5 Probiotics

If the live microorganisms benefit the host when they are given in sufficient quantities, they are defined as probiotics. Furthermore, these microorganisms can be considered probiotics if they are not only safe for humans but also should be able to live in the gastrointestinal tract and benefit their host's health [53]. The composition of the intestinal flora and resistance to pathogens are two health benefits that probiotic consumption has been linked to. Food items with probiotic bases have become more and more popular in recent years. The importance of probiotics in the treatment and prevention of some gastrointestinal disorders has been highlighted in a number of scientific studies. The large intestine in the human gut has a composition of bacteria that is made up of more than 50 genera, some of which are harmful

(producing toxins) and others of which are helpful (producing vitamins), some examples are shown below in Figure 10. Probiotic bacteria are administered to promote the development of beneficial gut flora, drive out harmful bacteria, and strengthen the defense mechanisms of the body [54]. Probiotics generally enhance human health by balancing the composition of the intestinal microbiota, enhancing the immune system, as well as boosting metabolic processes [55].

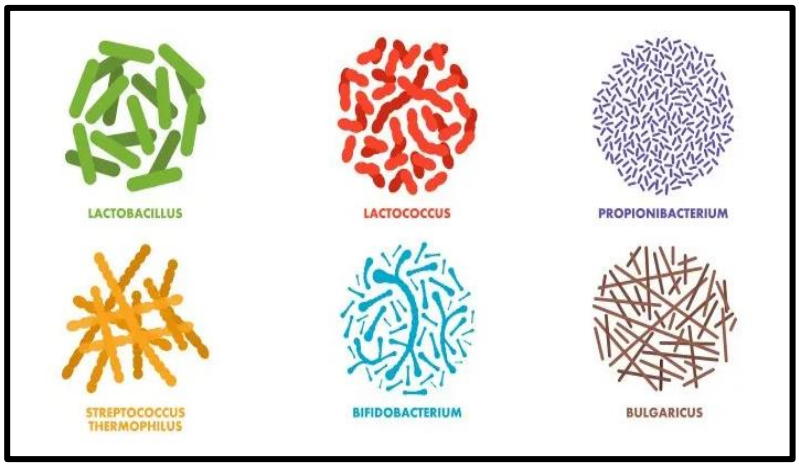

Figure 10: Examples of beneficial probiotics of the human gut [56]

Probiotics are available in markets as tablets, capsules, or even as powder [56]. Yet, dairy products are considered the main resource of probiotic administrations [57].

1.4.6 Streptococcus thermophilus

Gram-positive and gram-negative bacteria can be distinguished from one another by the layers of peptidoglycan that surround them. A common gram-positive bacterium called *Streptococcus thermophilus* is used to make yogurt and cheese. This particular species of Streptococcus was distinguished by the Food and Drug Administration (FDA) as a safe bacterium because humans have been using it for a very long time without showing any kind of harmful effects or diseases. *Lactococcus lactis* is another species of bacterium, which is the most important type of industrial Lactic Acid Bacteria (LAB), followed by *Streptococcus thermophilus*, which is considered the second most important species. A 3D colored illustration is shown below in Figure 11 [58].

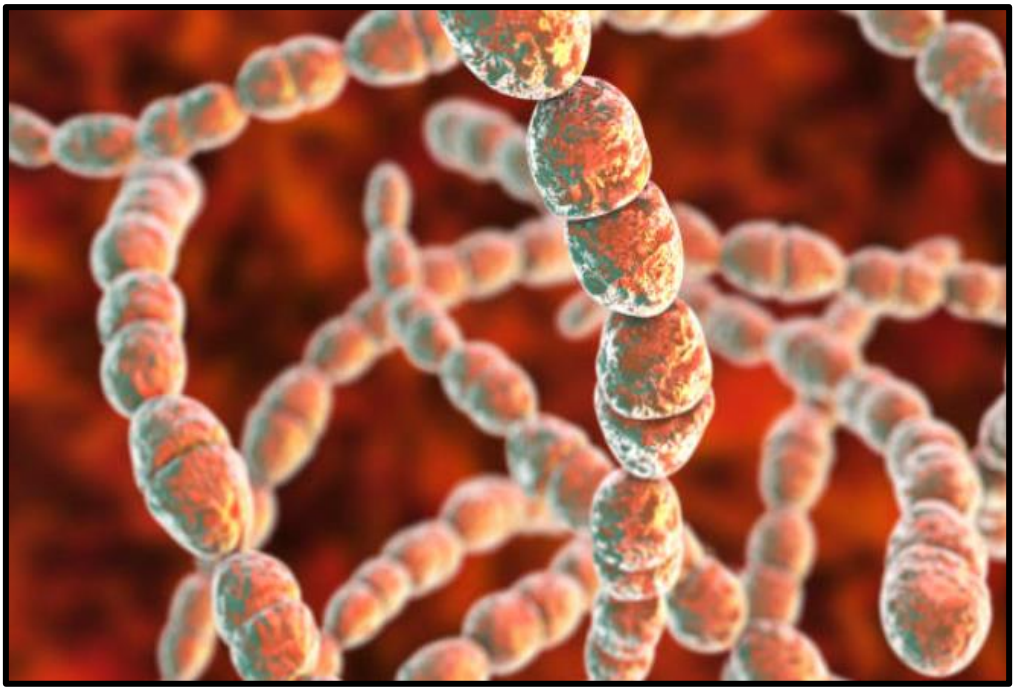

Figure 11: 3D illustration of Streptococcus thermophilus bacterium [59]

Many studies performed recently have shown that *Streptococcus thermophilus* have useful effects on host health, such as the alleviation of lactose intolerance, prevention of chronic gastritis, and prevention of diarrhea [60].

Chapter 2: Materials and Methods

2.1 Chemicals and materials

2.1.1 The date pit

The date pits used in this study were obtained from the UAE University farm in Al Foah, Alain. The date pits were dried for a week to get rid of any moisture and were then crushed using a commercial milling machine before being sieved to reach the size of 150 μm. This date pit's main components are cellulose, hemicellulose and lignin. Naptit Saif, Lulu, Raziz, Naghal, and Khadrawy are famous date types used in huge amounts during the year in the U.A.E. Each one of them has its own unique color and shape as shown below in Figure 12. Furthermore, the date pit and its color are also different in each date type, as well as different chemical composition.

Figure 12: Collection of the different varieties of date from UAEU farm in Al Foah

2.1.2 Chemicals

Sodium alginate that was used in this study is purchased from Sigma-Aldrich Company, U.S.A in powder form. It has the following specifications: the molecular weight is 398.316 g/mol, its molecular formula is $C_{14}H_{22}O_{13}$, and its melting point is > 300°C. Calcium chloride as well is purchased from Sigma-Aldrich Company, U.S.A. and its specifications are as the following: the molecular weight is 110.98 g/mol, the molecular formula is $CaCl_2$, and its melting point is 176°C.

Amylase, Ammonium Carbonate, Bile, Hydrochloric acid, hydrated Calcium Chloride, hydrated Magnesium Chloride, Pancreatin, Pepsin, Potassium chloride, Potassium sulfate were purchased from Sigma-Aldrich Company, U.S.A. in the highest purity grade possible and used as received unless otherwise stated.

2.2 Methodology

The methodology of the experiment was divided into three parts. Firstly, collecting and preparing the date pits that were used in the experiment. Secondly, prepare the sodium alginate beads that encapsulate the bacteria and the date pits. Finally, performing the simulated digestion system for the samples and followed by the enumeration of the viable probiotic cells see Figure 13.

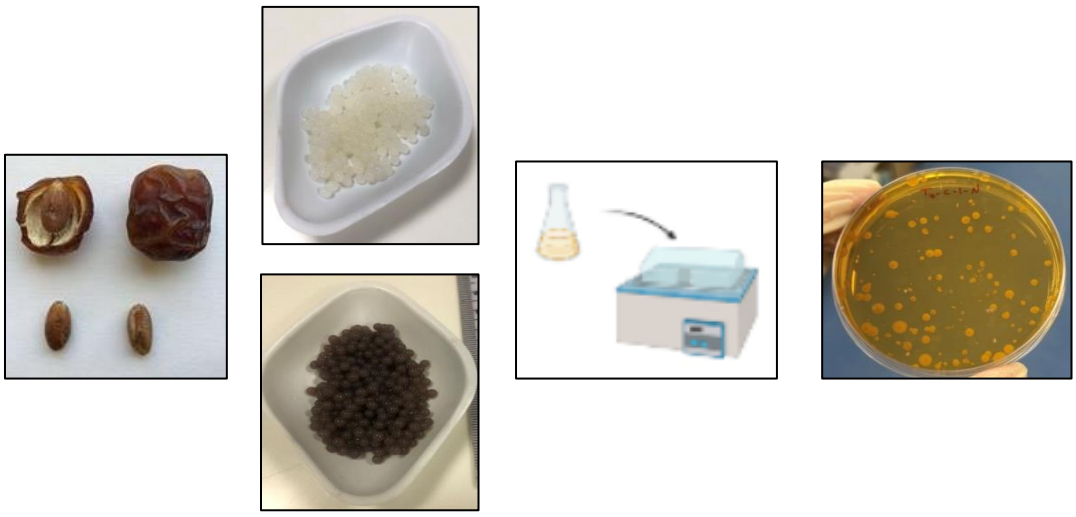

Figure 13: The three stages of the methodology starting from left to right, date pits were prepared, beads(with/without date pits) were formed, digestion protocol was performed and viable probiotic cells released were counted.

2.2.1 Preparing the date pit powder

Five different varieties of dates were used. The date pits were first separated from the flesh date, and then they were washed with normal water and kept to dry out. Date pits were ground into small sizes using a grinder shown below in and sieved to get the size of the particles 150µm.

2.2.2 Digestion Protocol

Regardless of the digestion model, researchers must adhere to a digestion protocol when preparing the enzyme solution and simulated digestion fluids. There are many different digestion protocols for creating simulated digestion fluids, but the COST INFOGEST network's standardized protocol simulates a wide range of digestion conditions. The oral, gastric, and intestinal exposure phases make up the three stages of the digestion study. And different enzymes and amounts of electrolytes should be included in the simulated digestion fluids for each stage [61].

Performing a simulation on digestion, particularly in the upper gastrointestinal tract, has become more and more important in gaining a mechanistic understanding of how food structure and composition affect human health. COST INFOGEST network has developed a standardized protocol that is used in this thesis as well as used in many types of research recently. By following this standardized protocol see Figure 14, a minimal amount of experience in digestion is needed, as well as no need for complex equipment that might limit the researchers from performing the experiment. Common laboratory tools and equipment can be enough. The described digestion protocol is done by using constant ratios of meal to simulated digestive liquids of the body with constant pH values in each step of the process [62].

The above-mentioned protocol is used in order to measure or evaluate the end product after each digestion stage and analyse the release of micronutrients or microorganisms from the food matrix.

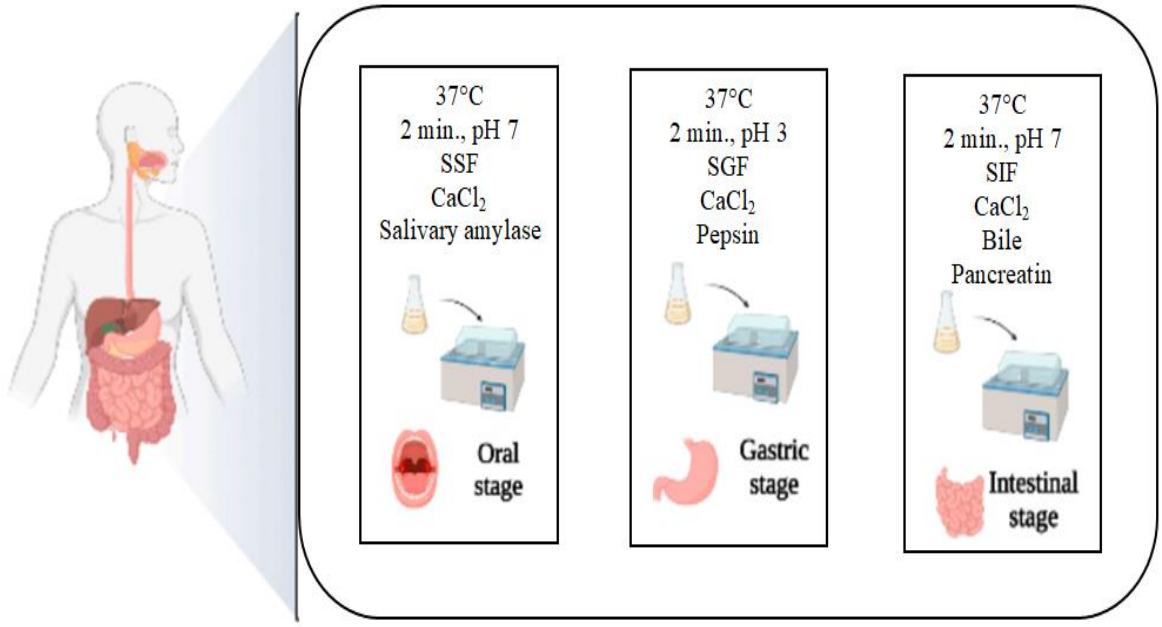

Figure 14: Schematic diagram of static simulation of digestion protocol. Adopted from [63]

The second part of the experiment is preparing the encapsulated probiotics in date pit-based beads made out of sodium alginate. So, under aseptic conditions, frozen stock solution (100 μL) of *Streptococcus thermophilus* was inoculated in MRS broth at 37ºC overnight see Figure 15; the cell pellets were harvested by centrifugation at 2000 rpm for 15 min using a centrifuge Digicen 21R Universal, Ortoalresa centrifuge as the pellets were used in the preparation of the encapsulated sodium alginate beads.

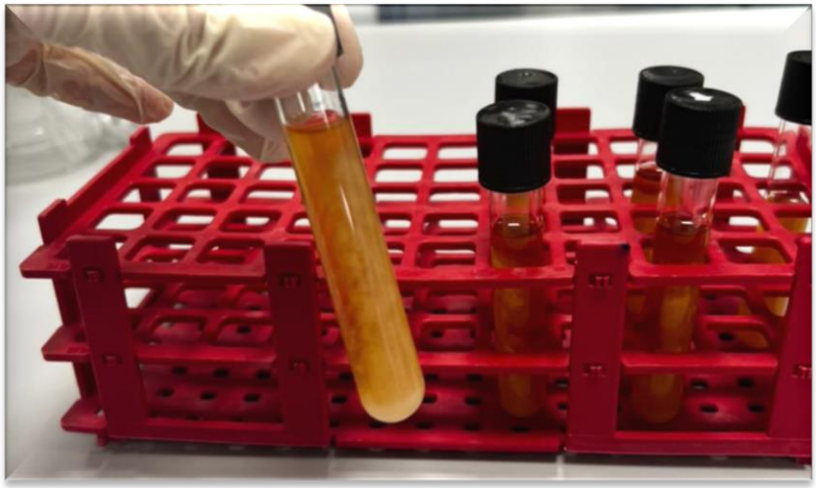

Figure 15: The resulted pellets after 24h incubation period

250 µL of Phosphate buffer with pH 7.0 was added to each tube of activated bacteria pellets. Activated probiotic bacteria pellets were added to sodium alginate solution and mixed for 15 minutes under aseptic conditions. The concentration of sodium alginate was constant; 0.2 g, while the concentrations of the date pits powder varied starting with 0.05, 0.1, 0.2, and 0.4 g, respectively, as shown below in Table 1.

