Survival enhancement of probiotic bacteria by encapsulation with chitosan succinate nano-particles and evaluation of survival in simulated gastrointestinal conditions

Najla Ali

A Thesis Submitted in Partial Fulfillment of Requirements for
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ABSTRACT

The present study describes chitosan succinate (as nano particals) to protect *Bifidobacterium lactis* Bb-12 from simulated gastric conditions (SGC) . Chitosan succinate polymer was synthesized and emulsification to prepare microspheres. Besides, the microspheres of *Bifidobacterium lactis* BB-12 and chitosan succinate particle size were determined by scanning electron microscopy, FT-IR spectroscopy.

The characteristic and the results of absorption peaks of chitosan succinate microspheres by used IR spectra was 1495 cm^{-1} and degree of electrostatic repulsion of chitosan succinate particle in the microspheres was -33 m using Zeta potential measurement, and chitosan succinate microspheres had a net negative charge. Moreover, that the size of chitosan succinate microspheres was chitosan succinate microspheres 482.8 nm. The width diameter of free cells was 2.144 µm (2144 nm), while the width diameter encapsulation of the microspher with and without Genipin were 2.925 µm (2925 nm) and 2.212 µm (2212nm), respectively were determined by Zeta potential

In vitro release chitosan succinate microspheres with Genipin croos-link were had protect effect of the degradation from gastric enzymes than Nanoencapsulation cell (NEC) without Genipin. The encapsulated Bb-12 was significant highest cells survival in simulated intestinal juice in pH 8 and simulated colon juice in pH 7.4, while the encapsulated BB-12 had low number of survival was released in simulated gastric juice (pH 2.0).

pH can be effect of release matrix form in chitosan succinate microsphere. Chitosan succinate microsphere cross-link with Genipin had significanly high level of survival which was observed under pH 7.4 (Colon) However, this can be resist in Gasteric conditions than chitosan succinate microsphere no cross-link with Genipin.

The stability of chitosan succinate cross-linked with Genipin was observed at a high stability in the acidic environment of simulated gastric juice with absent of pepsin enzyme ($2.60\pm3.60\times10^{5}$a in initial time of incubation). Furthermore, the simulated gastric juice with pepsin enzyme had reduced stability significantly ($3.20\pm2.50\times10^{3}$a after 90 min of incubation). The stability of chitosan succinate without cross-linked with genipin observed that there was no significant effect ($4.21\pm2.20\times10^{4}$d and $4.90\pm4.72\times10^{4}$a), of simulated gastric juice with or without

pepsin enzyme. While, the survival of free cell was reduced to <10 cfu/ml after 30 min of incubation gastric juice without pepsin enzyme and to $3.44\pm2.70\times10^{2}$c after 120 min of incubation in gastric juice with pepsin.

Genipin under concentration 2.5 mM cross-link might be enhance the resistance of chitosan succinate to degradation by pepsin enzyme for controlled release of probiotic cells. According to these results the new carrier system described can be a promising tool probiotic delivery from oral to colon.

Recently, foods with functional properties are needed. Tomato ketchup has high contents of total sugar and consumed in many fast food. The objective of this study was to monitor the growth and survival of probiotic bacteria in *Bifidobacetrium lactis* Bb-12 strain in local tomato ketchup. The NEC *Bifidobacetrium lactis* Bb-12 count increased gradually in tomato Ketchup up to 2 days of incubation. While, the count of free cell was decreased after 90 min of incubation.

However, the count of NEC of Bb-12 was increased significantly after initial time to tow days and then decreased to not count after 7 days of incubation time. While, the survival of free cell was increased until 90 min then decreased to <10 cfu/ml after 2 days of incubation in tomato ketchup. However, an inverse relationship was observed between the time of incubation and the survival rate of free Bb-12 cells. The study results showed a good protection activity of NEC of Bb-12 for rigors tomato Ketchup for 2 days.

Keyword : Bifidobacterium lactis Bb-12 Nanoencapsulation Techniques Nanoemulsions Nanoparticals

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Father… I’m sure you are proud of your daughter.

Najla Ali

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CHAPTER 1

INTRODUCTION

Background and significance of study

Probiotics bacteria such as *Bifidobacterium* spp, *Lactobacillus acidophilus* are being developed for these micoroorganisms in range of food associated with numerous health benefits, for examples: lowering cholesterol, improving lactose tolerance and preventing some cancers. Many experiments and reports find that there is a poor survival of probiotics in different products. Ecapsulation technique can help achieving longer shelf life of the product. The main reason to encapsulate bacteria is to enhance their survival during production and storage of delivery food and to protect them from the physical and chemical rigors of digestive tract.

There are also number of technologies that can be used to increase the survival of probiotics in products and digestive tract, including microenchapsulation and nanoenchapsulation.

Nanotechnology the world 'nano' 'dwarf' in Greek language refers to dimensions on the order of magnitude of 10^{-9}. (Nanotechnology is the dimensions of matter are understanding and control by roughly 1-100 nm). Optical effects, electrical conductance, magnetism, physical strength and chemical reactivity are the results of small size of nanomaterials (Muñoz et al., 2007); (Uskoković, 2007).

In biological systems, the first level of organization occurs at the nanoscale structure where all the fundamental properties and functions are systematically defined (Dutta et al., 2010). Some advances of synthesis and characterization of nanoscale materials are: nanoparticles, nanowires, nanotubes and nanofibers which are new applications and science.

Two major applications of nanotechnology in food safety, first, pathogens and toxins in food products are detected by using sensitive biosensors, second, antimicrobials are

used nanomaterials to enhance stability, activity and protection food (Doyle 2006; ENG 2006; IFST 2006).

Nanoencapsulation is a technology to deliver substances in miniature making use of techniques such as nanostructuration, nanocomposite and nanoemulsification. It will keep the final product functional which is expected to be maintained during storage and controlled release of the core. In the food engineering field, this technique is used for the production of functional foods with enhanced functionality and stability protection of bioactive compounds such as: carbohydrates, proteins, lipids, vitamins and antioxidants as well as benefits bacteria. Also, the main objective of nanoparticles is used in supramolecular assemblies that are critical to living cells in order to improve the quality of human health.

Types of nanoparticles

Recently Nanoparticles NPs are a type of colloidal drug delivery system comprising particles with a size range from 10 to 1000 nm in diameter. NPs may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials (Buzea et al., 2007). The advantages of nanoparticles idea are: first to improved bioavailability by enhancing aqueous solubility, second increasing resistance time in the body (increasing half-life for clearance/increasing specificity for its cognate receptors and the third advantage is, to target drug to specific location in the body (its site of action). This results in concomitant reduction in quantity of the drug required and dosage toxicity, enabling the safe delivery of toxic therapeutic drugs and protection of non-target tissues and cells from severe side effects (Irving et al., 2007). It is increasingly used in different applications, including drug carrier systems and to pass organ barriers such as the blood-brain barrier, cell membrane (Madhumathi et al., 2010). They are based on biocompatible lipid and provide sustained effect by either diffusion or dissolution (Goldstein et al., 1995); (Müller et al., 2000); (Parsa et al., 1999); (zur Mühlen et al., 1998).

Extensive libraries of nanoparticles, composed of an assortment of different sizes, shapes, and materials, and with various chemical and surface properties, have already been constructed. The field of nanotechnology is under constant and rapid growth and new additions continue to supplement these libraries. The classes of nanparticles

below is as developed to uses in nanoencapsulated of *Bifidobacterium lactis* Bb-12 are described here in this study.

Chitosan nanoparticles

Chitosan nanoparticles have been developed to encapsulated proteins such as bovine serum albumin, tetanus and diphtheria toxoid , vaccines , anticancer agents , insulin, and nucleic acids.The methods proposed to prepare chitosan nanoparticles are based on the spontaneous formation of complexes between chitosan and polyanions or the gelation of a hitosan solution dispersed in an oil emulsion . Various methods for producing chitosan nanoparticles are described in the literature.

Chitosan nanoparticles obtained by formation of a spontaneous complex between chitosan and polyanions such as tripolyphosphate and organic compound, which were in small diameters (200–500 nm) and showed a quasi spherical shape under transmission electron microscopy. Chitosan nanoparticles produced by a promoting gelation in an emulsification-based method , results in a diameter of 400 nm. This technique has a major disadvantage of involving organic solvents during the isolation of the particles; these are difficult to remove and may cause toxicity.

Recently, scientists investigated that, Polymer nanoparticles from biodegradable and biocompatible polymers are good candidates for drug carrier to deliver drugs, because they are expected to be adsorbed in an intact form in the gastrointestinal tract after oral administration (Florence et al., 1995).

Chitosan (CS) is the second abundant polysaccharideand a cationic polyelectrolyte presents in nature. CS has shown favorable biocompatibility characteris-tics (Knapczyk, 1993); (Miki et al., 1989), (Hirano et al., 1990) as well as the ability to increase membrane permeability, both in vitro ((Katz & Aspden, 1997); (Lehr et al., 1992); (Dumitriu & Chornet, 1998) and in vivo (Kaneko et al., 1996), and degraded by lysozyme in serum.