Table 1: Samples prepared for the experiments

Samples prepared	Samples Codes	Sodium Alginate / g	Date pit powder/ g	Bacteria added / µL
Free Cells Bacteria	Free Bacteria	none	none	1000 µL
Sodium Alginate Control	Control	0.2 g	none	500 µL
Naptit Saif	dpA-T1	0.2 g	0.05 g	500 µL
	dpA-T2		0.1 g	
	dpA-T3		0.2 g	
	dpA-T4		0.4 g	
Lulu	dpB-T1	0.2 g	0.05 g	500 µL
	dpB-T2		0.1 g	
	dpB-T3		0.2 g	
	dpB-T4		0.4 g	
Raziz	dpC-T1	0.2 g	0.05 g	500 µL
	dpC-T2		0.1 g	
	dpC-T3		0.2 g	
	dpC-T4		0.4 g	
Naghal	dpD-T1	0.2 g	0.05 g	500 µL
	dpD-T2		0.1 g	
	dpD-T3		0.2 g	
	dpD-T4		0.4 g	
Khadrawy	dpE-T1	0.2 g	0.05 g	500 µL
	dpE-T2		0.1 g	
	dpE-T3		0.2 g	
	dpE-T4		0.4 g	

The encapsulation of *Streptococcus thermophilus* was carried out by a simple green method technique, see Figure 16. The polymer solution with the date pit and probiotic bacteria was introduced dropwise via a syringe attached with a 22-gauge hypodermic needle into 6% hydrated Calcium Chloride solution (cross-linking agent) under gentle agitation at room temperature. The droplets from the polymer solution with the date pit instantaneously gelled directly upon contact with the hydrated calcium chloride solution. The formed beads were allowed to cure for another 15 min to enhance the rigidity of the beads, and then they were recovered by filtering through a filter paper (Whatman 42, 15 cm, England) and washed with distilled water 3 times to ensure the removal of $CaCl_2$ leftovers, see Figure 17.

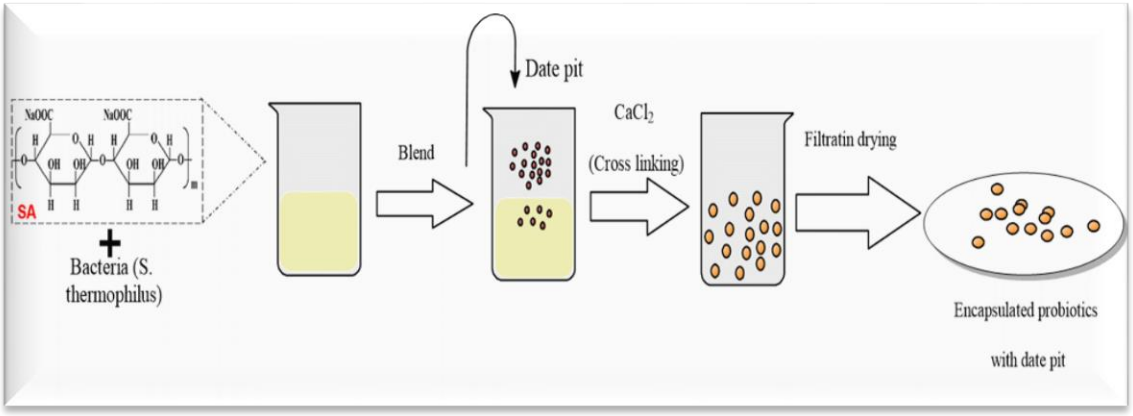

Figure 16: The process of preparing the loaded date pit encapsulated probiotics

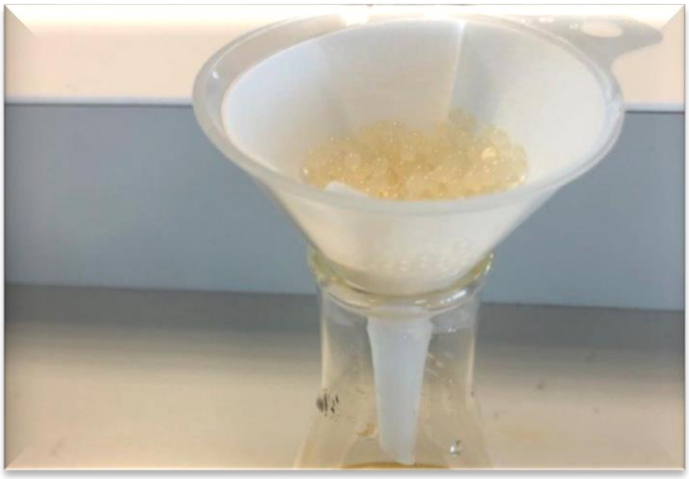

Figure 17: The final filtered products of beads

2.2.3 Performing the digestion protocol

The beads were dried and 2 grams of each sample were used in the INFOGEST digestion protocol that was explained above in chapter 1 and explained below in detail, while the rest of the beads were stored for further tests and kept in the fridge at 4°C.

2.2.3.1 Stock Solutions preparation

At the beginning of the INFOGEST protocol, stock solutions were prepared by mixing different previously prepared solutions in certain concentrations shown below in Table 2, which will produce the final simulated digestive fluids; Simulated Salivary Fluids (S.S.F), the Simulated Gastric Fluid (S.G.F.), and the Simulated Intestinal Fluid (S.I.F.) [63].

Table 2: The preparation values of the stock solutions [63]

			SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
	Stock concentration		Milimeters of Stock added	Final Salt concentration in SSF	Milimeters of Stock added	Final Salt concentration in SSF	Milimeters of Stock added	Final Salt concentration in SSF
Salt solution added	g/L	M	ml	mM	ml	mM	ml	mM
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

Figure 18 shows the final stock solutions of the simulated digestive fluids that were prepared to be used during the digestion protocol.

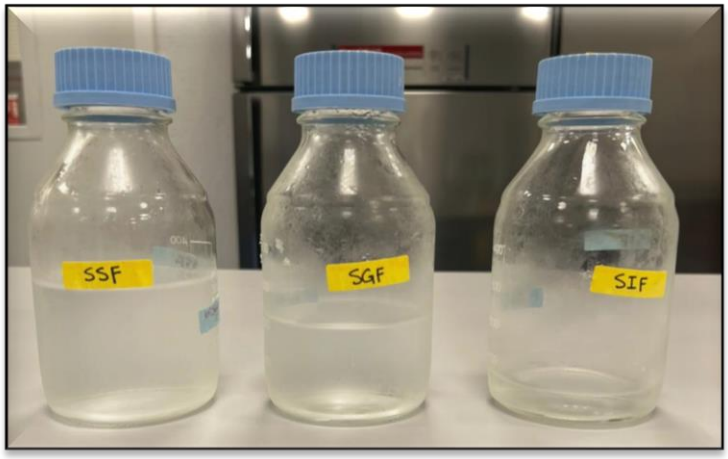

Figure 18: The simulated digestive fluids

2.2.3.2 pH adjustment experiment

One sample from the batch samples was used in order to check the required amount of 1 M HCl and 1 M NaOH to be added later in the digestion experiment to justify pH 3 and pH 7 at the gastric and intestinal phases, respectively. The steps were done as the following:

1. The contents of S.S.F. were added to the samples. In this test, no need to add Amylase.

2. The pH 3 of the solution mixed with the sample was checked by using a pH meter. The pH was 5.6, which is higher than 3. So, an adjustment of pH was performed by adding 1 M HCl to

the mixture by adding a very small quantity (1 µL) of HCl. The mixture reached pH 3 after the addition of 5 µL and the final amount of HCl was noted to be used in the real experiment.

3. S.I.F was added to the sample solution mixture, and no need to add Pancreatin and bile.

4. The pH was checked again by using a pH meter, and the pH actually was 7, so no further adjustment was needed here.

5. If it is less than 7, adjustment can be performed by adding 1 M NaOH to the mixture.

2.2.3.3 Enzymes Solutions preparation

Another preparation that was done is the preparation of the enzymes that are found in the mouth, the gastric and the intestine. Amylase, Pepsin and Pancreatin were prepared following the calculations shown below in Table 3 and kept in the ice box, see Figure 19.

Figure 19: Enzymes solutions prepared for the experiment

Table 3: Preparation of enzymes

Amylase	Pepsin	Pancreatin
The amount used was 75µL in 925 µL water.	The amount used was 0.004g in 1000 µL water.	The amount used was 0.04 g in 10000 µL S.I.F

2.2.3.4 Oral Phase

An oral master mix solution of S.S.F was first prepared. The master mix contains the S.S.F stock solution, $CaCl_2.H_2O_2$, water and amylase. Mastermix calculation was done by using a "Template for the harmonized *in vitro* digestion method from COST INFOGEST" [63] Figure 20 shows the initial entry quantity of our sample (2 grams), so the other components of the master mix stock solution will be calculated accordingly.

Oral phase composition

	Individual addition		Using Oral mastermix	
Quantity of food	2	g	2	g
Simulated salivary fluid (SSF)	1.6	ml		
Salivary amylase solution	0.2	ml		
0.3 M $CaCl_2$	10	µl		
Water	0.19	ml		
Oral mastermix			2	ml

Figure 20: Screenshot of the Oral phase composition in the online INFOGEST template

8 milliliters of Simulated Salivary Fluid (S.S.F.) was added into a falcon tube, as shown below in Figure 21.

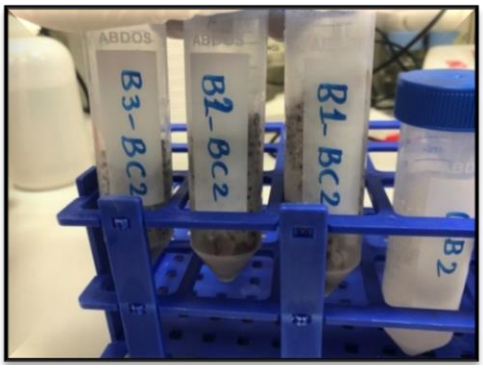

Figure 21: Falcon tubes

Then, 50 µL of $CaCl_2.H_2O_2$, 950 µL of water and 75 µL of the salivary amylase were added. The 2 grams of date pit-based beads were mixed with 2 ml of the master-mix Simulated Salivary Fluid (S.S.F.) at a ratio of 1:1 (wt/wt). The final digestion mixture of the beads and S.S.F was

measured by its volume. The 75 µL of the salivary amylase was added to achieve an activity of 75 U/ml in the final mixture. The mixture was incubated in the water bath for 2 min at 37°C.

2.2.3.5 Gastric Phase

Mastermix of simulated gastric fluid (S.G.F.) was prepared according to the values shown below in Figure 22. First, 16 ml of S.G.F stock solution was used. Then, 10 µL of $CaCl_2.H_2O_2$, 1965 µL of water, and 25 µL HCl were mixed. The pH was adjusted to 3.0 by adding a previously determined HCl amount during a pH-test adjustment experiment that was explained above.

Gastric phase composition

	Individual addition		Using Oral mastermix	
Volume of oral phase	4	ml	4	ml
Simulated gastric fluid (SGF)	3.2	ml		
0.3 M $CaCl_2$	2	µl		
Acid/base for pH3.0	0.005	ml		
Water	0.393	ml		
Gastric mastermix			3.6	ml

Figure 22: Screenshot of the Gastric phase composition in the online INFOGEST template

The pepsin solution was added after it was prepared in water to achieve an activity of 2,000 U/ml in the final digestion mixture. A 0.004 g of Pepsin is dissolved in 1 ml distilled water. 4.0 ml of the previously prepared master mix was added to the samples after the 2 minutes period of incubation in the water bath to achieve a final ratio of 1:1 (vol/vol). Finally, the samples were incubated in a 150 RPM shaking water bath at 37°C, and the digestive mixture was mixed sufficiently for 2 h from the point at which pepsin was added.

2.2.3.6 Intestinal Phase

A master mix of S.I.F. was first prepared by using the values shown below in Figure 23. A 17 ml of S.I.F stock solution was put in a falcon tube, 80 µL of $CaCl_2.H_2O_2$, 7920 µL of water, 5000 µL of bile solution, and 10,000 µL of Pancreatin were mixed.

Intestinal phase composition with pancreatin

	Individual addition		Using Intestinal mastermix	
Volume of gastric phase	8	ml	8	ml
Simulated intestinal fluid (SIF)	3.4	ml		
0.3 M $CaCl_2$	16	µl		
Acid/base for pH7.0	0	ml		
Water	1.584	ml		
Pancreatin solution	2	ml		
Bile solution	1	ml		
Intestinal mastermix			8	ml

Figure 23: Screenshot of Intestinal composition in the online INFOGEST template

The bile solution was prepared by dissolving 0.008 g of bile salt in 5 ml of S.I.F. stock solution. While the pancreatin solution was prepared, 0.04 g of pancreatin was dissolved in 10 ml of S.I.F. stock solution. 8 ml of the master-mix solution was put in each falcon tube on the above-mentioned mixture of beads in a final ratio of 1:1 (vol/vol). Samples were incubated for another 2 hours after the addition of Simulated Intestinal Fluid (S.I.F.). The pH value was adjusted to 7.0 by the mixture itself with no addition to NaOH. Surviving bacteria after the gastric phase is not the end of the challenges that face the probiotic to reach the intestinal phase [64]. Bile in the intestine is one of the key and main challenges, as it has properties of detergent-like antimicrobial activity, which can cause the dissolution of the membrane of bacteria [55],[66].

2.2.3.7 Serial Dilution

Peptone water of 0.1% concentration as shown below in Figure 24 was prepared and sterilized at 121°C for 50 minutes in the autoclave. One ml (1000 µL) of each solution of the finished samples was taken care of by using an Eppendorf pipette, as shown in Figure 25 under aseptic conditions and was mixed separately with Peptone water to obtain a degree of dilution.

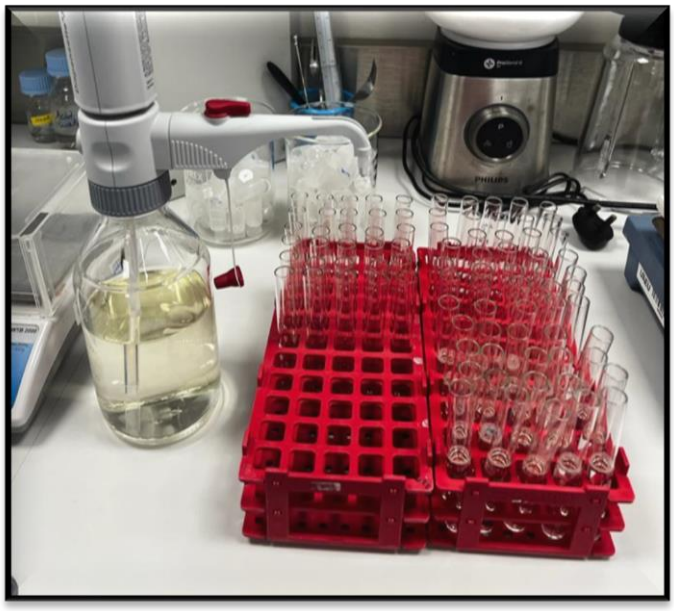

Figure 24: Peptone water prepared to be used in the serial dilution step

Figure 25: Eppendorf Pipette, and the transferred 1 ml of the aliquots to the petri dishes

Agar was prepared by following the manufacturer's instructions and sterilized at 121°C for 50 minutes in the autoclave, see Figure 26.