Probiotics bacteria and nanoparticles

In the beginning, there has been an explosion of probiotics bacteria health-based products. Many reports indicated that there is a poor survival of probiotics bacteria in these products. So the survival of these bacteria in the human gastro-

intestinal system is questionable. Providing probiotic living cells with a physical barrier against adverse environmental conditions is therefore an approach currently receiving considerable interest. The technology of nano-microencapsulation of probiotic bacterial cells evolved from the immobilised cell culture technology used in the biotechnological industry. Several methods of nano- microencapsulation of probiotic bacteria have been reported which includes spray drying, extrusion, emulsion and phase separation. None of these reported methods however, had resulted in large numbers of shelfstable viable probiotic bacterial cells necessary for use in industry for development of new probiotic products. The most commonly reported nano-microencapsulation procedure is based on the calciumalginate gel capsule formation. Kappa-carrageenan, gellan gum, gelatin, starch and chitosan are also used as excipients for the nano-microencapsulation of probiotic bacteria. The currently available equipment for nano-microencapsulation is not able to generate large quantities of uniform sized micro or nano capsules. There is a need to design and develop equipment that will be able to generate precise and uniform micro or nano capsules in large quantities for industrial applications. The reported food vehicles for delivery of encapsulated probiotics bacteria are yoghurt, cheese, ice cream and mayonnaise. The number of probiotic supplements will increase in the future. More studies, however, need to be conducted on the efficacy of nano-microencapsulation to deliver probiotics bacteria and their controlled or targeted release in the gastrointestinal tract.

Release in the gut

The mechanical of drug release from nanoparticle capsules in the gut is coated by polymer, and releases the drug by controlled diffusion or erosion from the core across the polymeric membrane or matrix.

The mechanism of release in the gut is affected by several conditions one of those conditions is: the strong peristaltic waves in the colon. The other mechanisms are to use the pH variations, digestive enzymes and the time of transport in the gastrointestinal tract. The third, and most promising method according to the present authors, is to take advantage of the precise, local activity of enzyme-systems produced by the microbiota (Kinget et al., 1998); (Kosaraju, 2005); (Shantha et al., 1995).

In general, drug release rate depends on (1) solubility of drug, (2) desorption of the surface bound/ adsorbed drug, (3) drug diffusion through the nanoparticle matrix, (4) nanoparticle matrix erosion/degradation and (5) combination of erosion/diffusion process (Mohanraj & Chen, 2006). Thus solubility, diffusion and biodegradation of the matrix materials govern the release process. So to develop a successful nanoparticulate system, both drug release and polymer biodegradation are important consideration factors.

Objectives

The objectives of this study are to:

1. To encapsulate of probiotics bacteria with nanoparticles to enhance their survival during shelf life of delivery food and to protect them from the physical and chemical rigors of digestive gut
2. To evaluative the survival probiotics bacteria with nanoparticles in simulated gastrointestinal conditions.

Expected outcome

Obtain the knowledge of ability effect of chitosan succinate microsphere cross-link with Genipin to protect *Bifidobacterium lactis* Bb-12 from the physical and chemical rigors of digestive gut

Obtain the knowledge of ability effect of chitosan succinate microsphere cross-link with Genipin to protect *Bifidobacterium lactis* Bb-12 from the physical and chemical rigors tomato ketchup as food products.

Hypothesis of research

The survival of *Bifidobacterium lactis* Bb-12 ability effect of chitosan succinate microsphere cross-link with Genipin to protect cell of SGJ with pepsin, without pepsin and delivered pass through gastric tract and delivered to the specific receptors in colon. The number of samples of each method was 5 times replicate and the period of experiments was six months which started from first of Jun 2013 to end of December 2013.

Scope of the research

Determine survival of encapsulate (with Genipin and Without Genipin) and free cells of probiotic bacteria BB.12 by used chitosan succinate as nanoparticles *in vitro* (Simulated Gastric Juice with 0.3% of pepsin, Simulated Gastric Juice without pepsin, Simulated Intestinal Juice, Simulated Colonic Juice) and in Tomato Ketchup during shelf life

The study is divided into 5 chapters as followed; Chapter 1: Introduction (to allow the readers to understand more in background and significance of study as well as the objectives of study), Chapter 2: Literature review (definition of probiotics bacteria, *Bifidobacterium lactis* Bb-12, Nanoencapsulation, nanoemulsification of probiotics bacteria, GT and genipin etc.), Chapter3: Methodology, Chapter 4: Results and Discussion, and Chapter 5: Conclusions.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics bacteria

In the 1900s, Ilya Metchnikoffs' discovery on the exceptional benefits of fermented dairy milk led him to a Nobel Prize in Medicine in 1908. (According to their nutritional effect discovered was due to the probiotics present in the products) (Vasiljevic & Shah, 2008) Living bioactive food components Probiotic bacteria are defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits affect to inherent basic nutrition" (Guarner & Schaafsma, 1998); (Knarreborg et al., 2002), however, before 100 years Metschnikov was interested in this area (Metschnikoff, 1907) and become increasingly popular during the last decade.

Most strains of probiotic microorganisms are Lactic Acid Bacteria (LAB), and the main strains are: *Lactobacillus* spp, *Bifidobacterium* spp and *Enterococcus* spp (Klein et al., 1998). Also other bacterial species, such as *Bacillus* spp (Batoni et al., 2001) and *Clostridium butyricum* (Takahashi et al., 2004). Moreover, the yeast *Saccharomyces boulardii* had been studied extensively (Elmer et al., 1999). But used *Enterococcus* spp as a probiotic had been questioned because in regards of safety aspects with regard to transfer of genes conferring antibiotic resistance (Lund & Edlund, 2001).

Most scientists and researchers agreed that probiotic strains shall be able to survive during the processing and shelf life of food and supplements, transit through the gastric tract environment as well as exposure to bile and pancreatic juice in the upper small intestine to be able to exert beneficial effects in the lower small intestine and the colon (Mottet & Michetti, 2005).

According to the International Dairy Federation (IDF) recommendations, that food containing probiotic bacteria should contain at least a level of $\geq 10^7$ cfu/g up to the date of minimum durability (Kirjavainen et al., 1998) at the time of consumption in order to produce therapeutic benefits (Capela et al., 2006); (Picot & Lacroix, 2004).

Also, although, the FAO/WHO standard recommended that any food sold with health claims from the addition of probiotics is that it must contain at least 10^6–10^7 cfu per gram of viable probiotic bacteria (FAO/WHO, 2001). In some countries such as Argentina, Prague and Brazil, the standard of $\geq 10^6$ cfu/g has been accepted in the case of *Bifidobacteria* spp. However, in Japan, this standard has been prescribed $>10^7$ cfu/g (Robinson & Samona, 1992). Also, various recommendations have been presented by different researchers such as $>10^6$ cfu/g by all probiotics in yogurt (Robinson & Samona, 1992); (Kurmann & Rašić, 1991) and $>10^7$ cfu/g in the case of *Bifidobacterium* spp (Redding et al., 1991).

Shah and many researchers reported that the minimum amount 'therapeutic minimum' of the latter index had been recommended as approximately 10^9 viable cells per day (Shah, 1997); (Kurmann & Rašić, 1991); (Mortazavian et al., 2006).
Clinical data resulting from *in vivo* studies will confirm the delivery of probiotics in the gut, besides providing an evidence of their health benefits. Legislation in the United State of America allows probiotics under dietary supplement health (Kailasapathy, 2009). In Europe, probiotics are defined by their application: drug or food (Ferreira & Chauvet, 2011). Probiotics that are used as dietary supplements or functional foods are regulated by food legislation. A positive list of health claims with their conditions of use is defined. For any drug claim, scientific evidence of the health benefits must be provided.

Analysis of probiotic products in many different countries has confirmed that probiotic strains poor survival by several factors that have been claimed to affect the viability of probiotic cultures including titratable acidity, hydrogen peroxide, oxygen toxicity (oxygen permeation through packaging), storage temperature, species and strains of associative fermented dairy product organisms, post-acidification (during storage) in fermented products, concentration of lactic and acetic acids and even whey protein concentration(lack of proteases to break down milk protein to simpler nitrogenous substances and compatibility with traditional starter culture during fermentation)and stability in dried or frozen form, poor growth in milk, (Østlie et al., 2005); (Vinderola et al., 2000); (Corcoran et al., 2005); (Samona et al., 1996); (Mättö et al., 2006); (Dave & Shah, 1997); (Kailasapathy & Rybka, 1997); (Shah, 2000).

Moreover, Oxygen plays a major role in the poor survival of probiotic bacteria (Brunner et al., 1993).

2.1.1 *Bifidobacterium* spp

LAB are the most important strains of probiotic microorganisms typically associated with the human gastrointestinal tract. LAB bacteria are Gram-positive, non-spore-forming, rod-shaped, catalase-negative organisms that are devoid of cytochromes and are of non-aerobic habit but are aero-tolerant, fastidious, acid-tolerant and strictly fermentative; lactic acid is the major end-product of sugar fermentation (Axelsson & Ahrné, 2000). Few of the known LAB that are used as probiotics are: *Lactobacillus acidophilus*, *Lactobacillus amylovorous*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus johnsonoo*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* etc. (Mäyrä-Mäkinen & Bigret, 1993).