Figure 26: The autoclaved Agar and Peptone water solutions

The sterilized agar solution was cooled to about 40-50°C before being poured into the 1 ml (1000 µL) sample solution in the 90 × 15 mm sterile Petri dishes. The plates were allowed to dry at room temperature, see Figure 27. All plates were incubated overnight to allow the growth of the released bacteria from the encapsulated beads, see Figure 28.

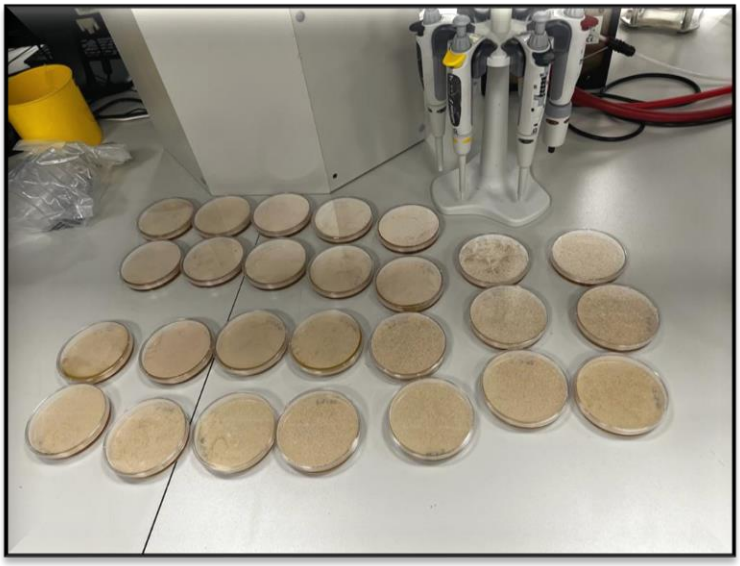

Figure 27: Samples with agar ready to be incubated

After 24 hours, the released viable probiotic bacteria were observed, as shown below in Figure 28.

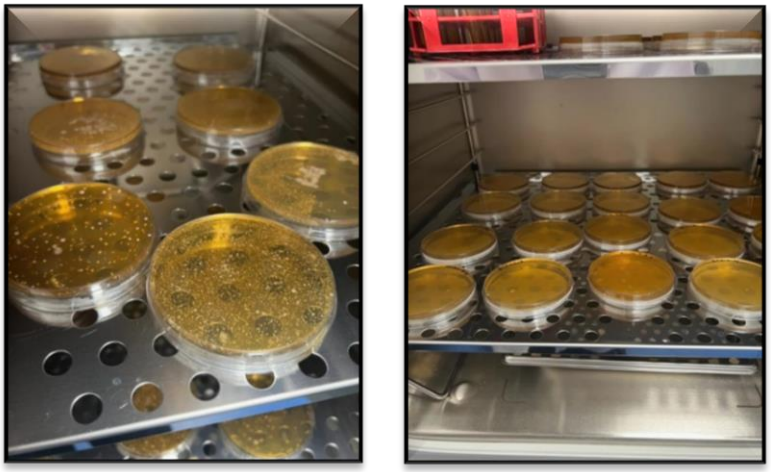

Figure 28: The results of the viable probiotic cells growth

2.2.3.8 Enumeration of encapsulated bacteria

After 48 h incubation at 37°C, samples (free cells and encapsulated probiotics) that were serially diluted in peptone water and plated on MRS agar were plate counted and the cell counts were expressed in $\log_{10}$ CFU/ml. Tests were conducted in triplicate. The count of the viable probiotic cells was examined by using Interscience Scan 1200; NY, U.S.A, shown below in Figure 29 to enumerate the released encapsulated bacteria, which is the main goal of the experiment.

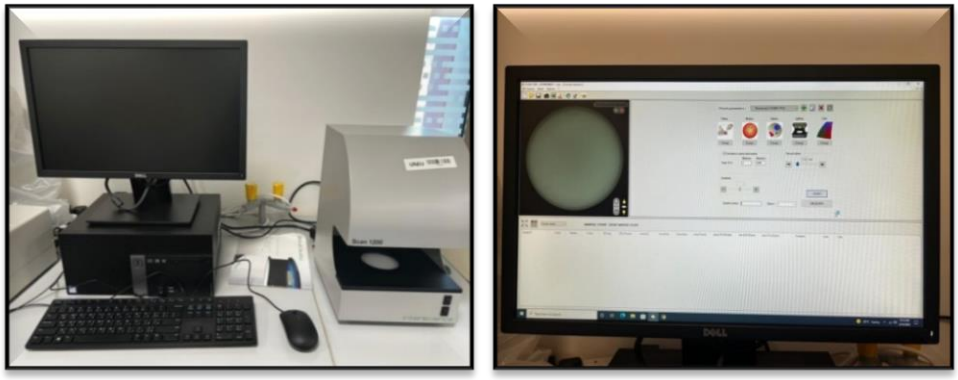

Figure 29: Interscience Scan 1200 device for viable probiotic cells count

2.3 Characterization

2.3.1 Chemical analysis

Date pit samples were ground to pass a 150 μm sieve and analyzed for moisture, protein, ash, and nitrogen contents[67]. Nitrogen and Protein were analyzed using the Kjeldahl method [68]**.** Carbohydrate content was estimated by the difference of mean values; 100 – (sum of moisture, ash, protein, nitrogen) [69].

2.3.2 FTIR

Fourier Transform Infrared test was used to determine the functional groups that are present in the beads samples and whether a chemical reaction occurred in the presence of date pit in the solution mixture, which might affect the characteristics of the beads. The test was done using FT/IR-4700 by JASCO. This test was done with the aim of confirming that the date-pit powder is combined with sodium alginate mixture and to check all the available functional groups produced, the stability and the integrity of the samples [70]. Peak adsorptions were recorded in the range of 400–4000 cm^{-1}.

2.3.3 Thermal properties

a. Differential Scanning Calorimeter (D.S.C.)

   DSC test determines the glass transition and melting behavior of the sample. It is used for polymer analysis. Since sodium alginate is considered a biopolymer, a DSC test can be performed to determine the melting point.

   Samples were tested by differential scanning calorimetry (DSC Q10, MDSC Q1000, TA Instruments, New Castle, DE, USA). A mechanical refrigerated cooling system with a capacity to cool the sample up to -45°C was used. Tzero Hermitic Aluminum pan of 30 ml, which could be sealed with a lid, was used in all experiments with an empty sealed pan as a reference. Nitrogen at a flow rate of 50 ml/min was used as a carrier gas. Samples from 6-9 mg of beads were placed in a sealed aluminum pan and allowed to be cooled to 30°C at 10°C/min and equilibrated for 1 min. After equilibration, the samples were heated up to 200°C.

b. Thermogravimetric Analysis (TGA)

TGA test provides data regarding thermal degradation and thermal stability. The test was done using TGA Q500 by TA Instruments. Samples of weight ranging from 5 to 10 mg were placed in the instrument, and the temperature was raised from 25℃ to 700℃. The TGA data can be interpreted in two graphs, which are weight percentage versus temperature and derivative weight versus temperature.

2.3.4 Morphological study

a. Microscope: Carl Zeiss light microscope attached with Stemi 508 camera and Zeiss Zen blue software was used in order to have clear pictures for the samples.
b. Scanning Electron Microscopy: SEM was also used to allow for high-resolution inspection of the date pit powder and the beads. An SEM model JSM-5600 (JEOL, Akishima, Japan) was used at 15 kV beam energy. To prepare the samples before analysis, Na-Alg./dpA/dpB/dpC/dpD/dpE beads were freeze-dried and sputter-coated with gold (10 nm) before loading onto the microscope. Images of the beads were represented. The freeze-dried beads were pre-frozen in a −80°C freezer for two hours and then lyophilized in a freeze dryer at a temperature of −50°C for 24 h (Micromodulyo, Thermo Fisher, US) after that was used. The beads were mounted on aluminum stubs by means of sticky carbon tape supplemented with silver paste and then coated with a thin layer of gold to increase the surface conductivity by sputtering in an argon atmosphere.

2.3.5 Releasing Efficiency RE

The efficiency of released, viable cells during the encapsulation process was calculated by the following expression:

$$RE = \frac{X_R}{X_i}\ x\ 100\ \ldots\ldots Eq.\ 1$$

Where X_R is the amount of probiotic released, and X_i is the initial amount of probiotic added in the preparation process [71].

2.3.6 Water Content

The beads were weighed before and after freeze-drying to estimate their water content. According to Eq.2, the water content was calculated, where m_w is the mass of the wet beads and m_d is the mass of the dry beads. Measurements were performed in triplicate.

$$Water\ \% = \frac{m_w - m_d}{m_w} \times 100 \ldots\ldots Eq.2$$

2.3.7 Density and Volume

The mass and radius of 10 beads were measured carefully by the weighing balance and the microscope. Using the formulas of density and volume of the sphere, the results were calculated. Observations of the size or diameter of the encapsulated beads were done by random selection of the beads.

$$V = \frac{4}{3}\,\pi\, r^3 \ldots\ldots Eq.3$$

Equation 3 is used to calculate the average volume of the spherical beads, where r is the average measured radius.

$$D = \frac{m}{V} \quad \ldots\ldots Eq.4$$

Equation 4 is used to calculate the average density of the beads, where m is the average mass and V is the average volume.

2.4 Treatment of Lulu date pit samples

After performing the experiments and according to the readings mentioned above, encapsulated probiotics with Lulu date pit-based beads showed the second highest viable probiotic cells count after the Naptit Saif values. But, because Lulu dates are the most famous dates used in U.A.E., further test was performed on the Lulu date pit.

20 grams of Lulu date pit were put in 100 ml distilled water and heated to 80°C for 1 hour. Aliquot from the solution was taken for characterization. The Lulu date pit was kept in the distilled water for 24 hours at room temperature and another aliquot from the solution was taken as well. The digestion protocol was done again for the treated lulu date pit and the viable probiotic cells were measured at the end of the experiment.

Chapter 3: Results and Discussions

3.1 Overview of the Main Findings

Three main findings were observed in our work. The released viable probiotic cells after the digestion experiment were the main and critical concern, which is directly related to the physical and chemical properties of the encapsulated date pit based beads. What should be mentioned is that the viable probiotic cells at the end of the experiment of the encapsulated probiotics with date pit-based beads are showing promising values to consider the date pit as a part of the sodium alginate beads solution mixture. The modification that was taken into consideration done to the sodium alginate beads by adding the date pit powder showed positive results as the main findings are showing in details via charts and tables.

The encapsulated date pit sodium alginate beads were characterized and it has been shown in FTIR and DSC tests that the beads confirmed their stability and integrity by not forming new peaks, as well as no peak was removed accordingly. This also confirms to us that our beads are safe to be used, as no chemical reaction has occurred, which means no presence of toxics is confirmed.

3.2 Characterizations of the beads

3.2.1 Chemical analysis of date pit

Moisture, ash, nitrogen, protein, fat and carbohydrate contents of date–pits varied from 7.73-8.93, 1.13–1.33, 0.82–1.12, 5.13-7.03, 6.67-8.17 and 74.34–76.82% respectively, see Table 4. These changes in the chemical compositions are caused by differences in the kind of date palms, the stage of ripening, soil fertility, weather patterns, and other environmental factors.

Table 4: Chemical analysis of date pit powder of 5 varities

Sample	Moisture %	Ash %	Nitrogen %	Protein %	Fat%	Carbohydrate %
Naptit Saif	8.32	1.17	1.01	6.32	6.67	76.52
Lulu	8.93	1.17	0.98	6.16	6.83	75.93
Raziz	8.29	1.13	1.12	7.03	8.08	74.34
Naghal	8.05	1.30	1.11	6.97	6.83	75.73
Khadrawy	7.73	1.33	0.82	5.13	8.17	76.82

It can be shown from our work, that moisture content in the samples actually formed the pattern of probiotic viability count. By taking into account the encapsulated date pit based beads of (1:1) ratio, where the mass of date pit is 0.2 g as well as the mass of sodium alginate in the polymeric mixture. The probiotic viability count from highest to lowest was as the following: Log_{10} 6, 5.8, 5.7, 4.9, and 4.4 CFU/ml representing Lulu, Naptit Saif, Raziz, Naghal, and Khadrawy respectively, which matches the presence value of moisture in the date pit samples with Lulu the highest 8.93% and Khadrawy the lowest with 7.73%. It can be concluded that having moisture in the samples of date pit would protect the viable probiotic and would increase the bacterial count accordingly.

3.2.2 FTIR Analysis

FTIR concentrates on specific bands or groups to distinguish and characterize the samples to accurately identify bonding types, functional groups, and intermolecular interactions. FTIR tests of the optimum samples of beads before digestion were performed, and their spectra peaks are shown below in Figure 30.

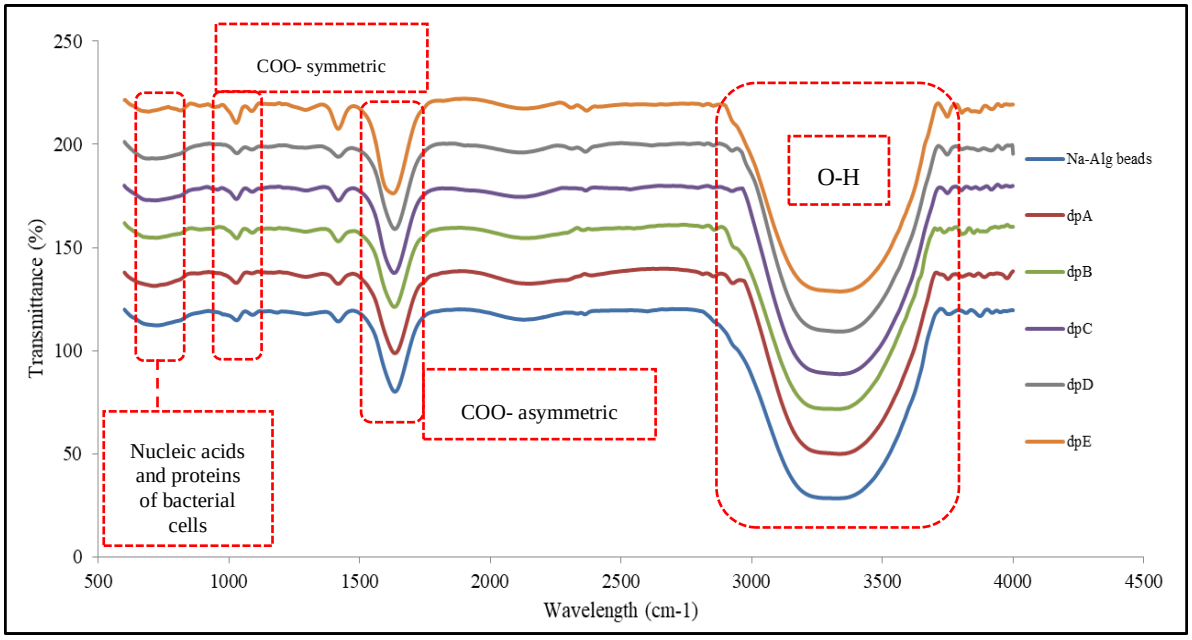

Figure 30: FTIR results of encapsulated date pit based beads. dpA represents Naptit Saif, dpB represented Lulu, dpC represented Raziz, dpD represented Naghal and dpE represented Khadrawy

a. Encapsulated date pit beads

The spectrum can be mainly divided into two areas, summarized in Table 5. The functional group zone, which spans the wavelength range of 4000–1200 cm^{-1}, is where the majority of functional groups exhibit absorption bands. The fingerprint region is the second region, spanning a distance of 1200 to 400 cm^{-1}. The compound's overall characteristics can be found in the fingerprint section [72]. Four main peaks can be observed in the spectra. One broad peak between 3200 and 3300 cm^{-1}, another two peaks are observed between 1500 and 1650 cm^{-1}, a peak between 1000 – 1100 cm^{-1}, and one between 600 and 900 cm^{-1}. The broad band around 3325.64 cm^{-1} can be explained in our samples for the content of water for the formation of hydrogen bonds. It is related to O-H vibrational stretching. That could be intra-, inter-molecular bonding and free hydroxyl groups [73]. The carboxylic [COO-] asymmetric stretching is indicated by the peak that appeared at 1634.86 cm^{-1}. The peak at 1418.39 cm^{-1} is illustrative of the symmetric stretching of carboxylic [COO-] groups in the polymer backbone. The last peak appeared at 725.104 cm^{-1} and corresponded to nucleic acids and proteins of bacterial cells [74].

Table 5: Summary of the FTIR peaks

Peak wavenumber(cm^{-1}) Date Pit	Band origin (assignment) with comments
3320.82	O-H stretching vibrations in hydroxyl groups [75]
2922.59	Asymmetric C-H stretch in methyl and methylene groups [76], [77]
2853.65	Symmetric C-H stretch in methyl and methylene groups, cutin, and waxes [78]
1743.33	C=O, due to either the acetyl and uronic ester groups of hemicelluloses or the ester linkage of carboxylic groups of the ferulic and p-coumaric acids of lignin and/or hemicelluloses [79], [80]
1526.38	C=C stretching of aromatic skeletal mode [81]
1454.55	C=C stretching of aromatic skeletal mode [81]
1369.69	C-H stretch of cellulose [82]
1237.11	C-O-H deformation and C-O stretching of phenolic [83], [84]
1153.22	C-O-C vibration in cellulose and hemicellulose [85]
868.292	C-H rocking vibration of cellulose [86]

b. Treated and untreated date pits

The broad band around 3320.82 cm^{-1} is always taken as hydrogen bonding, and it can be exhibited in aqueous solutions, hydrates, alcohols, and amino and ammonium compounds [70]. Nitrogen or nitrogenous base substances were not revealed earlier in any of the research about the history of date pit powder, which excludes the presence of ammonium and amino compounds. Moreover, the hydroxyl (alcohol) functional group is of intense bands such as 1526.38, 1454.55 and 1369.69, which are due to hydrogen-bonded OH adsorption. The (C–H) stretching vibration is interpreted as the band around 2922.59 cm^{-1} due to the methylene carbon-hydrogen bond [87]. In the absorption range of 1750 – 1700 cm^{-1}, the carbonyl functional group is expected to fall in.

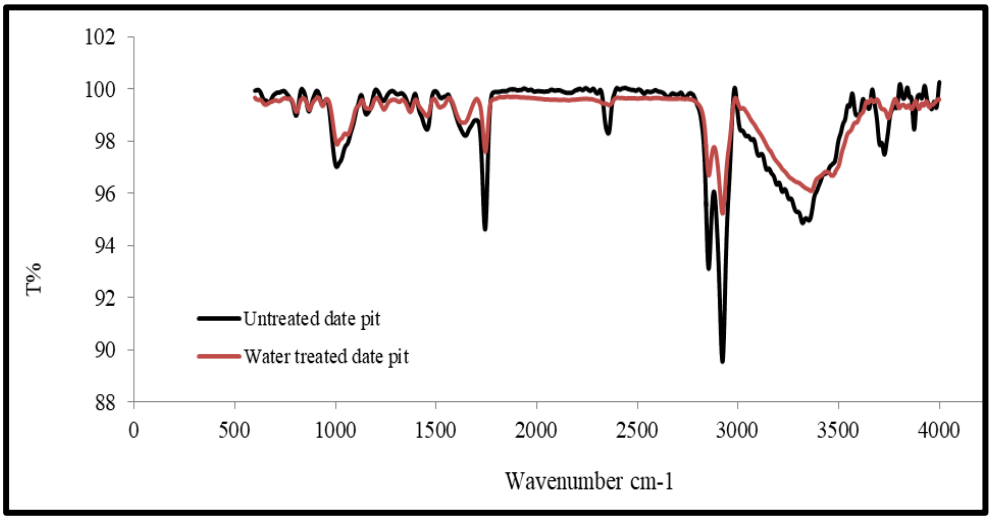

Figure 31: The FTIR peaks of the treated and untreated Lulu date pits

So, the major band found in the figure shown in the region of 1645.46 cm^{-1} is usually the presence of carbonyl compounds such as aldehyde, carboxylic acid, ester and ketone [88]. From prior knowledge, it has been reported that carbonyl absorption may be lowered by 30 – 50 cm^{-1} due to conjunction with an aromatic ring or double-bound or conjugation with another carbonyl group [89]. The bands 1526.38 and 1454.55 cm^{-1} are consistent with aromatics compounds. What can be seen as the main peak observed above in is 1640 cm^{-1}, which represents mainly the C=O functional group. This explains the presence of carbonyl compounds such as aldehyde, ester, carboxylic acid, and ketone. But in this case, the ester is the proper justification; because from the intense peak shown in that region in Figure 31 and the SEM result shown below in

Figure 44, waxes is the part that was extracted when date pit powder was soaked in water overnight. And waxes are what are needed to keep in the date pit powder to protect the survival of the viable bacterial cells during the digestion system protocol.

3.2.3 Thermal properties

3.2.3.1 DSC

The thermal stability of the beads was analyzed using the DSC. The melting point (Tm) was one of the points taken into consideration in this test. Figure 32 below shows the peaks of the sodium alginate control beads encapsulated viable probiotic cells with no loaded date pit powder, before and after the INFOGEST. It is shown clearly that the melting point of the bead after INFOGEST digestion decreased by 5 degrees. Before the simulated digestion was performed, the Tm was 161°C, while after the experiment, this value decreased to 156°C. This can be explained by the effect of the simulated digestive fluids added to samples within the experiment and the changing of pH in each stage; gastric stage pH of 3 and intestinal phase with pH of 7.

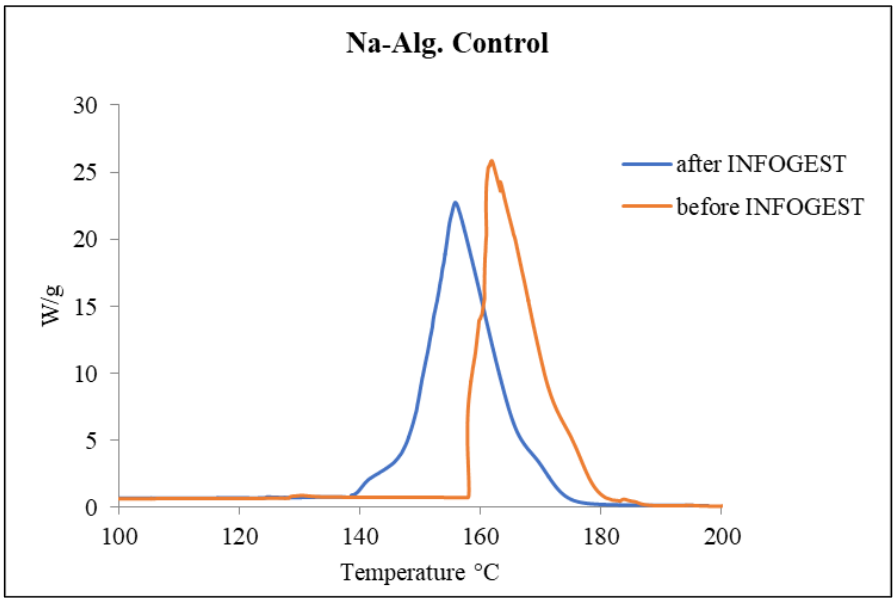

Figure 32: DSC analysis result of the encapsulated sodium alginate control bead before and after INFOGEST

Furthermore, the DSC test was performed to all the optimum samples of beads before the digestion. DSC was measured to detect the possible interactions occurring between sodium alginate, date pit powder, and the probiotic and the stability of the samples by checking the

melting points. Table 6 below shows the melting temperature (Tm) of the formed beads. As it is observed, the addition of date pit powder made a difference in the Tm of the formulated sodium alginate beads. Naptit Saif date pit powder showed the highest melting point temperature compared to all other samples, and this shows that date pit can improve the beads' stability, which is approved with the DSC results of the viable probiotic cells formed in INFOGEST explained above. Based on these results, the Tm of the alginate beads was reported to be 161.0°C, while incorporating Naptit Saif and Naghal date pits into the capsules' solution increased their Tm to 166.3°C and 163.2°C respectively. On the other hand, Lulu date pit and Khadrawy incorporation showed very close Tm to the control sample, as shown below in figure 46. Raziz date pit showed the least Tm compared to the control sample with a Tm of 147.8°C. It could be concluded from these results that incorporating the date pit powder of Naptit Saif and Naghal into the microcapsules improves the thermal stability of the microcapsules in food products.

Table 6: Melting temperature of the loaded encapsulated beads

Sample Code	Tm °C
Na-Alg. Control	161
dpA	166.3
dpB	157.7
dpC	147.8
dpD	163.2
dpE	158.2

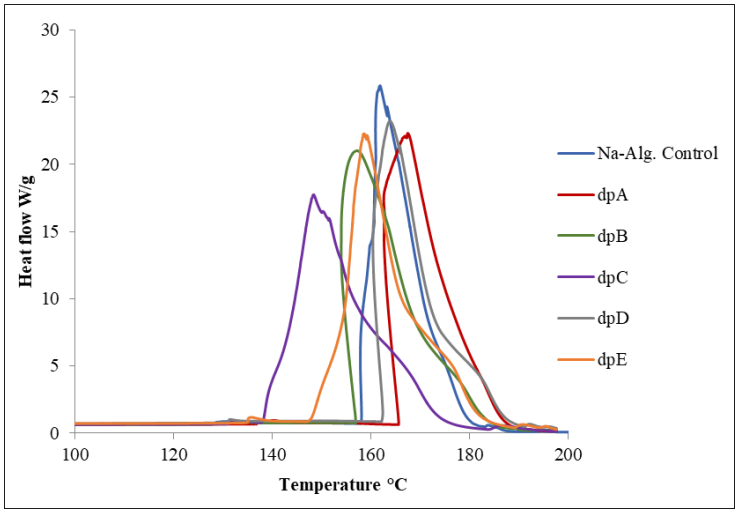

Figure 33: DSC peaks of the loaded encapsulated beads before digestion

Although DSC data show that there are physical interactions, between the polymer and the date pit, as the presence of the date pit shifted the melting point, the FTIR spectra did not reveal the formation of new chemical entities. These results confirm the bead's chemical stability, the permanence of its biological activity, and the possibility of a sustained delivery system profile. Absorption bands showed no prominent interaction between sodium alginate and date pits. Furthermore, neither new peaks appeared, nor existing peaks disappeared in the loaded beads of date pits. So, in conclusion, adding date pit into the formulation of the encapsulated beads is advantageous to our experiment. This is an indication that no date pit alginate interaction occurred in the alginate beads, which confirms the results of the FTIR above.

3.2.3.2 TGA

In accordance with the DSC data, the alginate TG curve shows a first dehydration phase followed by decomposition under nitrogen. Water desorption is what caused the initial weight loss that starts just above room temperature. At 220°C, the encapsulated beads only displayed one degrading step. Similar behavior was seen, and the carbonaceous material produced by the decomposition at about 400°C was identified.

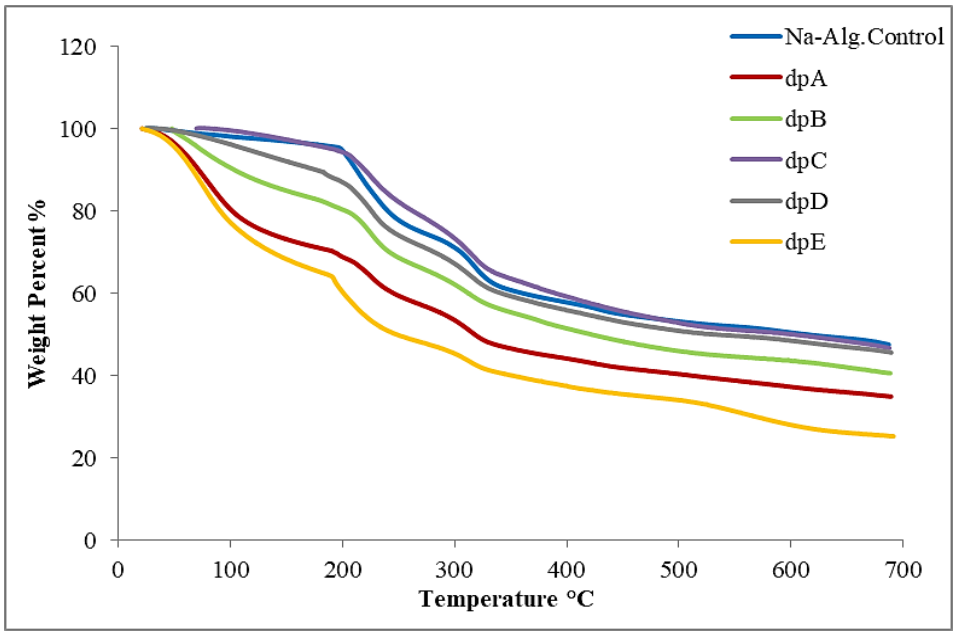

Figure 34: TGA analysis results of the encapsulated beads before digestion

It can be seen in Figure 34 that in the first stage, about 20% of weight loss occurs in the temperature range of 0–100°C, and this happened because of the evaporation of absorbed water present in the beads. In the second stage, polyene formation occurred because of the removal of the side –OH groups when (ca. 40% of mass loss) with a maximum at 250°C is assigned to the alginate polymer by chain-stripping. Lastly, the final phase of weight loss would be explained by the degradation of carbonaceous elements (ca. 50%) at 350°C.