Other common probiotic microorganisms are the *Bifidobacterium* spp. In the early 1900s, *Bifidobacteria* spp were classified as *Lactobacillus* subspecies. However, now these microorganisms are classified as a separate genus, *Bifidobacterium* spp. recently, only 30 species of *Bifidobacterium* spp, isolated from human, animal, insect, and environmental sources, have been identified. Of these species, six species isolated from human origins: *B. adolescentis*, *B. breve*, *B. bifidum*, *B. lactis*, *B. infantis*, and *B. longum*, have been used in dairy products.

2.1.1.1 Characteristics of *bifidobacterium* spp

Bifidobacterium spp are Gram-positive, non-motile, non-spore forming anaerobic rods with variable cellular morphology (Scardovi, 1986). (Under size from 1 to 5 mm which is reported by (Kailasapathy, 2002) and (McClements et al., 2009).

In 1969, De Vries and Stouthamer, classified *Bifidobacterium* spp as strict anaerobes, because they are incapable of respiration using oxygen or growth under aerobic conditions. However, the degree of tolerance of oxygen depends on the species and culture medium (De Vries & Stouthamer, 1969) and even on the

morphology of the strains, whether they are branched or not (Norris et al., 1950); (Boylston et al., 2004).

The initial optimum growth pH for *Bifidobacterium* spp is between 6.5 and 7.0, and the growth of *Bifidobacterium* spp is inhibited above 8.5 or below 4.5 (Scardovi, 1986); (Lourens-Hattingh & Viljoen, 2001). In a liquid media with compounds typical of fermented dairy products, *Bifidobacterium* spp are decreases from 0.1 to 7.6 log10 at pH 4 (Vinderola et al., 2002). Under conditions typical of gastric tract (pH 1.5–3.0), *B. longum* 1941 and *B. pseudolongum* 20097 were more tolerant to the acid conditions than the 7 other strains of *Bifidobacterium* evaluated. However, after 3 hours of incubation time, the cell counts of these 2 acid-tolerant strains decreased less than 1 log10, while the cell counts of the other *bifidobacterium* strains evaluated decreased up to 7 log10 (Lankaputhra, 1995).

(J. Rasic & J. Kurmann, 1983), were reported that the optimum temperature growth for most species of the *Bifidobacterium* spp of human origin is between 36ºC and 38ºC, whereas the animal species have growth optima at 41ºC to 43ºC and there is no growth below 20ºC or above 46ºC (Rasic and Kurmann, 1983).

About ferment carbohydrates, (Tamime, 2002), found, in different *Bifidobacterium* spp are able to ferment different carbohydrates. *Bifidobacterium* actively ferment carbohydrates, producing mainly acetic acid and lactic acid in a molar ratio of 3:2 (v/v), but not carbon dioxide, butyric acid or propionic acid (Boylston et al., 2004).

The growth of the *Bifidobacterium* is also inhibited by lactic acid and other metabolic products produced by LAB during processing and storage of the cultured dairy products (Blanchette et al., 1996).

Most of *Bifidobacterium* spp depends on other lactic acid bacteria to ensure their growth. Out of 17 *Bifidobacterium* strains grown in pure milk, 15 failed to survive as reported by (KLAVER et al., 1993). Because these strains have lack proteolytic activity, they could be grown by adding casein hydrolysates or by coculturing with proteolytic species such as *Lactobacilli acidophilus.* Therefore, *L. acidophilus* strains live in excellent symbiosis with *Bifidobacterium* providing the necessary growth stimulants (Hansen et al., 2002). (J. L. Rasic & J. A. Kurmann, 1983), confirmed that when he studied the growth rate of *L. acidophilus* there was no

affected by *B. bifidum*, but the growth of *B. bifidum* was suppressed unless the initial inoculums are in the ratio of 10^4: 10^3 (*B. bifidum: L. acidophilus*) (RasicaKurmann,1983).

2.1.1.2 Beneficial effects of *Bifidobacterium spp*

Bifidobacterium spp and *Lactobacilli*, are part of the normal intestinal and colon microflora of the human and animals. The indigenous *Bifidobacterium* spp are dependent on the age of the individual. *Bifidobacteria infantis* and *B. breve* are predominant in infants while *B. adolescentis* is predominant in adults, and *B. longum* is present throughout life (Gomes & Malcata, 1999) and play an important beneficial role in the host's health, when present in sufficient numbers {Hoover, 1993 #141}; (Yaeshima, 1996); (Holzapfel et al., 1998) and (Alander et al., 1999).

Bifidobacterium maintain a balance between the population of beneficial and potentially harmful microorganisms in the gastrointestinal tract (Gibson & Roberfroid, 1995). But, many factors can disturb the balance of intestinal microflora, and significantly decrease the number of beneficial *Bifidobacterium* such as antibacterial drugs, stress, gastrointestinal disorders, and cause gastroenteritis symptoms like abdominal cramps, fever, vomiting, diarrhea, and other chronic gastrointestinal diseases (MITSUOKA, 1982); (Gothefors, 1989); (Hoover, 1993).

To perform good functions of *Bifidobacterium* spp, it must be viable at the time of consumption and maintain their viability throughout the gastrointestinal tract (Robinson & Samona, 1992; Blanchette *et al*., 1995; Arunachalam, 1999; Brassert and Schiffrin, 2000). So the minimum suggested level for *bifidobacterium* in the food to attain this viability are quite variable (Charteris, Kelly, Morelli, & Collins, 1998). Levels of 10^5 cfu/g^{-1} (Shah, Lankaputhra, Britz, and Kyle, 1995; Shah, 1997), 10^6 cfu/g^{-1} (Robinson & Samona, 1992; Arroyo, Cotton, & Martin, 1994; Rybka & Kailasapathy, 1995; Pagano, 1998) and 10^7 cfu/g^{-1} (Samona and Robinson, 1994) have been suggested to be achieved with a daily consumption of at least 100 g of a product containing between 10^6 and 10^7 viable cells g^{-1},. In general, the food industry has applied the recommended level of 10^6 cfu/g^{-1} at the time of consumption for *Lactobacillus acidophilus* to *Bifidobacterium* and other probiotic bacteria. This

standard appears to have been adopted to provide bacterial concentrations that were technologically attainable and cost-effective rather than to achieve a specific health effect in humans (Mao et al., 2001).

Several studies were done to achieve a balanced intestinal microflora by inclusion of *Bifidobacterium* spp into food (Ishibashi and Shimamura, 1993; Naidu *et al*., 1999). It has been recognized that selected strains for oral delivery must be of human origin, available in sufficient number in the food products at the time of consumption and resistant to the gastric acidity in the upper gastrointestinal tract before reaching the colon (Gilliland, 1989 and Holzapfel *et al*., 1998). Yogurt is commonly used as a vehicle to deliver *Bifidobacterium* spp (Hughes and Hoover, 1991 and Katz, 1999). However, a high number of viable *Bifidobacterium* spp in yogurt may not always be maintained because of the acidity of yogurt and the low acid tolerance of *Bifidobacterium* spp (Reuter, 1990, Iwana *et al*., 1993 and Micanel *et al.*, 1997). Furthermore, delivery of viable *Bifidobacterium* spp to the large intestine, where they would be able to function, has been limited because of the extreme acidity found in the human stomach (Berrada *et al*., 1991; Clark and Martin, 1993 and Mizota, 1996).

In addition, *Bifidobacterium* spp can improve the intestinal flora by preventing colonization of pathogens, activating of the human immune system, increasing protein digestion and amelioration of diarrhea or constipation (Ishibashi and Shimamura, 1993). Therefore, *Bifidobacterium* spp have been widely incorporated in various food products and are among the leading candidates for use as a probiotic or dietary adjunct (Gomes & Malcata, 1999).

Also, many scientists documented the benefits of *Bifidobacterium* spp in the health like: enhanced immune response, reduction of serum cholesterol, vitamin synthesis, anti-carcinogenic activity, and anti-bacterial activity (Robinson and Samona, 1992; Blanchette, Roy, and Gauthier, 1995; Gomes & Malcata, 1999; Arunachalam, 1999; Brassert and Schiffrin, 2000; Lourens-Hattingh and Viljoen, 2001).

Thus, the success of *Bifidobacterium* spp-containing food products depends on the viability of *Bifidobacterium* spp in the product during its shelf life as well as on the resistance of the bacteria to the conditions existing in the upper gastrointestinal tract.