3.2.4 Morphological tests

3.2.4.1 SEM

a. SEM of beads:

Different magnifications of 50x, 250x, 1000x and 2000x were measured for each sample, as shown below in Figure 35. The spherical shape with the porous structure of the bead is observed with a magnification of 50x. It can also be observed the formation of wrinkles after the freeze-drying step, which changed the smooth spherical surface into a rough one. In Figure 35 the shape of S. *thermophilus* cells can be observed clearly that the bacteria are distributed. This type of observation can be confirmed in the micrographs earlier stated [90].

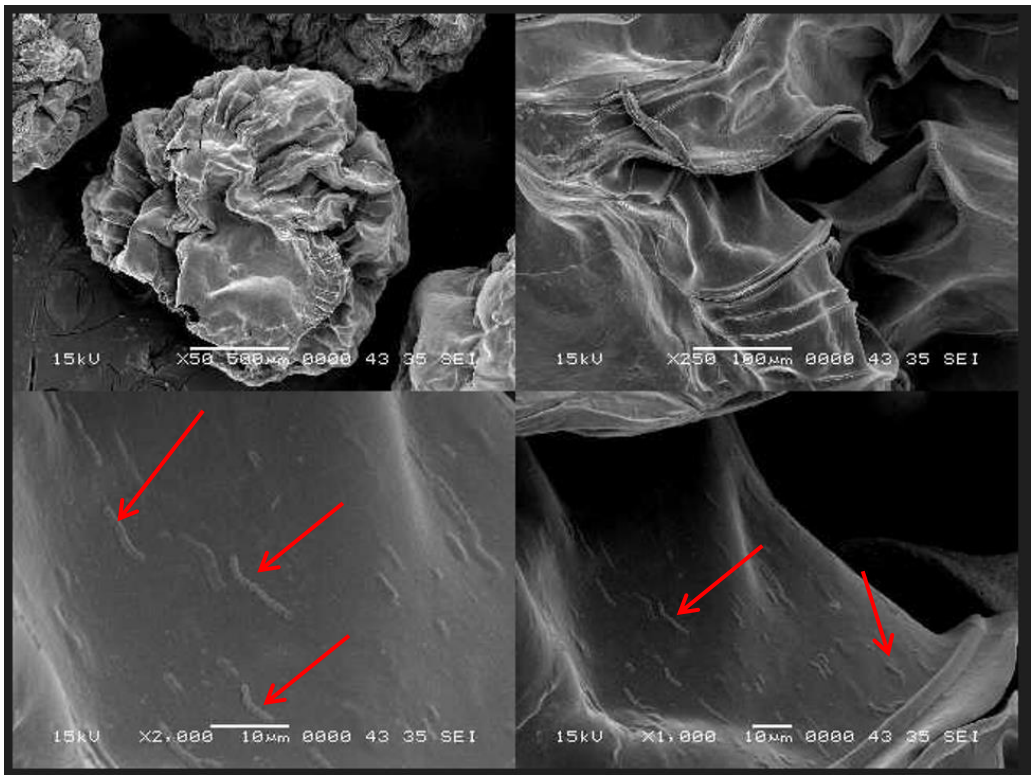

Figure 35: SEM images of control sodium alginate bead before the digestion. Visible bacterial cells included in the polymeric matrix of the beads. Arrows stand out *S. thermophilus* cells within the polymeric matrix.

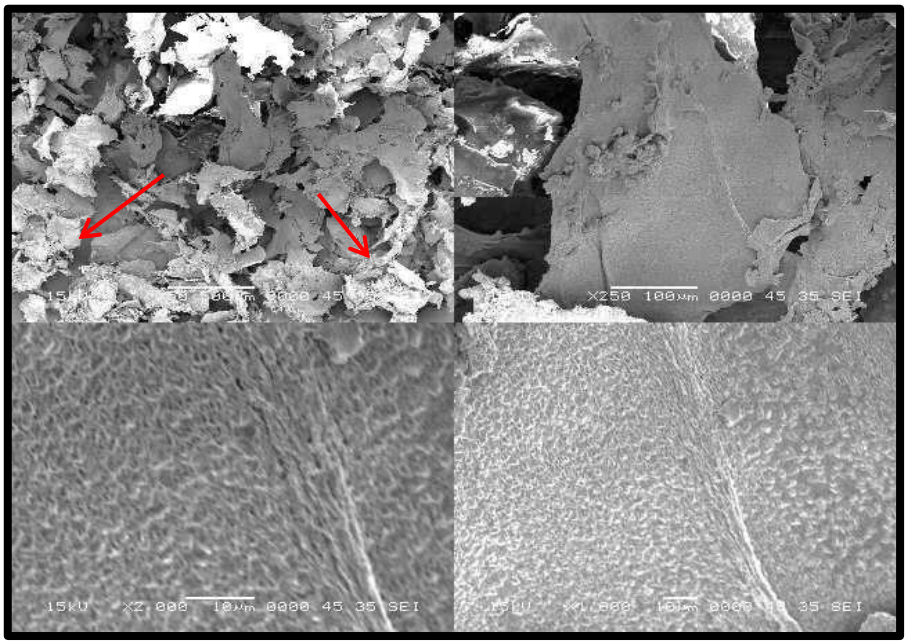

Figure 36: SEM images of control sodium alginate bead after the digestion. Less visible bacterial cells included in the polymeric matrix of the beads. Arrows stand out the digestive salts.

It can be clearly observed that the bead was not only ruptured but also broken. The salts of the digestion system are also shown using the SEM, as in Figure 36 above. What can be seen is the huge difference in the structure of the bead before and after the digestion protocol, which shows how harsh the gastric phase is on the beads, shown below in Figure 37.

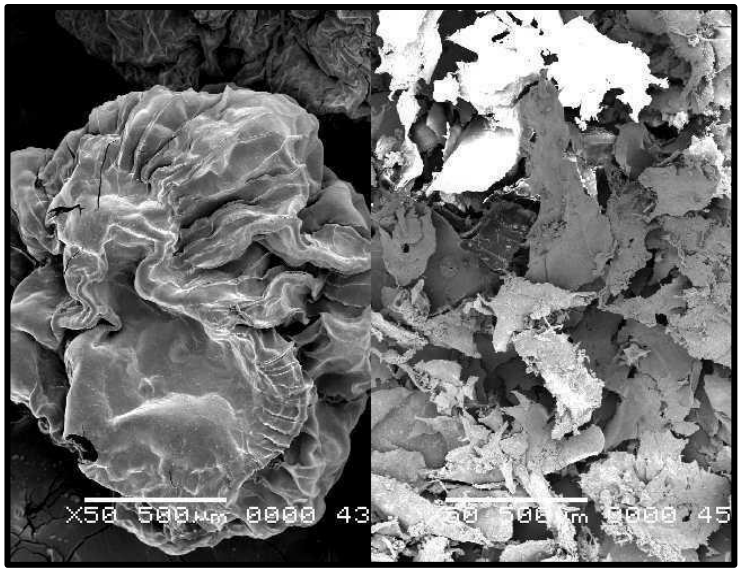

Figure 37: Na-Alg. beads before and after the digestion system protocol

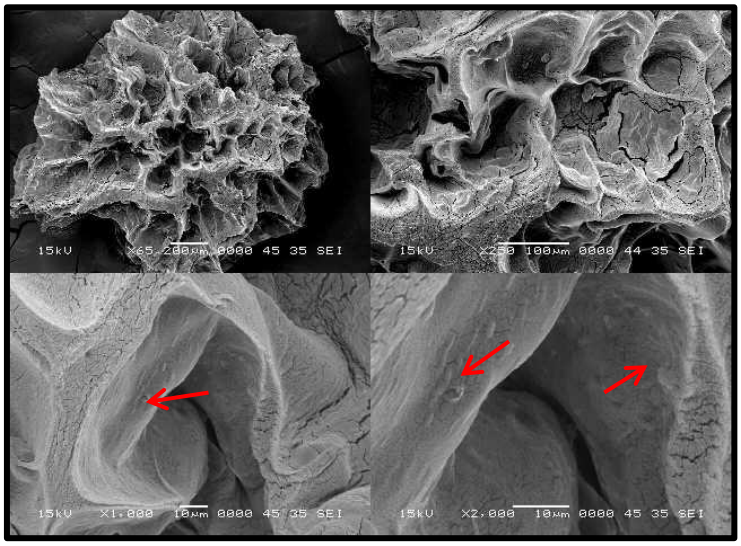

Figure 38: SEM images of Naptit Saif date pit powder bead before the digestion. Arrows stand out *S. thermophilus* cells within the polymeric matrix

It can be seen in Figure 38 that the bead itself is cracked a bit because of the freeze drying, and this is observed in 50x and 250x magnification, while the visible viable bacterial cells are shown at 1000x and 2000x. On the other hand, Figure 39 shows the ruptured bead and less viable bacterial cells in the polymeric matrix. SEM images of Lulu, Raziz, Naghal, and Khadrawy are shown below in Figure 40, 41, 42 and 43 respectively.

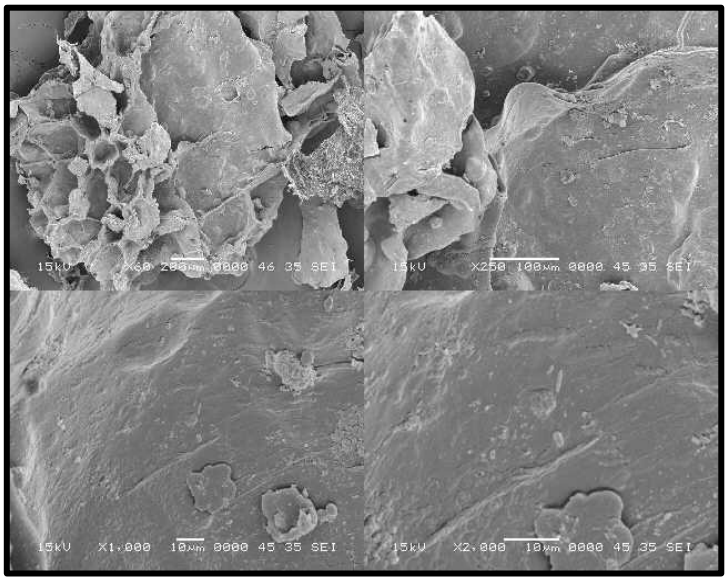

Figure 39: SEM images of Naptit Saif date pit powder bead after the digestion

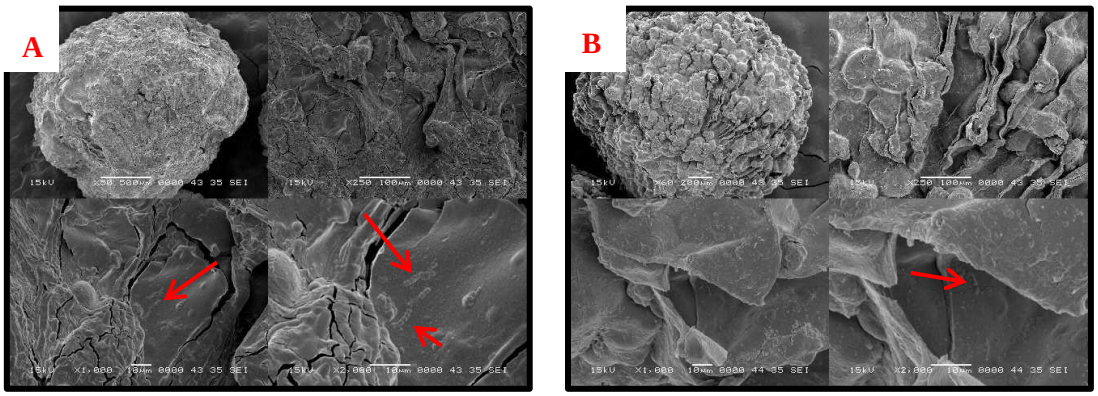

Figure 40: SEM images of Lulu date pit powder bead A: before the digestion, B: after the digestion, arrows stand out *S. thermophilus* cells

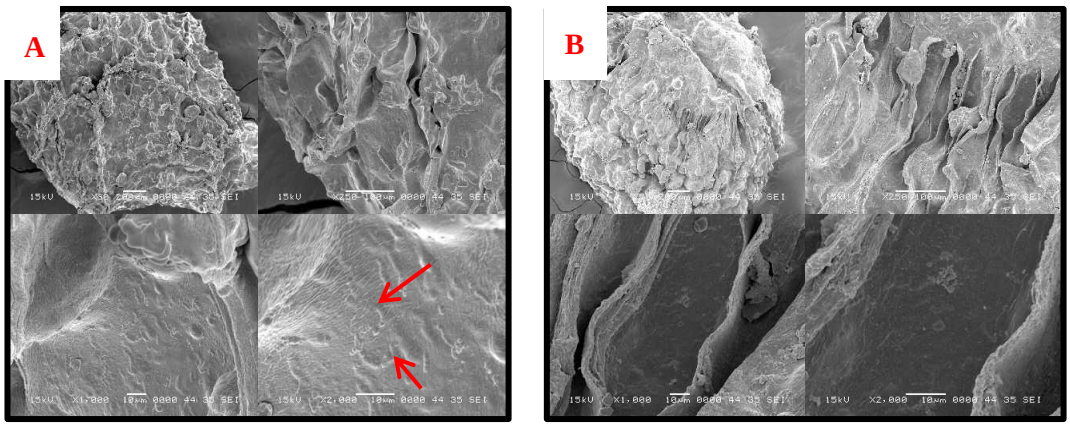

Figure 41: SEM images of Raziz date pit powder bead A: before the digestion. B: after the digestion, arrows stand out *S. thermophilus* cells

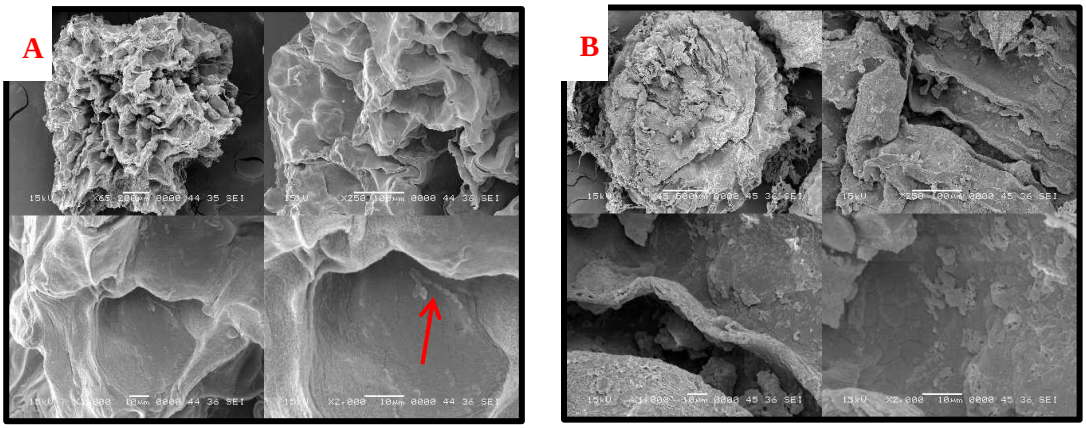

Figure 42: SEM images of Naghal date pit powder bead A: before the digestion. B: after the digestion, arrows stand out *S. thermophilus* cells

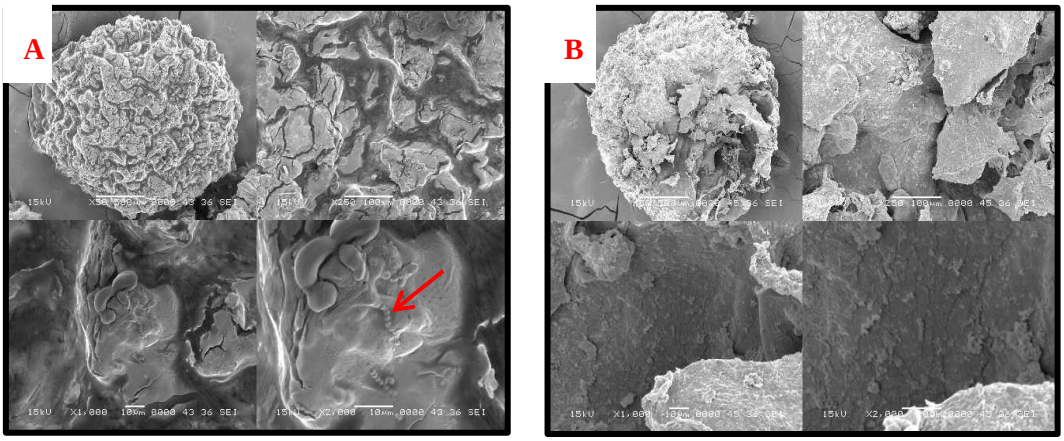

Figure 43: SEM images of Khadrawy date pit powder bead A: before the digestion. B: after the digestion, arrows stand out *S. thermophilus* cells

b. SEM of treated date pit beads:

The viable probiotic cells of the encapsulated beads when the date pit was treated with water were different from the enumerated viable cells when the date pit was raw. A decrease in 2 cycle log value was measured, so FTIR and SEM were performed on the beads to check the differences. In Figure 44, the structures of the treated date pit showed flake structure powder, while the untreated date pit formed a combined-like structure. This layer that bonded the powder together is the main difference observed via SEM. It can be explained as the reason behind the higher viable probiotic count, as this layer protected the bacteria more from the harsh gastric conditions, while the raw powder of date pit has a higher surface area, so the rate of the reaction between the powder, bacteria and the simulated gastric fluid was more, which caused a decrease in the survival bacteria.