2.1.1.3 *Bifidobacterium animalis* sub spp *lactis*

One of the *Bifidobacterium* species commonly used in the food industry is *Bifidobacterium animalis* sub spp *lactis* strain Bb-12 (previously known as *Bifidobacterium bifidum* Bb-12, and also as *Bifidobacterium lactis* Bb-12, but subjected to reclassification), which is marketed around the world under a variety of labels in dairy products and infant formulas (Satokari et al., 2003); (Saxelin et al., 2005). In German they deposit the strain culture collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) under number DSM 15954 was reported by (Kajander et al., 2008). In addition, several authors consider the Bb12 strain to be also equal to the strain DSMZ 10140 (Masco et al., 2004). Bb-12 is a widely used commercial probiotic strain isolated in 1983 by Chr. Hansen A/S. The strain is included in a variety of food applications and dietary supplements and is ascribed various probiotic effects (Ouwehand & Vesterlund, 2003). The taxonomy of Bb-12 has been controversial since its original description by Meile *et al*., in (1997), and several studies have investigated its similarity with the closely related species Bb-12 (Scardovi, V., and Trovatelli, L., 1974).

New genotypic evidence reported by (Ventura & Zink, 2002) (Ventura et al., 2003), (Zhu et al., 2010), (Masco et al., 2004), and (Kwon et al., 2005), indicated that Bb-12and *B. animalis* subsp. *animalis* should be considered two separate taxonomic entities at the subspecies level. Bb-12 exhibits properties such as elevated oxygen tolerance (Prasad *et al*., 1998), differential growth in milk-based media (Ventura, M., and Zink. R., 2002), and hydrolysis of milk proteins (Janer, *et al*., 2005); these properties differ from *B. animalis* subsp. *animalis* and facilitate its growth in commercial products under nonanaerobic conditions. Traditional bacteriological and biochemical identification techniques, such as selective growth of species in differential media, cannot be routinely used to differentiate *Bifidobacterium* species.

Bb-12 is widely used in the form of probiotic yoghurt. Probiotic yoghurt containing Bb-12 is reported to have beneficial effects on metabolism including lowered serum LDL-cholesterol in patients with type 2 diabetes (Ejtahed et al., 2011),increased HDL cholesterol in adult women (Sadrzadeh-Yeganeh et al., 2010)

and improved glucose tolerance during pregnancy (Luoto et al., 2010). Bb-12 administration has also been shown to increase faecal secretory IgA excretion in preterm infants (Mohan et al., 2008).

In India the dietary practices are different from that in the developed countries where evidence of probiotic efficacy has been gathered. This, together with the occurrence of frequent gastrointestinal infections in childhood and the widespread consumption of home-made yoghurt containing Bb-12 in the diet (Kabeerdoss et al., 2011)

2.1.2 LAB and survival within the gastrointestinal tract

2.1.2.1 Gastrointestinal strains of human origin

There are 400 species of microorganisms in the human gut (Goktepe et al., 2005). The LAB found likely to be associated with the human host are: in the large intestine is densely populated by *Bacteriodes* and the Gram-positive, anaerobic genera *Eubacterium* and *Bifidobacterium. Lactobacilli* are the predominant species in the vagina and are also normally present in the oral cavity (10^3-10^4 cfu/g), the ileum (10^3-10^7 cfu/g), and colon (10^4-10^8 cfu/g), where they play an important role in maintenance of a stable gut mucosa (Lidbeck *et al*., 1993). Long and Swenson. (1977), showed that *Bifidobacteriurm* spp and *Lactobacilli* the dominant bacterial species found to be present in the feces of breast-fed infants. There is a lack of research evidence that has demonstrated a single dominant species in the human gastrointestinal tract. However, *L. acidophilus* (commonly referred as simply "*acidophilus*") has been recovered in relatively high numbers from the gastrointestinal tract (Molin *et al*., 1993). Strains of *L. acidophilus* have been isolated from the intestinal tract of humans as well as animals such as rodents and birds.

Charteris *et al*., (1998), found, that both *Lactobacillus* spp and *Bifidobacterium* spp show a moderate tolerance to acid pH after 90 min incubation while a decrease occur after 2 h but individual strains vary considerably (Table 2.1).

Table 1. Survival of Lactic Acid Bacteria (LAB) in the gastrointestinal tract.

Table 1 Survival of Lactic Acid Bacteria (LAB) in the gastrointestinal tract.
Resistance to pH 4 for 1 hour Resistance to 20% human bile for 1 hour Adhesion to mucin Binding of Extracellular Matrix (ECM) proteins (fibronectin, collagens, vitronectin, laminin) Expression of cell surface hydrophobicity Production of antioxidants Production of antimicrobial susbtances (bacteriocins and others)

Acid tolerance can be mediated by membrane ATP ases as described for *L. acidophilus* (Lorca & Font de Valdez, 2001), *B. lactis* and *B. animalis* (Matsumoto et al., 2004). In the presence of milk or other food products the resistance was significantly higher as documented by Saxelin *et al*., 1999. Most of LAB is susceptible to bovine and porcine bile *in vitro*. However, they were resistant to human bile which correlated with the survival in the human GIT (O'Mahony, 2001). In *L. reuteri*, bile resistance appeared to be mediated by bile salt hydrolysis. (De Boever et al., 2000) This also resulted in precipitation of cholesterol. Similar effects were seen after deconjugation of bile salts by *L. acidophilus* strains (Ahn et al., 2003); (Ashar and Prajapathi, 1998).

Therefore, *Bifidobacterium* strains that tolerate low pH of the stomach and survive the effects of bile produced by the small intestine of human are selected if they are to be used as dietary adjunct (Lian et al., 2003).

Pancreatic juice inhibits growth of some probiotic bacteria, while, individual strains tolerate growth in media supplemented with pancreatic juice independent of proteolytic activity (Kruszewska et al., 2004). *S. boulardii* survived transit in the GI tract well but better in the presence of dietary fibers (Elmer *et al*., 1999). *Bacillus* spp as probiotics survive the transit very well since they are in the form of spores (Duc et al., 2004).

(Lian et al., 2003), Documented that, the *bifidobacterium* strains that tolerate low pH of the stomach and survive the effects of bile produced by the small intestine of human are selected if they are to be used as dietary adjunct.

To be successful, manufacturers must consider the effects of the environment of the food during processing and storage to ensure that the concentration of *Bifidobacterium* spp at the time of consumption provides a therapeutic dose to consumers. In addition, the food with added *Bifidobacterium* spp must maintain the characteristic sensory attributes of the traditional food. So, (encapsulation techniques have been widely used to protect *Bifidobacterium* spp cells from environmental and physiological degradation) (Lim and Moss, 1981; Kim *et al*., 1988; Kwok *et al*., 1992), encapsulation such as: against adverse effects of acid and bile salts, which have been reported by many researchers (Rao *et al*., 1989; Modler *et al*., 1990; Hughes and Hoover, 1991; Cui *et al*., 2000; Sun and Griffiths, 2000; Siuta-Cruce and Goulet, 2001).

2.1.2.2 Acid resistance of probiotics bacteria

Gastric juice with a pH as low as 1.5 is secreted from cells lining the stomach each day, providing a normally-effective, high-acid barrier against entrance of viable bacteria into the GIT. The effect of gastric pH on bacterial viability and in preventing bacterial colonization of the small intestine is well-studied (Simon and Gorbach, 1987; Heatley and Sobala, 1993). So, any probiotic organism that is to survive transit through the stomach must have a high acid tolerance. In typical acid tolerance tests, the viability of candidate probiotic organisms is determined by exposing them to low pH in a buffer solution or medium for a period of time, during which the number of surviving bacteria remaining is determined.

In *vivo*, the studies by (O'Mahony, 2001) suggested that *lactobacilli* isolated from human ileal samples could successfully transit the human stomach conditions and function effectively. In these studies, *Bifidobacterium* spp were found to be less resistant to stomach conditions like low pH etc. than *Lactobacilli*. Studies by Conway *et al*. (1987) showed that yogurt-producing species of *lactobacilli* were more sensitive to gastric juice while enteric species were more resistant. The best-performing among the two *L. acidophilus* strains (strain ADH) used in the study were reclassified as *Lactobacillus gasseri*, this is a homo fermentative lactobacilli (Morelli, 2000). *Lactobacillus rhamnosus* GG was unable to survive at pH 1.0, but remained viable at

pH 3.0 and higher (Goldin *et al*., 1992). *In vitro* studies, Hood and Zottola (1988) and Charteris *et al*., (1998a) showed that enteric *lactobacilli* had a lower pH tolerance limit of 2.0 for several min. The number of surviving bacteria was decreased from the inoculated level of 7.4-7.6 log cfu/mL to < 4 log cfu/mL at pH 1.0 and pH 2.0, whereas pH 4.0 and 5.0 did not affect the viability (Erkkilä & Petäjä, 2000).

The bile resistant isolates of *Bifidobacterium* strains displayed considerably higher survival at 90 min of exposure at pH 2.0, with a concentration of final surviving bacteria ~ 6.5 log cfu/mL, than their 19 corresponding strains of origin (Noriega et al., 2004). Survival of *Bifidobacterium animalis* strains BLC-1, Bb-12, and Bo, *Lactobacillus acidophilus* strains LAC-1 and Ki, *Lactobacillus paracasei* subsp. paracasei strain LCS-1 and *Lactobacillus brevis* strain LMG 6906 inoculated into whey cheese was studied by (Madureira et al., 2005). Except *L. paracasei* subsp. *Paracasei* LCS-1 and *B. animalis* Bb-12, all bacteria were resistant to the action of artificial gastric juice (pH 2.5–3.0) and maintained their initial viable cell numbers (~ 8 log cfu/mL) after both 60 and 120 min of exposure.