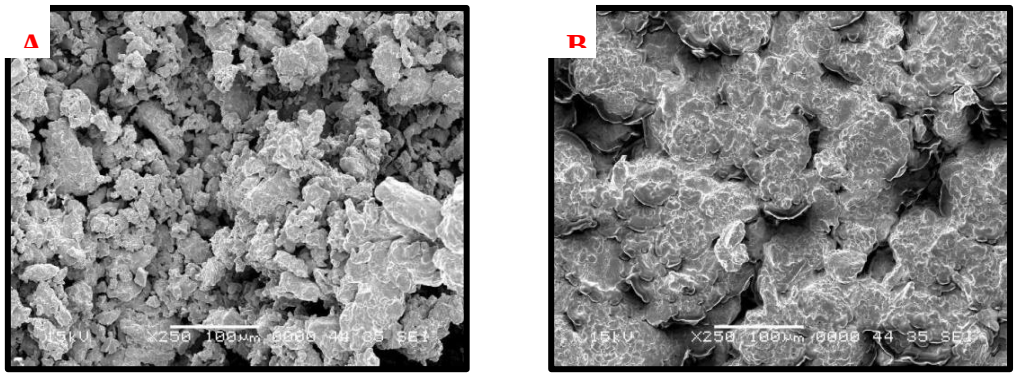

Figure 44: A:SEM of treated date pit powder at x250. B: SEM of untreated date pit powder at x250

3.2.4.2 Microscope

A clear morphology and appearance were observed by the microscope. Figures are shown below.

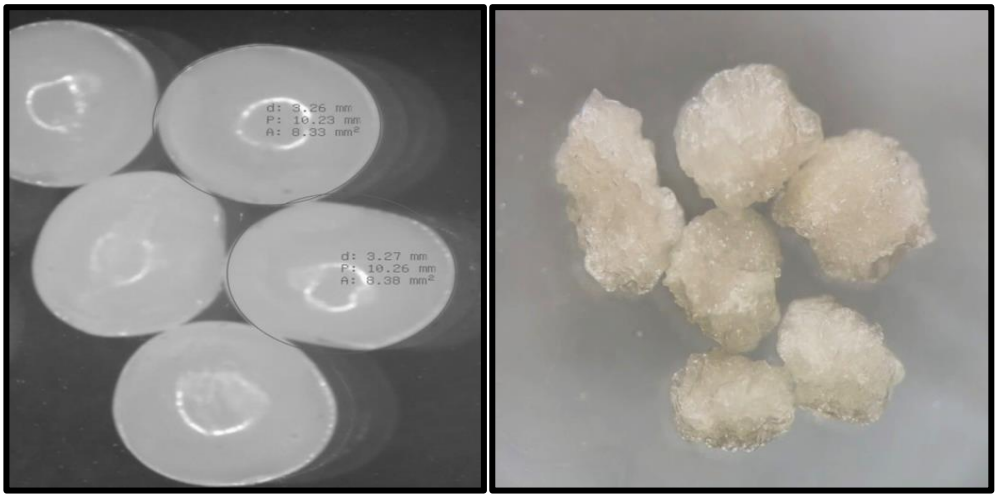

Figure 45: The encapsulated control beads unloaded with date pit powder wet and dried (before and after the freeze drying)

From Figure 45 and Figure 46; it can be seen clearly that the freeze-drying affected the size of the beads and made them smaller. Also, the beads were not smooth anymore; they formed a lot of wrinkles.

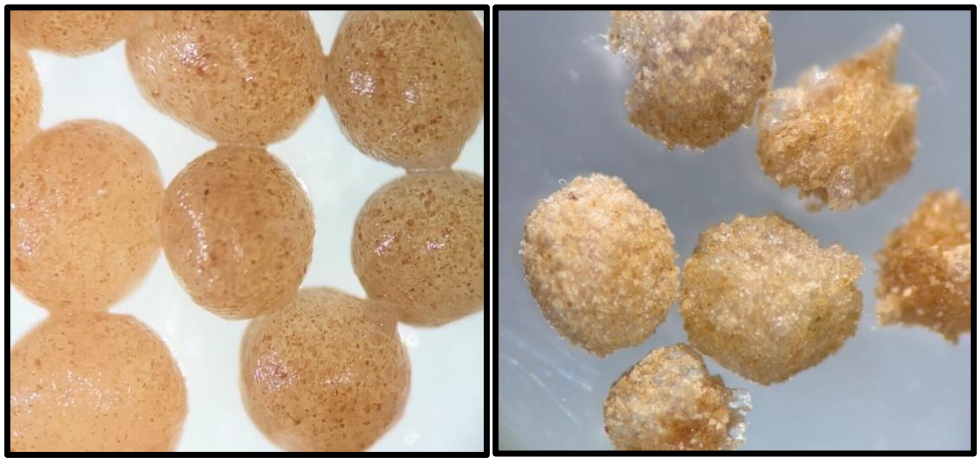

Figure 46: The encapsulated loaded beads with date pit powder; wet and dry

3.3 Viability of the probiotic cells

3.3.1 The probiotic viability in the 5 varieties of date pits

Variations of date pits showed different results, as well as the amount of date pit needed from each type also affected the final results. Table 7 and Figure 47 summarize the main findings that are discussed below in details. First, it was observed that free cells didn't survive after the simulated digestion experiment, which provides strong evidence of the importance of encapsulation technology to protect the probiotic from harsh conditions of the human body, especially the low pH of the gastric phase and the bile salt activity of the intestinal phase. This conclusion actually matches with many previous studies mentioned earlier [45–47]. Another finding in this study was that, using sodium alginate alone as a polymer matrix in encapsulation is not efficient enough to protect the microorganisms compared to mixing the sodium alginate with date pit, especially the Naptit Saif and Lulu date pit. S. Kim et al. [91], concluded that encapsulated *L. acidophilus* in sodium alginate maintained above 10^4 CFU/ml at gastric phase. While in our study, the bacteria encapsulated in sodium alginate with Naptit Saif or Lulu date pit mixture maintained above 10^6 CFU/ml. Furthermore, N.A. Mohammad [92] observed that *L. bulgaricus* encapsulated in alginate and protected in milk microspheres can survive the gastric phase with more than 8 Log_{10} CFU, but the viability decreased in bile salt and completely released after 45 minutes only, as well as another study by Pan et al. [93], when they found that bacterium viability in intestinal phase is less in 1% and 2% bile salt. On the other hand, our encapsulated date pit based beads showed stability and the bacteria viability was encountered at the end of the 2 hours intestinal phase in SIF. What also was observed that, the viability of probiotic in the water treated date pit beads is less than the untreated date pit beads.

Table 7: Viable probiotic cells count summary of the samples

	T1	T2	T3	T4
Free Cell	0			
Na-Alg. Control	4.4			
Naptit saif	6.2	6	5.8	5.3
Lulu	5.1	5.3	6	5.5
Raziz	5.6	5.8	5.7	5.1
Naghal	5.3	5.2	4.9	4.7
Khadrawy	4.2	4.7	4.4	4.3

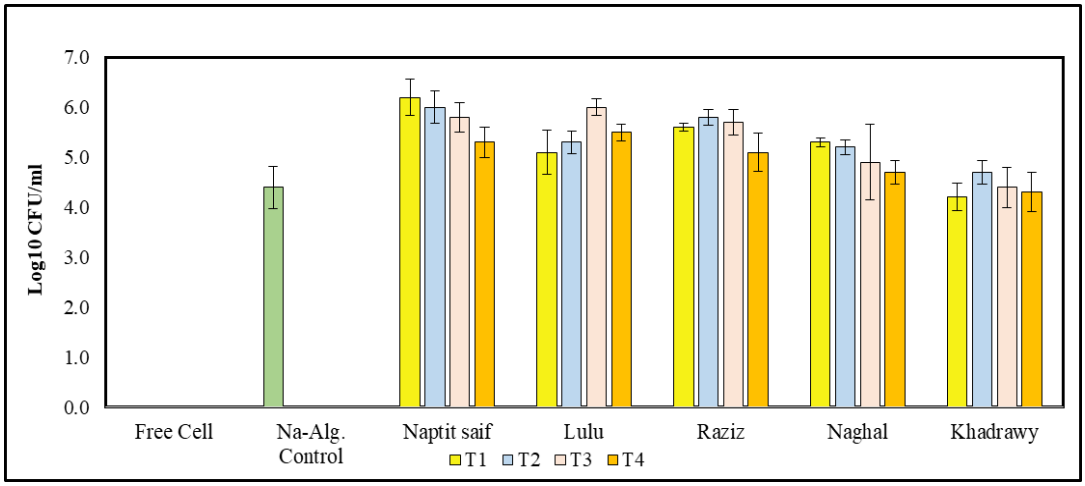

Figure 47: Viable probiotic cells count summary of the samples

The beads that were formed with Naptit Saif date pit showed viable probiotic cell growth of a range from 10^5-10^6 CFU/ml, as shown below in Figure 48 and Table 9. Different concentrations of date pit powder with a constant concentration of sodium alginate (0.2 g) showed different results. T1, as discussed earlier in Table 1 above, contains 0.05 g date pit, while T2 is 0.1 g, followed by T3 with 0.2 g, and finally, T4 is the highest mass with 0.4 g. The relationship between the concentration of date pit and the viable probiotic cells is inversely proportional, where 0.05 g only of date pit showed the highest count of a Log_{10} 6.2 CFU/ml, while 0.4 g of date pit showed viable probiotic cells of Log_{10} 5.3 CFU/ml.

Table 8: Calculations of the viable probiotic cells count of Naptit Saif samples after the simulated digestion

	replicates	Count on dilution factor (-3,-4)		-3	-4	Logs		Average
Free Cell	1	0	0	0	0	0	0.0	0.0
	2	0	0	0	0	0	0.0	0.0
	3	0	0	0	0	0	0.0	0.0
Sodium alginate - Control	1	14	9	14000	90000	4.1	5.0	4.6
	2	12	8	12000	80000	4.1	4.9	4.5
	3	8	5	8000	50000	3.9	4.7	4.3
dpA-T1	1	472	646	472000	6460000	5.7	6.8	6.2
	2	836	260	836000	2600000	5.9	6.4	6.2
	3	1120	222	1120000	2220000	6.0	6.3	6.2
dpA-T2	1	782	296	782000	2960000	5.9	6.5	6.2
	2	504	234	504000	2340000	5.7	6.4	6.0
	3	433	160	433000	1600000	5.6	6.2	5.9
dpA-T3	1	387	164	387000	1640000	5.6	6.2	5.9
	2	332	133	332000	1330000	5.5	6.1	5.8
	3	320	112	320000	1120000	5.5	6.0	5.8
dpA-T4	1	140	40	140000	400000	5.1	5.6	5.4
	2	110	38	110000	380000	5.0	5.6	5.3
	3	80	48	80000	480000	4.9	5.7	5.3

Table 9: Final viable probiotic cells count results of Naptit Saif samples

Sample Code	Log CFU/ml
Free Bacterial Cells	0.0
Na-Alg. control	4.4
dpA-T1	6.2
dpA-T2	6.0
dpA-T3	5.8
dpA-T4	5.3

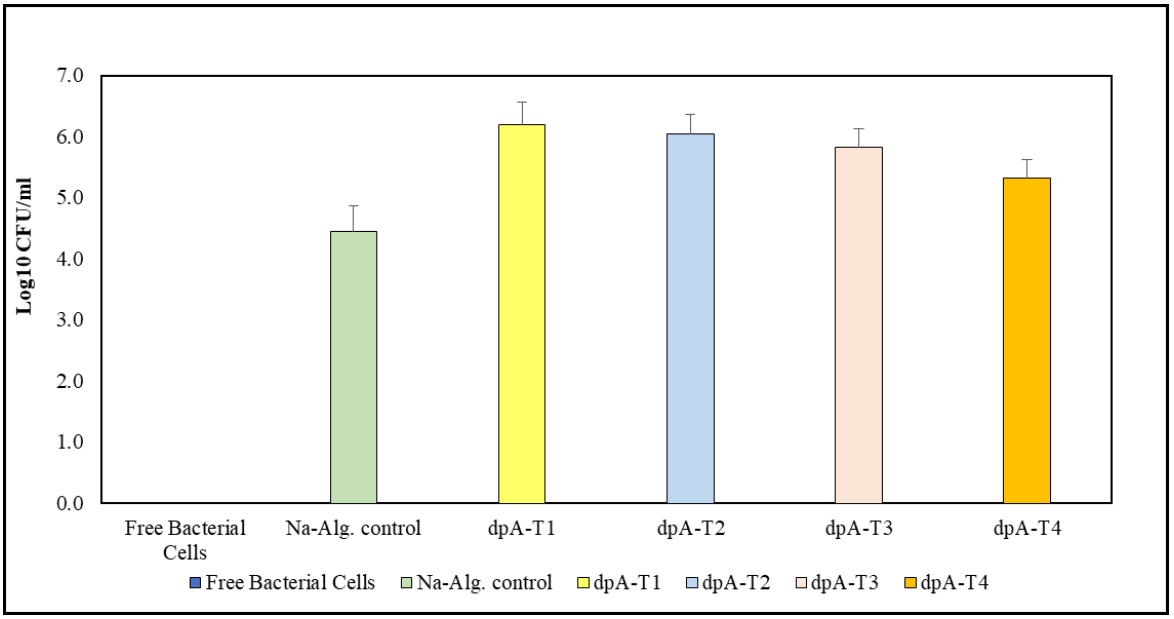

Figure 48: Comparison between the final viable probiotic cells counts of Naptit Saif samples that were formed with different date pit concentrations

The trend of viable probiotic cell count of the beads formed by using the Lulu date pit was different from Naptit Saif. The trend here showed that a ratio of (1:1) concentration of date pit and sodium alginate released the highest viable probiotic cells, 10^6 CFU/ml, as shown below in Table 11 and Figure 49.