In order to evaluate the survival of *lactobacilli* in the low pH conditions of the human stomach, (Corcoran et al., 2005) were studied that, five *Lactobacillus* strains were compared in simulated gastric juice (SGJ, pH 2.0) for 90 min. *Lactobacillus rhamnosus* GG had the highest survival rate and maintained their initial viable cell numbers (~ 9 log cfu/mL), while the poorest survivor was *L. paracasei* NFBC 338, whose concentration declined to undetectable levels after only 30 min of exposure.

Probiotics have been incorporated into a range of dairy products, including yoghurts, soft-, semi-hard and hard cheeses, ice cream, milk powders and frozen dairy desserts. However, there are still several problems with respect to the low viability of probiotic bacteria in GIT and food environments. Probiotics of intestinal origin are difficult to propagate and high survival is important for both economic reasons and health effects. Due to that reasons, there is a demand for new technologies such as encapsulation to enhance probiotic viability.

2.1.2.3 Bile resistance of probiotics bacteria

Bile defined by Hofmann and Roda (1984), is an aqueous solution made up of bile acids, cholesterol, phospholipids, and the pigment biliverdin, which gives the bile its yellow-green color. About 500-700 ml/day of bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum, after food intake by an individual. Bile plays an essential role in lipid digestion; it emulsifies and solubilizes lipids and functions as biological detergent.

Prior to secretion into the duodenum, bile acids are conjugated either with glycine (glycoconjugated) or taurine (tauroconjugated) reported by (Begley et al., 2006). Furthermore, (Begley et al., 2006) reported that, in the colon conjugated bile undergoes various chemical changes including deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation, almost just by microbial activity. The antimicrobial nature of bile is mainly because of its detergent property, which dissolves bacterial membranes. Bile salt hydrolases (BSHs) are generally intracellular, oxygen insensitive enzymes that catalyze the hydrolysis of bile salts. Hydrolysis of bile salts is mediated by various genera of the intestinal microflora, including *Clostridium* (Gopal *et al*., 1996), *Bacteroides* (Kawamoto *et al*., 1989), *Lactobacillus* (Lundeen and Savage, 1990; Christiaens *et al*., 1992), *Bifidobacterium* (Grill *et al*., 2000a) and *Enterococcus* (Franz *et al*., 2001). A number of BSHs have been identified and characterized in probiotic bacteria, and the ability of probiotic strains has often been included among the criteria for probiotic strain selection (Begley *et al*., 2006). Bile tolerance of probiotic bacteria can be investigated by incubating them for 24 h in a milk-yeast medium containing different concentrations of bile extracts and monitoring cell viability and pH before and after incubation (Goktepe et al., 2005).

Most studies reported a growth delay of *Lactobacilli* spp in the presence of oxgall that was strain- and not species-dependent. It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and BSH enzymes play a role in bile tolerance of probiotic organisms in the GIT (Savage, 1992). In 1986, Stewart *et a*l found, both conjugated and deconjugated bile acids have been determined to inhibit the growth of *Klebsiella* spp., *Enterococcus* spp. and *Escherichia coli* strains in *vitro*. However, deconjugated forms of bile acids were found to be more inhibitory against Gram-positive than Gram-negative bacteria

(Stewart *et al.,* 1986). Studies by Smet *et al*., (1995) suggested that deconjugation of bile acids decreases their solubility and thus diminishes the detergent's activity and makes it less toxic to bacteria in the intestine. It was assumed that the conjugated form of the bile salts exhibits toxicity by causing intracellular acidification through the same mechanism as organic acid. In contrast, Tannock *et al.,* (1989) stated that deconjugated bile salts are inhibitorier than conjugated bile salts to anaerobes including *Lactobacilli*. Similarly, deconjugated bile was reported to be involved in growth inhibition of *Bifidobacterium* spp. including *B. breve*, *B. longum*, and *B. coryneforme,* where the viable counts were reduced by approximately 6, 7 and 2 log cfu/ml respectively, after 2 h incubation in the presence of 1 mM deconjugated bile (Grill *et al*., 2000b). Another hypothesis states that certain *Clostridium* spp. utilize the amino acid taurine as an electron acceptor and have demonstrated improved growth rates in the presence of taurine and taurine-conjugated bile salts (Moser and Savage, 2001). However, taurine or taurine conjugates did not affect the growth of *Lactobacillus* spp. tested (Tannock *et al.,* 1989). Cholic acid was found to accumulate in *lactobacillus* cells by means of a transmembrane proton gradient (Kurdi *et al*., 2000). Whereas studies by Boever *et al*., (2000) reported that cholic acid was highly deleterious for the viability of *lactobacilli*. It has also been suggested that the BSH enzymes are detergent shock proteins (Adamowicz *et al*., 1991) that protects the *lactobacilli* from its toxic effects and may have a competitive advantage over the non-BSH producing bacteria. However, studies of (Moser & Savage, 2001) reported that, the deconjugation and resistance are unrelated activities. Finally, studies done by Gopal *et al.,* (1996) showed no relationship between the ability of 6 strains of *L. acidophilus* and 8 strains of *Bifidobacterium* spp. to grow in bile (0.3% oxgall) and their ability to hydrolyze bile salts (glycocholic acid or taurocholic acid).

2.1.2.4 Viability of encapsulated probiotics in gastrointestinal tract

(Mortazavian et al., 2007), noted that, the efficiently encapsulation technique was confirmed by various studies that increases the probiotics viability through the gastric tract from acidic-enzymatic-bile conditions. Rao *et al*., (1989), used freeze-dried method to encapsulate *B. pseudolongum* by cellulose acetate phthalate (CAP)

coated with beeswax, showing that encapsulated *B. pseudolongum* survived the simulated gastric environment in larger numbers than non-encapsulated cells. CAP is an enteric coating material used for control of drug release in the intestine. However, encapsulation of *Bifidobacterium* spp in butter oil was ineffective in preventing acid injury to bacteria in both low acid yogurt (pH 5.8) and high acid yogurt (pH 4.4) (Modler and Villa-Garcia, 1993). Calcium alginate and k-carrageenan–Locust bean gum gel beads are the two most commonly used polymers for immobilizing viable cells during fermentation which a reported by (Audet *et al*., 1988).

Moreover, Sultana, and others, reported that the microencapsulation of *B. pseudolongum* with CAP has also been claimed to have a suitable effect on the viability of *B. pseudolongum* after being exposed to the simulated gastric conditions. According to the same research, the free cells were completely destroyed after 1 h. It should be pointed out that apart from the type of capsulation materials; diameter of capsules or coats is also a determinable factor for improving the viability of probiotics. Excessive reduction in diameter can weaken or remove the protective function of encapsulation. For example, it has been reported that survivability of encapsulated probiotics with alginate capsules under the acidic-bile conditions showed no significant difference when the diameter of gel-beads were 20 and 70 μm compared with the bigger sizes (Sultana et al., 2000). Also, microencapsulation of *Bifidobacterium* spp. did not significantly increase their viability when the cells encountered the simulated gastric juice (Chandramouli et al., 2004).

Experiments of (Lee et al., 2004) showed that survivability of *B. longum* encapsulated with calcium alginate in the simulated conditions of gastric juice (pH 1.5) could be considerably increased. Experiments indicated that coating of the calcium chloride on sodium alginate capsules containing *L. acidophilus* increased the tolerance of the mentioned bacteria against harsh acidic (pH 2) and bile (1%) conditions (Chandramouli et al., 2004). Simulated conditions of the stomach (pH 1.5) led to a dramatic loss in the viable counts of *B. infantice* (from 1.23×10^9 to <10 cfu/ml after 30 min), nevertheless, its viability loss under the same conditions after microencapsulation did not exceed the 0.67% of the first viable cell amount (Sun & Griffiths, 2000).

2.2 Nanotechnology

In 1974, Nario Taniguchi was represent the term of nanotechnology as used it to describe the manipulation of particles of less than one micrometer, and in 1959, the physicist, Richard Feynman spread the concept within the atomic engineering field (Sekhon, 2010). A nanometer is 10^{-9} meter, 1 nanometer is about 60,000 times smaller than a human hair in diameter or the size of a virus, a red blood cell is about 2,000 to 5,000 nm in size, and the diameter of DNA is in the range of 2.5 nm. Therefore, nanotechnology deals with matter that ranges from one-half the diameter of DNA up to 1/20 the size of a red blood cell (Dingman, 2008). Further, it is interesting to note that nanomaterials are so small, even bacteria would need a microscope to see them according to IOM in 2010. Nanoparticles are generally accepted as those with a particle size from 1 to 100 nm. Where is applied to creating structures with special properties that strongly depend on size and structure (Muñoz et al., 2007); (Uskoković, 2007).

Nanomaterials on which most of the research has been carried out are normally powders composed of nanoparticles which exhibit properties that are different from powders of the same chemical composition, but with much larger particles (Chaudhry et al., 2008). Research is in progress into a number of advances in the food processing fields have been made on nanotechnology grounds such as food production, processing, packaging and supplements due to their unique functions and applications of nanomaterials.

(Elder & Oberdörster, 2006) a preparation of nano-sized materials can be carried out using ‘‘Top down’’ methodologies which consist in decreasing the size of macrostructures down to the nano-size scale or by ‘‘Bottom up’’ techniques in which arrangements of atoms, molecules, or single particles are induced. The large surface area per unit mass of nano-sized biomaterials may increase their biological activity, as in the formulations of herbaceous plants as a result of nanotechnology-based studies (Quintanilla-Carvajal et al., 2010).

(Graveland-Bikker & De Kruif, 2006), documented that, in food nanostructures have been used for the preparation of milk protein nanotubes with potential applications as carrier materials and enhancers of properties like jellification

and viscosity has received special attention. Also, to inhibition of thermal polymerization and for eliminating bad odors by catalytic refining of frying oil using nanoceramics (Oilfresh. 2005).

FDA approved methodologies and techniques supporting the inclusion of nanotechnology-based ingredients for mass consumption, within the nutraceutical and food safety fields that applications of nanotechnology have made important progress (Chau et al., 2007) such as: the production of vitamins in the form of micelles and of small (nano) detectors of viruses.

2.2.1 Regulation

Legal aspects related to the handling and consumption of nano-sized materials in food applications are important for deciding on the consumption of these composites for years come. It will play an important role in mass production of nanofoods. Currently available information of risks associated to manipulation of nano-sized products is limited, and there are not internationally agreed regulatory parameters related to manipulation of nano-sized food materials, and thus, many products reach markets without prior evaluation on safety aspects related to their manipulation (Nanotechnology and food, Nanotechnology in packaging., 2010). Stones M. (2009), noted, here is a lack of information on aspects related to development of foodstuffs which contain manufactured nanoparticles and on physicochemical properties of nanosized particles used as additives during their transit through the gastrointestinal tract.

United States of America body led the regulatory aspects related to nanoproducts. The National Nanotechnology Initiative (NNI) describes the uses and the applications of nanotechnology in three main areas: first, research and technology on the development of products at the atomic, molecular or macromolecular scales in the length of 1–100 nm. Second, creating and using structures that has novel properties and functions because of their small sizes and, ability to control or manipulate on the atomic scale (Nanotechnology and food).

Improved functionality may be observed in food applications at particle sizes somehow larger than the 100 nm range. For example, (Wulff-Pérez et al., 2009), demonstrated that the colloidal stability of some nanoemulsions can be precisely

controlled by the chemical structure of its interface. In their work, they used the amphiphilic uncharged tri-block copolymer Pluronic F68 as surfactant and natural oils from soybean, sesame, and olive as the organic phase. The more stable resulting emulsions resulted very appropriate for parenteral administration and had an average particle size, determined by dynamic light scattering (DLS), of approximately 500 nm. Chi-Fai *et al*., (2007) reported 42 bodies worldwide that support development of nanotechnology and involved in regulatory aspects.

2.3. Probiotics in food nanotechnology

What have been previously reported, probiotics are products aimed at delivering living, potentially beneficial, bacterial cells to the gastric tract of humans and other animals. For example, calcium and Dr Kim's probiotic nanofood combination reduces risk of osteoporosis. Probiotic- containing foods are the best way to get Dr Kim's probiotic nanofood for most people. A variety of capsules, liquids, and powders are available. Powders can be stirred into food but should not be added to food warmer than room temperature, because heat will kill the bacteria. Another option is to add to your diet. These foods include yogurt, kefir (a cultured milk beverage), tempeh (made from soybeans), and kimchi (a Korean fermented cabbage dish) (Renton, 2006).

2.3.1 Nanoencapsulation of probiotics

Probiotics can be used in food for examples: in the form of yoghurts and yoghurt-type fermented milk, cheese, puddings and fruitbased drinks. However, encapsulated forms of ingredients achieve longer shelf life of the product. Nanoencapsulation is desirable to develop designer probiotic bacterial preparations that could be delivered to certain parts of the gastric tract where they interact with specific receptors. These nanoencapsulated designer probiotic bacterial preparations may act from the beginning vaccines, with the capability of modulating immune responses (Vidhyalakshmi et al., 2009). Biopolymer assemblies stabilized by various types of noncovalent forces have recently shown considerable progress (Sletmoen et al., 2008).

2.3.2 Nanoencapsulation

(Weiss et al., 2006), were defined nanoencapsulation as a technology to pack substances in miniature and refers to bioactive packing at the nanoscale. Nanocapsules can release their content under controlled rates, such as, Niu *et al.*, 2007 who reported that the effects of tissue engineering scaffold on the controlled releasing of a synthetic peptide from porous nano-hydroxyapatite/collagen/poly (L-lactic acid)/chitosan microspheres (nHAC/PLLA/CMs) containing different quantities of chitosan which were prepared by a thermally induced phase separation method.

The main methods to prepare the nanocapsules are development of nano-sized capsules, structuring nano or micro capsules with nano-sized pores (Friends of the Earth), and encapsulation of nano-size particulate compounds. Encapsulation of nanoparticles prepared from nanoemulsions of different sources of oils and produced by different homogenization methodologies followed by spray drying has received a good results in this field (Sorrentino et al., 2007); (Kuzma & VerHage, 2006).

In general nanoemulsions methodologies to produce nanocapsules can be divided into three main groups (Morris, 2008): first group, Physical processes such as spray drying–coating, extrusion, and spray drying; Then, Physiochemical processes such as simple or complex coacervation and entrapment into liposomes; finally Chemical processes: interfacial polymerization and molecular inclusion.

When selecting the most suitable encapsulation process, it is necessary to know the required size of the particles, the physicochemical properties of the core material as well as the nature of the substance to encapsulate and releasing mechanism and associated costs (Morris, 2008). Nanoencapsulation Techniques are more complex than those used for microencapsulation chiefly due to the fact that it is, in general, more difficult to attain a good nanoencapsulation process given the complex morphology of the capsule and core material and the demands of releasing rates (Nanotechnology and food).

Nanoencapsulation can help to solve difficulties such as loss of functionality during processing or in storage, incompatibilities between core and shell materials, generation of bad odors and flavors, deterioration of texture, and in the case of enzymes, lose of activity (Huang et al., 2010).

2.3.2. Nanoencapsulation Procedures

2.3.2.1 Nanoemulsions

The emulsions are defined as mixtures of immiscible fluids, usually, one of the fluids, are in the form of small droplets, forms the dispersed phase, and although the natural tendency of the emulsions is to coalesce, the rate and the extent of these phenomena will depend mainly on the droplet size of the emulsion (DSE) and composition of the phases. Emulsions can be classified into 3 groups depending on the DSE: micro (10–100 nm), mini (100–1,000 nm), and macroemulsions (0.5–100 *l*m) (Bayer AG., 2010). Nano-submicron emulsions are kinetically stable systems that can be either transparent (DSE\200 nm) or ‘‘milky’’ (DSE&500 nm) (Sekhon, 2010); (Nanotechnology can enhance food packaging. 2010).

Nanoemulsions have now used in food industry due to their high clarity. These enable the addition of nanoemulsified bioactive and flavors to a beverage without a change in the appearance of the products (Centre for Biologic Nanotechnology., 2010). Different types of nanoemulsion such as: single-layer, double-layers and triple-layers nanoemulsions, could be produced, depending on the polyelectrolytes, like alginate and chitosan (Choi et al., 2008).

2.3.2.2 Formation of Nanoemulsions

The preparation methods and principles of nanoemulsions are based on processes available for the fabrication of traditional-sized emulsions and on the applications of flow fields, and by using membranes and micro-channel (Boom R., 2008). Table 2, show some of the work describing nanoemulsification techniques and applications in food-related areas lots of methods are used for the preparation of emulsions is using Flow Fields by inducing a very strong flow by agitation, intense mixing, or by flow through a small opening. Flow around a droplet induces a shear force that makes the droplets to break up, forming smaller droplets (Koupantsis & Kiosseoglou, 2009).

There are main methods to prepared nanoemulsification of flow fields such as:

• High-pressure homogenizers: the mixture is pumped through a very small hole or gap. The extensional flows break up the large droplets into small ones. Valve and nozzle systems are used in these cases (Lee et al., 2009).

• Ultrasound systems: high frequency sound waves follow a pattern which propagates turbulence and induces fluctuation of pressure generated by an actuator which vibrates at specific frequencies. This technology has not yet been proved as efficient for industrial scale applications (Wagner et al., 2008).

The relationship between radiuses of conducts for different fluids subjected to processing are, besides working pressure, key factors for obtaining good quality nanoemulsions with the desired size distribution (Skurtys & Aguilera, 2008).