Table 10: Calculations of the viable probiotic cells of Lulu samples

	replicates	Count on dilution factor (-3,-4)		-3	-4	Logs		Average
Free Cell	1	0	0	0	0	0	0.0	0
	2	0	0	0	0	0	0.0	0
	3	0	0	0	0	0	0.0	0
Sodium alginate - Control	1	14	9	14000	90000	4.1	5.0	4.6
	2	12	8	12000	80000	4.1	4.9	4.5
	3	8	5	8000	50000	3.9	4.7	4.3
dpB-T1	1	40	30	40000	300000	4.6	5.5	5.0
	2	44	32	44000	320000	4.6	5.5	5.1
	3	48	37	48000	370000	4.7	5.6	5.1
dpB-T2	1	130	36	130000	360000	5.1	5.6	5.3
	2	122	34	122000	340000	5.1	5.5	5.3
	3	134	38	134000	380000	5.1	5.6	5.4
dpB-T3	1	657	126	657000	1260000	5.8	6.1	6.0
	2	578	117	578000	1170000	5.8	6.1	5.9
	3	643	147	643000	1470000	5.8	6.2	6.0
dpB-T4	1	256	56	256000	560000	5.4	5.7	5.6
	2	240	49	240000	490000	5.4	5.7	5.5
	3	220	46	220000	460000	5.3	5.7	5.5

Table 11: Final bacterial count results of Lulu samples

Sample Code	Log CFU/ml
Free Bacterial Cells	0.0
Na-Alg. control	4.4
dpB-T1	5.1
dpB-T2	5.3
dpB-T3	6.0
dpB-T4	5.5

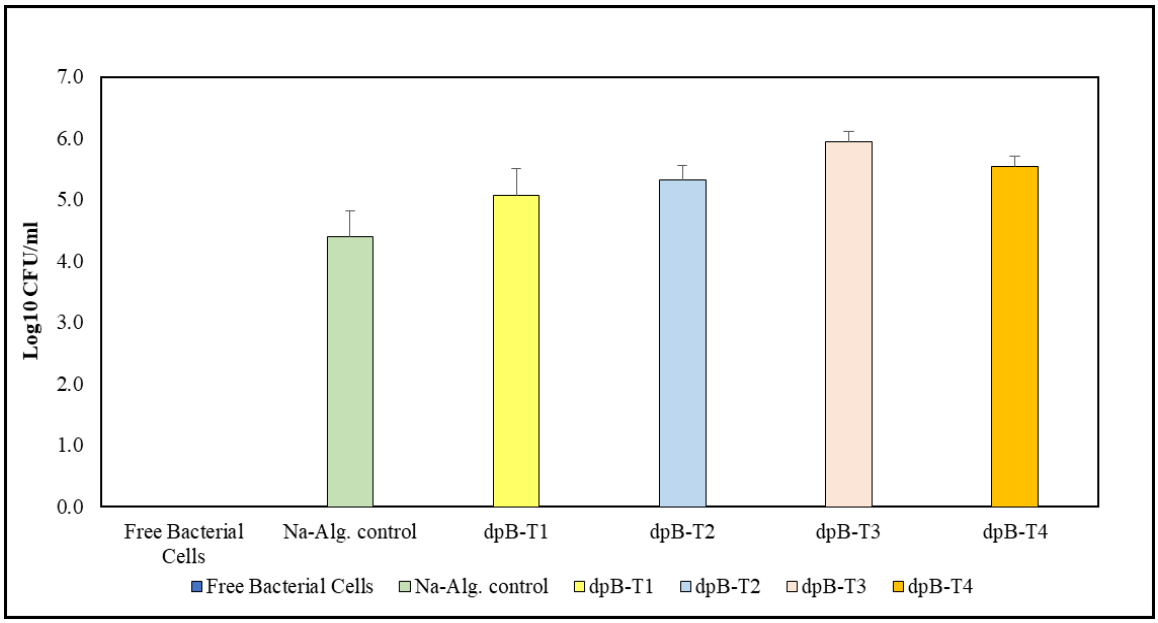

Figure 49: Comparison between the final viable probiotic cells counts of Lulu samples that were formed with different date pit concentrations

Raziz date pit viable probiotic cells were less than Naptit Saif and close to Lulu, as shown below in Table 13 and Figure 50. The range was 10^5-10^6 CFU/ml. The optimum value among the 4 samples was to produce beads with 0.1 g of date pit powder and 0.2 grams of sodium alginate, where it shows a Log_{10} of 6.0 CFU/ml.

Table 12: calculations of the viable probiotic cells count of Raziz samples after the simulated digestion

	replicates	Count on dilution factoer (-3,-4)		-3	-4	Logs		Average
Free Cell	1	0	0	0	0	0	0.0	0.0
	2	0	0	0	0	0	0.0	0.0
	3	0	0	0	0	0	0.0	0.0
Sodium alginate - Control	1	14	9	14000	90000	4.1	5.0	4.6
	2	12	8	12000	80000	4.1	4.9	4.5
	3	8	5	8000	50000	3.9	4.7	4.3
dpC-T1	1	340	52	340000	520000	5.5	5.7	5.6
	2	350	54	350000	540000	5.5	5.7	5.6
	3	365	50	365000	500000	5.6	5.7	5.6
dpC-T2	1	640	120	640000	1200000	5.8	6.1	5.9
	2	670	147	670000	1470000	5.8	6.2	6.0
	3	720	138	720000	1380000	5.9	6.1	6.0
dpC-T3	1	290	95	290000	950000	5.5	6.0	5.7
	2	250	78	250000	780000	5.4	5.9	5.6
	3	280	82	280000	820000	5.4	5.9	5.7
dpC-T4	1	52	26	52000	260000	4.7	5.4	5.1
	2	50	28	50000	280000	4.7	5.4	5.1
	3	46	29	46000	290000	4.7	5.5	5.1

Table 13: Final viable probiotic cells count results of Raziz samples

Sample Code	Log CFU/ml
Free Bacterial Cells	0.0
Na-Alg. control	4.4
dpC-T1	5.6
dpC-T2	6.0
dpC-T3	5.7
dpC-T4	5.1

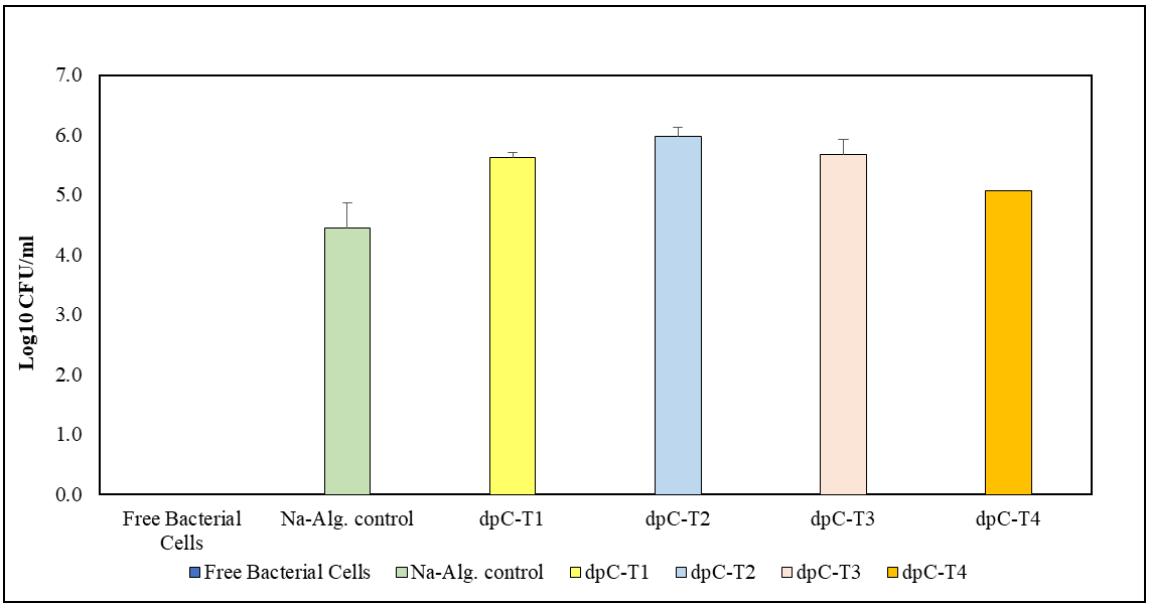

Figure 50: Comparison between the final viable probiotic cells counts of Raziz samples that were formed with different date pit concentrations

Naghal date pit beads also showed a good range of viable probiotic cells 10^4-10^5 CFU/ml, see Figure 51, Table 14 & 15 compared to the Khadrawy date pit with a $\log_{10}$ 4.0 CFU/ml only. Khadrawy showed the least number of released viable probiotic cells as shown below in Table 17 and Figure 52.

Table 14: calculations of the viable probiotic cells count of Naghal samples after the simulated digestion

	replicates	Count on dilution factors (-3,-4)		-3	-4	Logs		Average
Free Cell	1	0	0	0	0	0	0.0	0.0
	2	0	0	0	0	0	0.0	0.0
	3	0	0	0	0	0	0.0	0.0
Sodium alginate - Control	1	14	9	14000	90000	4.1	5.0	4.6
	2	12	8	12000	80000	4.1	4.9	4.5
	3	8	5	8000	50000	3.9	4.7	4.3
dpD-T1	1	170	28	170000	280000	5.2	5.4	5.3
	2	179	27	179000	270000	5.3	5.4	5.3
	3	185	25	185000	250000	5.3	5.4	5.3
dpD-T2	1	123	22	123000	220000	5.1	5.3	5.2
	2	114	26	114000	260000	5.1	5.4	5.2
	3	137	22	137000	220000	5.1	5.3	5.2
dpD-T3	1	13	47	13000	470000	4.1	5.7	4.9
	2	14	37	14000	370000	4.1	5.6	4.9
	3	11	41	11000	410000	4.0	5.6	4.8
dpD-T4	1	35	12	35000	120000	4.5	5.1	4.8
	2	32	7	32000	70000	4.5	4.8	4.7
	3	30	8	30000	80000	4.5	4.9	4.7

Table 15: Final viable probiotic cells count results of Naghal samples

Sample Code	Log CFU/ml
Free Bacterial Cells	0.0
Na-Alg. control	4.4
dpD-T1	5.3
dpD-T2	5.2
dpD-T3	4.9
dpD-T4	4.7

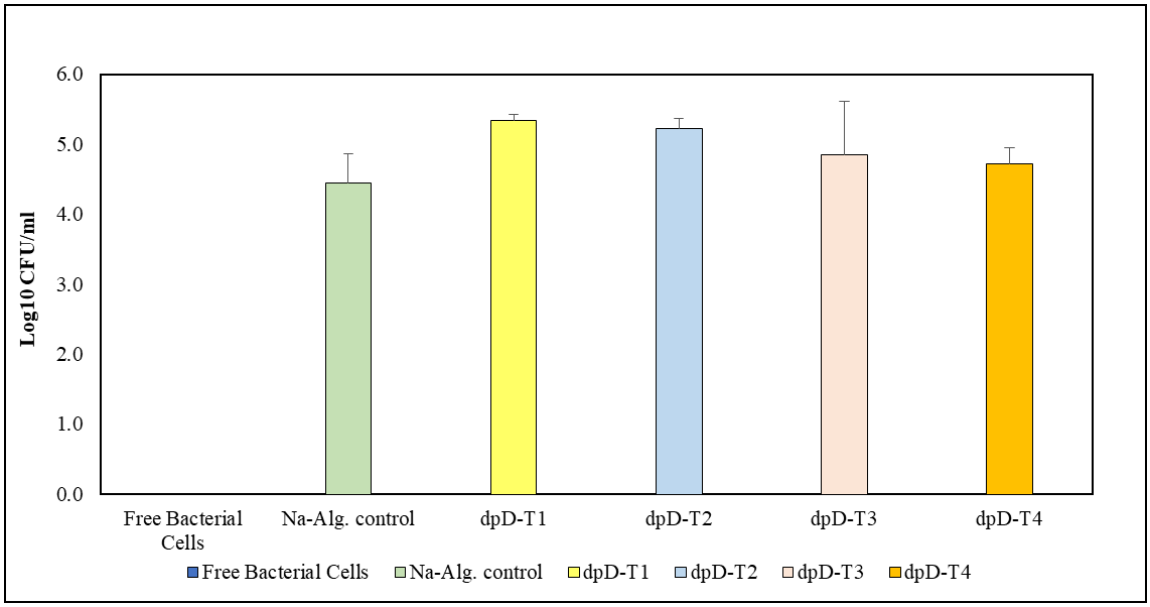

Figure 51: Comparison between the final viable probiotic cells counts of Naghal samples that were formed with different date pit concentrations

Table 16: Calculations of the viable probiotic cells count of Khadrawy samples after the simulated digestion

	replicates	Count on dilution factors (-3,-4)		-3	-4	Logs		Average
Free Cell	1	0	0	0	0	0	0.0	0
	2	0	0	0	0	0	0.0	0
	3	0	0	0	0	0	0.0	0
Sodium alginate - Control	1	14	9	14000	90000	4.1	5.0	4.6
	2	12	8	12000	80000	4.1	4.9	4.5
	3	8	5	8000	50000	3.9	4.7	4.3
dpE-T1	1	9	2	9000	20000	4.0	4.3	4.1
	2	6	2	6000	20000	3.8	4.3	4.0
	3	10	4	10000	40000	4.0	4.6	4.3
dpE-T2	1	22	6	22000	60000	4.3	4.8	4.6
	2	32	10	32000	100000	4.5	5.0	4.8
	3	29	7	29000	70000	4.5	4.8	4.7
dpE-T3	1	10	7	10000	70000	4.0	4.8	4.4
	2	12	6	12000	60000	4.1	4.8	4.4
	3	11	8	11000	80000	4.0	4.9	4.5
dpE-T4	1	9	5	9000	50000	4.0	4.7	4.3
	2	8	4	8000	40000	3.9	4.6	4.3
	3	9	7	9000	70000	4.0	4.8	4.4

Table 17: Final viable probiotic cells count results of Khadrawy samples

Sample Code	Log CFU/ml
Free Bacterial Cells	0.0
Na-Alg. control	4.4
dpE-T1	4.2
dpE-T2	4.7
dpE-T3	4.4
dpE-T4	4.3