Table 2 Examples of different methods of nanoemulsification in food-related areas

Method	Studied system	Reference
Phase inversion composition	Meyhod used to prepare O/W nanoemulsions in the W/oleylammonium chloride–oleylamine–C12E10/hexadecane ionic system, where the oleylammonium acted as a cationic surfactant. Nanoemulsions were prepared by continuous addition of HCl watery solutions to the mixtures formed by hexadecane, oleylamine, and C12E10 or by progressive addition of potassium hydroxide watery solutions to hexadecane–oleic acid–C12E10 mixtures. Progressively the pH decreased, resulting in a progressive ionization of the oleylamine. Likewise, the oleic acid was gradually ionized when an increase in pH is produced by the addition of KOH. The preparation conditions were maintained constant at a stirring rate of 750 rpm and an addition rate of 1.6 mL/min	Maestro *et al*., 2008
High pressure homogenization	Oil-in-water (O/W) emulsions were prepared using b-carotene as the dispersed phase and Milli-Q water as the continuous phase. The premix was homogenized using a high-speed homogenizer at 5,000 rpm for 10 min to form a coarse emulsion, followed by a two-stage high pressure homogenization. Temperature and pressure, b-carotene concentration, the quadrics of emulsifier concentration and the interactions between b-carotene and emulsifier concentrations and between homogenization temperature and emulsifier concentration p\0.05 had a significant effect on the stability of the emulsions	Yuan *et al*., 2008
Ultrasonic	Nanoemulsion was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein, and 0.5 mg cholesterol	Pires *et al*., 2009
	Emulsification of lipids by prolonged ultrasonic irradiation in aqueous media and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain the nanoemulsion	Choi *et al*., 2008

2.3.2.3 Probiotic bacteria and Emulsification technique

Emulsion technique has been successfully applied for the microencapsulation of lactic acid bacteria reported by Audet *et al*., (1988) and Lacroix *et al*., (1990). But, microencapsulation with the extrusion technique, can be easily scaled up and the diameter of produced beads is considerably smaller (25 μm-2 mm). On the other hand, emulsification and ionic gelification is a chemical technique to encapsulate probiotic living cells and use hydrocolloids (alginate, carrageenan, chitosan and pectin) as encapsulating materials. This technique work on the relationship between the discontinuous and the continuous phases which needed all: the an emulsifier and a surfactant. A solidifying agent (calcium chloride $CaCl_2$) is then added to the emulsion (Chen and Chen, 2007; Kailasapathy, 2009; De Vos *et al*., 2010). In this technique, a small volume of cell/polymer slurry (as a dispersed phase) is added to the large volume of vegetable oil (as a continuous phase) such as soy-, sun flower-, corn-, millet or light paraffin oil (Groboillot *et al*., 1993). Resulting solution becomes well homogeneous by proper stirring/ agitating, till Water-in-oil emulsion forms.

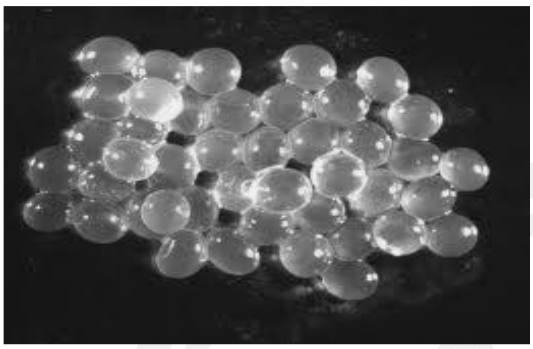
Sheu and Marshall, (1993) and Sheu *et al*., (1993), found, that Tween 80 can be used at the concentration of 0.2% for better emulsion formation and it has been recommended as the best choice. Once W/O emulsion a form, the water soluble polymer becomes insoluble after addition of $CaCl_2$, by means of cross linking and thus makes gel particles in the oil phase.

Smaller particles of the water phase in W/O emulsion will lead to the formation of beads with smaller diameters.

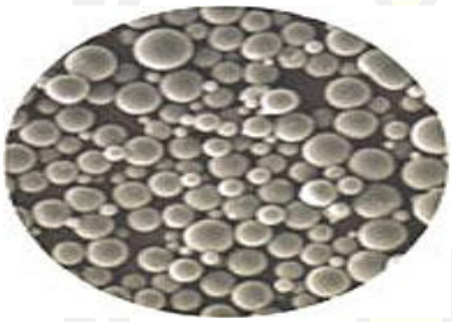
This technique is easy to scale-up and gives a high survival rate of the bacteria (Chen & Chen, 2007). The produced capsules have a small diameter. However, the main disadvantage of this method is that it provides large size range and shape. The emulsion procedure enables the production of the targeted microcapsules size by variation of agitation speed and the water/oil ratio (Kailasapathy, 2009). The gel beads can be introduced into a second polymer solution to create a coating layer that provides added protection to the cell or maybe give improved organoleptic properties (Kailasapathy, 2009).

Emulsification by interfacial polymerization technique is an alternative technique which is complete in a single step. This technique requires the formation of an emulsion: the discontinuous phase contains an aqueous suspension with the probiotic cells and the continuous phase is an organic solvent. To initiate the polymerisation reaction, a biocompatible agent which is soluble in the continuous phase, is added. The droplets obtained containing probiotic cells are enveloped in a thin and strong membrane (Kailasapathy, 2002). Interfacial polymerisation is used to encapsulate microorganisms in order to improve their productivity in fermentation (Yáñez-Fernández et al., 2008).

(Shima et al., 2006), reported that, in the emulsification technique, the mixture represents the discontinuous phase. This phase is dispersed in a large volume of vegetable oil (continuous phase). The water-in-oil emulsion being formed is continuously homogenized by stirring. The stirring speed is a critical step because it affects the size and the shape of the droplets formed (Sultana *et al.,* 2000). Once the emulsion has been broken, the droplets are collected by settling (Sultana et al., 2000); (Cui et al., 2000). Emulsification generates oily or aqueous droplets commonly named capsules, while the extrusion gives gelled droplets called beads. The core of the capsule is liquid while the core of the bead presents a porous network which reported by Gentile *et al*., (1995). Furthermore, the capsules have sizes that are at least 100 times lower than those of the beads (Krasaekoopt *et al*., 2003). The difference between capsules and beads is shown in Figure 2.1. Capsules have unequal size and shape compared to beads whose shape is uniform. This can affect mouth feel and will therefore not be suitable for incorporation into food (Kailasapathy, K. 2009).



a



b

Figure 2.1 (a) Photographs of alginate gel beads and (b) Photographs of alginate capsules (Gouin, S. 2004).

Emulsification is more expensive because it requires additional raw materials such as vegetable oil and emulsifiers to stabilize the emulsion need for vigorous stirring which can be detrimental to cells survival, random incorporation of cells into the capsules, and inability to sterilize vegetable oil if you have to work under conditions of strict asepsis. Among these improvements are coating with others biomaterials such as: (Krasaekoopt *et al*., 2004) cross-linking with organic solvents (Cui *et al*., 2001) or adding additives or cryoprotectants in the mixture (Carvhalo *et al*., 2002).

In emulsification technology, emulsifier or surfactant added in vegetable oil was used to promote the capsule. This technique may not be suitable for food product development because the residual oil in the encapsulated material is detrimental to texture and organoleptic characteristics, and may not be suitable for the development of low-fat dairy products as documented by (Kailasapathy, 2009). Moreover, the residual oil, emulsifier and surfactant in the encapsulated material can be toxic to probiotic cells and may interact with food components (Rokka & Rantamäki, 2010). Probiotic encapsulation technique (PET) has been applied to dairy products such as

yogurt, milk, frozen dessert and cheese. The selection is now expanding to fruit juices, cookies and chocolate (Rokka & Rantamäki, 2010).

Furthermore, another challenge will be to determine the physicochemical characteristics of encapsulation materials to predict their mechanisms of disintegration or dissolution under varying conditions of pH and salinity and their interactions with probiotic cells or other components present in the digestive tract. PET will be of importance in delivering viable strains of probiotic to consumers in the near future.

To proof this delivering must firstly be provided by the results of *in vitro* studies, through simulation of simple and reproducible gastrointestinal tract models. At this level, the lack of standard protocol in the conduct of these tests remains a concern. Efforts should be made in this direction by the scientific community. A model of gastro-intestinal tract has been recently published by Gbassi *et al*., (2011).

2.3.2. 4 Polymerization

(Hentschel et al., 2008) reported, the polymerization of miniemulsions causes the encapsulation by separation of phases during the process. Amphiphilic oligomers are used as surfactant agents to prepare the miniemulsion (oil particles from 50 to 500 nm dispersed in water). Due to the properties of amphiphilic oligomers, the molecules can auto assembly in the water/oil interface through ultrasonication. To form these products, a water soluble initiator such as potassium per sulfate may also be added, and from the mixture, the water-soluble radicals will emerge. After several additions of monomers, oligoradicals begin to be active on the surface and are captured by mini packets of oil, causing that the active radicals of the surface link to the interface of those mini packets and water. Polymerization is carried out on the interface where the chains of the polymer gradually grow, allowing the formation of the core. Important factors during this process are the hydrophobicity and the structure of the oligomers which also have an influence on the morphology of the nanocapsules. (Ren et al., 2008) prepared a liquid-core nanocapsules by cross linking an amphiphilic copolymer at an oil–water interface. The hydrophilic copolymer poly[(ethylene oxide)-co-glycidol] was first obtained by anionic polymerization of ethylene oxide and ethoxyethyl glycidyl ether, then the hydroxyl groups on the backbone were recovered

after hydrolysis and partially modified by hydrophobic conjugated linoleic acid. The copolymer with multiple linoleate pendants was absorbed at an oil–water interface and then cross linked to form stable nanocapsules. The mean diameter of the nanocapsule was below 350 nm, and the particle size could be adjusted by manipulating the emulsification conditions. It was found that the nanocapsules were stable in water for at least 5 months.