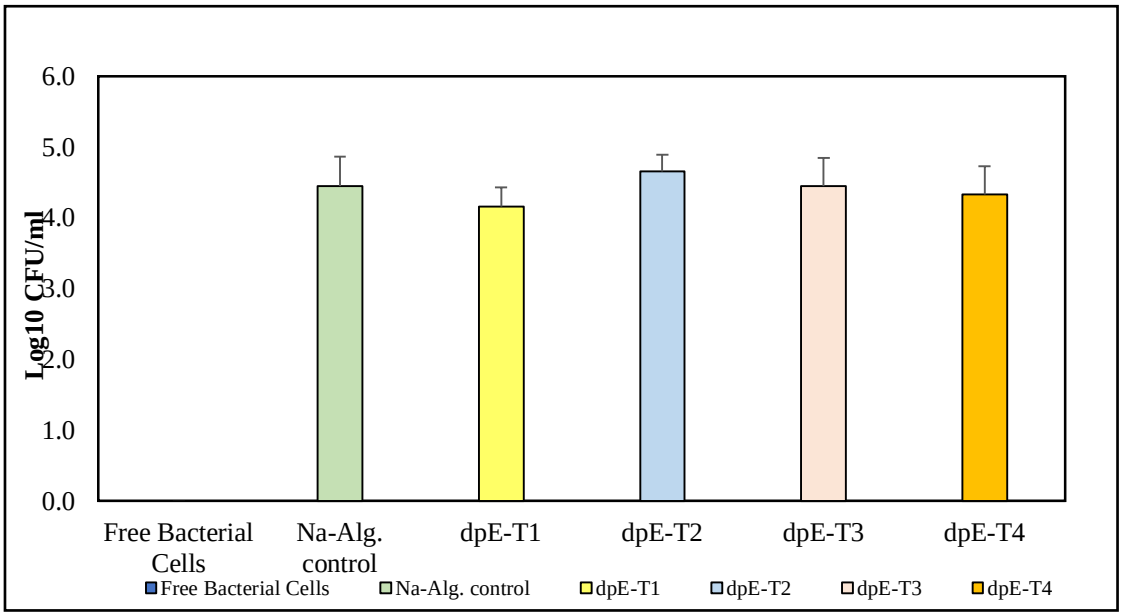

Figure 52: Comparison between the final viable probiotic cells counts of Khadrawy samples that were formed with different date pit concentrations

The encapsulating polymer breaks down in high-pH solutions, resulting in a rapid release of cells from the beads in the intestinal sector [94]. Poor survival rates were seen for cells that were not encapsulated. The findings demonstrated that probiotics are shielded by encapsulation under simulated intestinal conditions [95].

3.3.2 Optimum values of viable probiotic cells in different date pits varieties

In each experiment, it was observed that there was an optimum amount of powdered date pit to add to the polymeric solution mixture for the formation of the beads. The results are shown below in Figure 53. Naptit Saif date pit results showed that 0.05 g powder date pit is enough in a ratio of (0.25:1) of sodium alginate to give the highest value of viable probiotic cells, which equals Log_{10} of 6.2 CFU/ml. Three types of date, Raziz, Naghal, and Khadrawy, showed that 0.1 g for each 0.2 g sodium alginate, which means a ratio of (0.5:1) is the best scenario to follow for the highest viable probiotic cells desired, Log_{10} 5.8, 5.3 and 4.7 CFU/ml respectively. While Lulu date pits showed that equal mass of date pit and sodium alginate in the solution, which was 0.2 g of date pit powder and 0.2g of sodium alginate in the mixture, formed the highest count of bacteria, 10^6 CFU/ml, as shown below in Table 18.

Table 18: Optimum values among the four samples of viable probiotic cells growth in Log_{10} CFU/ml

Date type	Naptit saif, T1	Lulu, T3	Raziz, T2	Khdrawy, T2	Naghal,T2
Bacterial count Log10 CFU/ml	6.2	6	5.8	5.3	4.7

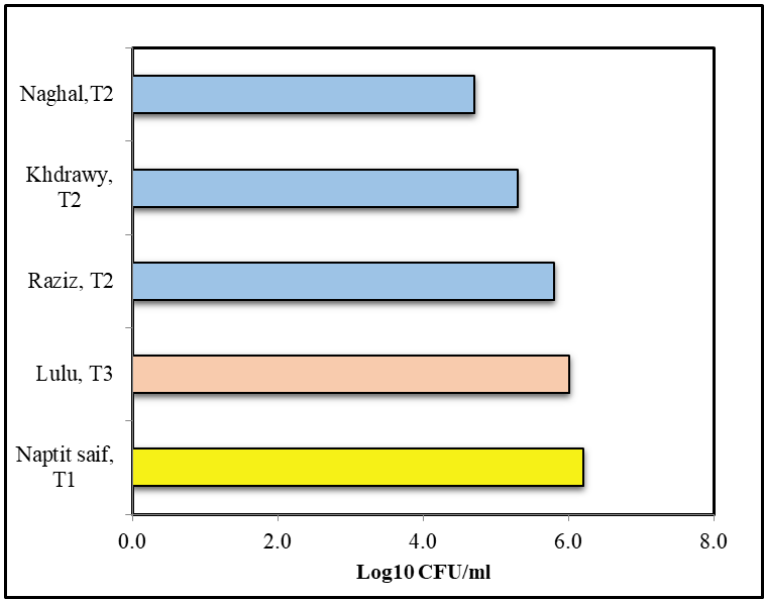

Figure 53: Optimum values of viable probiotic cells growth among the four trials of each date pit sample

3.3.3 Comparison between the treated and untreated date pit

Because Lulu date pit is one of the most famous dates in the U.A.E., furthermore, it showed a high viable probiotic cells count on a ratio of (1:1), a further test was conducted on it. What was observed clearly that water treated date pit powder produced less viable probiotic cells at the end of the INFOGEST digestion experiment compared to the untreated date pit. Results are shown below in Table 19 and Figure 54. According to the FTIR analysis and the SEM, it was shown that a layer of waxes was removed because of the water treatment step, which caused on less survival of the viable probiotic cells. The water treated date pit powder formed a higher surface area and this formed a higher rate of a reaction and higher exposure between the probiotics and the simulated digestive fluids, which caused the death of the viable bacterial cell.

Table 19: Final results of viable probiotic cells count on Lulu treated and untreated date pit

	Treated Dp Log10 CFU/ml	Untreated Dp Log10 CFU/ml
dpB-T1	3.9	5.1
dpB-T2	4.1	5.3
dpB-T3	4.7	6
dpB-T4	4.4	5.5

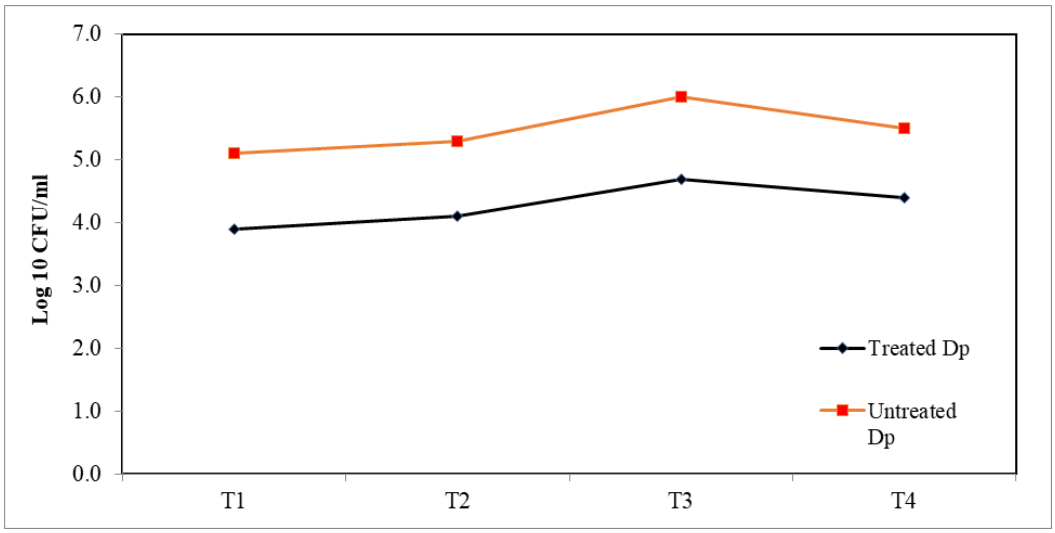

Figure 54: Final results of viable probiotic cells count on Lulu treated and untreated date pit

3.4 Release Efficiency

It was observed that the efficiency of the released probiotic is high, shown below in Table 20 where X_i is the initial loaded value of 8.3 $\log_{10}$ CFU and X_R is the released viable probiotic cells in each optimum sample of the experiment.

Table 20: The efficiency of the released probiotic cells after the digestion ended

Sample	Xi / CFU	XR / CFU	Release Efficiency %
Na-Alg.	0.92	0.64	70.01
dpA	0.92	0.79	86.22
dpB	0.92	0.78	84.67
dpC	0.92	0.76	83.06
dpD	0.92	0.72	78.80
dpE	0.92	0.67	73.13

3.6 Water Content

The beads (ca. 2 g) were weighed before and after freeze-drying as shown below in Table 21 to estimate their water content. According to the below-mentioned Eq.1, the water content was calculated, where m_w is the mass of the wet beads and m_d is the mass of the dry beads. Measurements were performed in triplicate.

$$Water\ \% = \frac{m_w - m_d}{m_w} \times 100\%$$

Table 21: Average water content of the loaded and unloaded encapsulated beads

	Weight of beads		
Sample	before freeze drying / mg	after freeze drying / mg	water content %
Na-Alg.	0.1600	0.0365	77.19
dpA	0.0871	0.0240	72.45
dpB	0.0913	0.0248	72.84
dpC	0.0827	0.0240	70.98
dpD	0.0874	0.0252	71.17
dpE	0.0828	0.0236	71.50

3.7 Density and Volume

The mass and radius of 10 encapsulated date pit beads were measured carefully by the PR series explored analytically by Ohaus Carl Zeiss microscope. By using the following formulas, the density and volume of the beads were calculated.

$$V = \frac{4}{3}\,\pi\, r^3$$

$$D = \frac{m}{V}$$

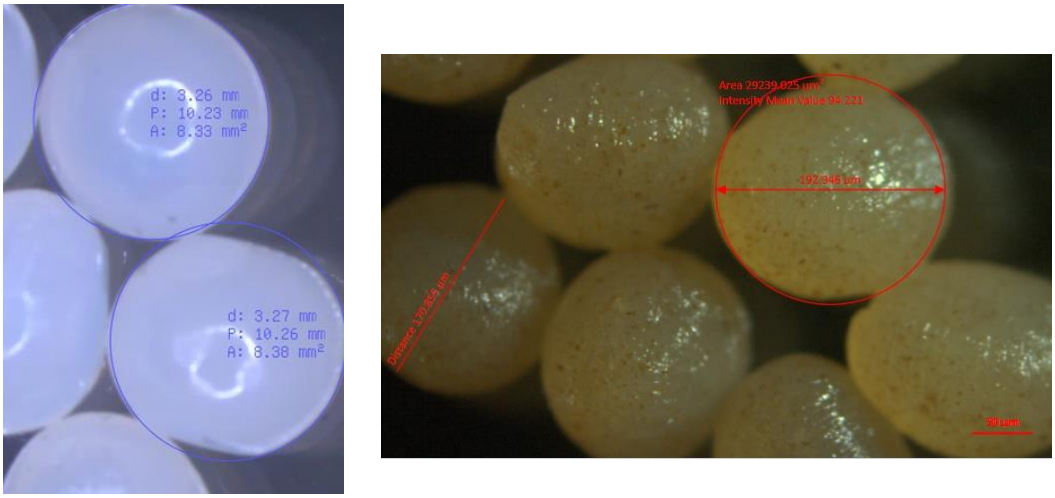

Figure 55: The diameter measurement of samples to calculate the volume and density

3.7.1 Volume and density of unloaded date pit encapsulated beads

The average radius measured of the beads was 1.55 mm as shown above in Figure 55, and the average mass was 0.15 mg, accordingly the average volume of the beads is:

$$V = \frac{4}{3}\,\pi\, 1.55^3 = 15.60\ mm^3$$

Furthermore, the average density of the beads is

$$D = \frac{0.15}{15.60} = 9.6\ x\ 10^{-3}\ \ mg/mm^3$$

3.7.2 Volume and density of date pit loaded encapsulated beads:

The average radius measured of the loaded encapsulated beads was 1.38 mm and the average mass was 0.088 mg, accordingly the average volume of the beads is:

$$V = \frac{4}{3}\ \pi\ 1.38^3 = 11.00\ \text{mm}^3$$

Furthermore, the average density of the loaded beads is

$$D = \frac{0.088}{11} = 8\ x\ 10^{-3}\ mg/mm^3$$

Chapter 4: Conclusion

Microencapsulation is one of the most effective techniques to enhance the stability and viability of live probiotic strains from the gastrointestinal environment. The results of this study showed that some types of date pits were capable and suitable candidates to be used in the formulation of sodium alginate beads to encapsulate probiotics. *Streptococcus thermophilus* were successfully encapsulated in date pit-based beads by the gravimetrical method. The viable cells of encapsulated *Streptococcus thermophilus* in beads showed better survival ability than that of free cells in simulated gastrointestinal conditions. Encapsulation has proved to be a good method to protect probiotics in gastrointestinal environments. A variety of date pit powders showed different results, and for each formula, the probiotics' viability was evaluated to find the most potent combination. It was concluded that the encapsulated probiotics with date pit powder in the matrix of sodium alginate solution have been released and survived after the gastrointestinal conditions more than encapsulating them without date pit powder. So, date pit powder beads show the potential as a new delivery carrier for the oral administration of probiotics. These beads can meet commercialization as an economically inexpensive source of the material used and hence can be used in functional food applications. The beads were evaluated for particle size, density, morphology, thermal stability, water content. Beads were also characterized by FTIR, DSC, and SEM, the results of which indicated the successful development of encapsulated probiotics in sodium alginate beads with date pit powder.

The possibility of using date pits in medicine manufacture will help to start a new market for what is known to be a waste in huge amounts or low-value products in our countries. Therefore, from an economic perspective, the accessibility to this powder of date pit at a very low cost makes the production of these beads economically practical. Consequently, sodium alginate beads mixed with date pit display superior characteristics in term of delivery carrier for oral administration of probiotics. Accordingly, the future work can be extended and the following can be implemented: Using different biopolymers in the mixture of the beads that can produce beads with even better physical and chemical properties. Furthermore, implementing further treatments to date pits before utilizing them in the formation of beads, which can show exact chemical composition needed for optimum beads and bacterial viability. Producing beads with different types of probiotics that can benefit the human body more than the *Streptococcus thermophilus* used in this project.

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This work deals with the medicinal use of date pit powder, which turns it from a waste product to a useful stabilizer for the release of beneficial probiotics in the human intestine. The main objective of this work is to encapsulate probiotics with date pit beads and their subsequent release in the human intestine. The beads were formed using gravitational dripping technique, while INFOGEST protocol was used to test the digested beads and the release of viable probiotic cells.

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