2.3.3 Types of nanomaterials and nanostructures

In fact, many natural foods contain nanoscale components and their properties are determined by their structure. For example, some of food's most important raw materials such as: proteins, starches, and fats, undergo structural changes at the nanometer and micrometer scales during normal food processing. In 2008, Morris VJ and Parker R documented that, Food proteins (for example, native beta-lactoglobulin, which is about 3.6 nm in length) can undergo denaturation (via pressure, heat, pH, etc.) and the denatured components reassemble to form larger structures, like fibrils or aggregates, which in turn can be assembled to form even larger gel networks (eg, yogurt). The dairy industry utilizes three basic microsized and nanosized structures (casein micelles, fat globules, whey proteins) to build all sorts of emulsions (butter), foams (ice cream and whipped cream), complex liquids (milk), plastic solids (cheese), and gel networks (yogurt) (Aguilera & Stanley, 1999). In fact, dairy technology is not just a microtechnology but also a nanotechnology, and it has existed for a long time.

The novel properties of nanomaterials offer many new opportunities for the food industry. (Choi et al., 2008). Different types of functional nanostructures can be used as building blocks to create novel structures and introduce new functionalities into foods. These include: nanoliposomes, nanoemulsions, nanoparticles and nanofibers. Weiss has described several of these structures, their actual and potential uses in the food industry (Weiss et al., 2006); (McClements et al., 2009). According to the currently available information, nanomaterials used in food applications include both inorganic and organic substances.

2.3.3.1 Chitosan (CS) nanoparticles

Chitosan (CS) is a linear polysaccharide with negative charge arising from its amine groups which are obtained by deacetylation of chitin and it is in turn interacts with many negatively charged substances (Fig.2.2). CS is a polysaccharide found in bone, cartilage, connective tissue, crustacean shells, insect cuticles and the membranes of fungi, composing of acetylgalactosamine and glucuronic acid residues alternately linked to each other by the b1–4 and b1–3 bonds, respectively (Huang et al., 2010).

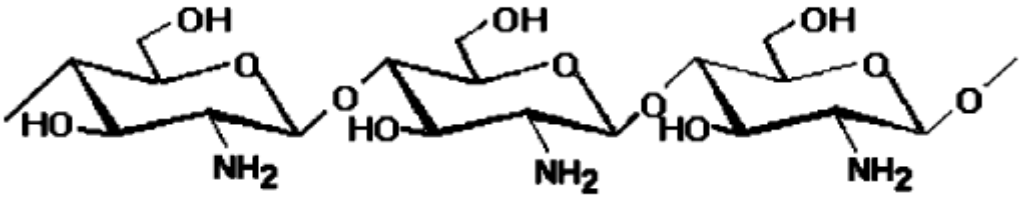


Figure 2.2 Structure of chitosan

It is soluble at pH < 6, makes a gel structure by ionotropic gelation. Chitosan polymers can further polymerize by means of cross-link formation in the presence of anions, polyanions (Klien *et al*., 1983), polyphosphates (Anal & Stevens, 2005) and sodium alginate (Anal et al., 2003).

CS is gaining importance in the food and pharmaceutical field because of its unique polymeric cationic character, good biocompatibility, non-toxicity and biodegradability.

Chitosan has been shown to possess mucoalhesive properties (Lehr *et al*., 1992; Needleman and Smales, 1995; Rillosi and Buckton, 1995; He *et al*., 1998; Shimoda *et al*., 2001; Kockisch *et al.,* 2003) due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces. These properties may be due to: strong hydrogen bonding groups like –OH,–COOH (Schipper et al., 1997); strong charges (Dodane *et al*., 1999); high molecular weight (Schipper *et al*., 1996; Kotze *et al.,* 1998); sufficient chain flexibility (He *et al*., 1998); and surface energy properties favoring spreading into mucus (Lue_en *et al*., 1994).

Commercially, chitosan is available in the form of dry flakes, solution and fine powder. It has an average molecular weight ranging between 3800 and 2,000,000 and is from 66 to 95% deacetylated (removal of the acetyl group) (Kas, 1997). Particle size, density, viscosity, degree of deacetylation, and molecular weight are important characteristics of chitosan which influence the properties of pharmaceutical formulations based on chitosan.

Zhou *et al*., in 1998 reported that, chitosan has been used for coating of gelatin capsules according to its efficiency for increasing viability of probiotic cells is not satisfactory, it is most often used as a coat/shell, but not capsule. And, low-concentration 0.4% of chitosan solution is applied for shell-making on capsules such as gelatin (Zhou *et al*., 1998).

Other researchers has been reported, that mixture of chitosan and hexamethylene diisocyanate or chitosan and glutaraldehyde make stronger coats compared to chitosan alone and that withstands the low pH in the stomach and upon arrival in the small bowel it is degraded by the rich colonic microbiota (Groboillot *et al*., 1993). In order to coat chitosan on alginate capsules, solutions of microbeads with alginate capsules should be dripped into a chitosan-calcium chloride mixture, because the presence of calcium ions is necessary for proper coating (Krasaekoopt et al., 2004).

In fact, (de Vos et al., 2010) reported, chitosan is being applied for the targeted release of both bioactive molecules and living cells. So, in food industry chitosan used to release probiotics because of its high compatibility with living cells. However, chitosan has some diadvantages like having inhibitory effects on LAB (Groboillot *et al*., 1993).

2.3.3.2 Encapsulation of probiotics in CS

Krasaekoopt, Bhandari, and Deeth (2003, 2004) evaluated the survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt and in UHT- and conventionally treated milk during storage. They used *L. acidophilus* 547, *L. casei* 01 and *B. bifidum* 1994 as model organisms for their study. The survival of the encapsulated bacteria was higher than that of the free cells by approximately 1 log. The number of probiotic bacteria was maintained above the recommended therapeutic

minimum (10^7 cfu/g) throughout storage for the lactobacilli but not for the *Bifidobacterium* spp. Lee *et al*., (2004) carried out a similar study and compared various chitosans (different molecular weights) for coating conventional alginate beads. They investigated the effects of chitosane alginate microparticles on the survival of *L. bulgaricus*

KFRI763 in simulated gastric juices and simulated intestinal fluid and on their stability during storage at 4 and 22 ºC. The probiotic loaded in alginate microparticles was prepared by spraying a mixture of sodium alginate and cell culture into a $CaCl_2$ chitosan solution using an air atomizing device.

When the microorganism was exposed to gastric fluid (pH 2.0) for 1 h, none survived. In contrast, an impressive and high survival rate was obtained when the sprayed particles were coated with chitosan. They concluded that the microencapsulation of LAB with alginate and a chitosan coating offers an effective means of delivering viable bacterial cells to the colon and maintaining their survival during refrigerated storage.

Krasaekoopt *et al*., (2006), also studied the survival of probiotics encapsulated in chitosan-coated alginate beads in yogurt and found that the survival of the encapsulated probiotic bacteria was higherc thanthat of free cells by approximately a factor of ten. Furthermore, Zhou *et al*., (1998) reported that suspending alginate capsules in a low molecular weight chitosan solution reduced cell release by 40%. Low molecular weight chitosan diffused more readily into the calcium alginate gel matrix, resulting in a denser membrane than with high molecular weight chitosan which reported by McKnight *et al*., 1988.

Liserre *et al*., (2007), observed that, the *Bifidobacterium animalis* subsp. *lactis* was entrapped in alginate (as control), alginate-chitosan, alginate-chitosan-Sureteric and alginate-chitosan-Acryl-Eze (Liserre et al., 2007). Survival *in vitro* and release of *Bifidobacteria* from the microparticles were investigated under conditions simulating gastrointestinal fluids covering the pH range from 1.5 to 7.5, with and without pepsin, pancreatin, and bile. All types of microcapsules protected *B. animalis*, but the use of chitosan and enteric polymers in the formulation of the beads, especially Acryl-Eze, enhanced the beneficial effects of the microencapsulation technique.

Besides promoting the controlled release of *Bifidobacterium* spp in simulated gastrointestinal juices, the microencapsulation with enteric polymers improved the survival rate of these microorganisms.

2.3.3.3 Chitosan nanoparticles by used emulsification technique

CS nanoparticles have been developed to encapsulated proteins such as bovine serum albumin, tetanus and diphtheria toxoid (Soppimath et al., 2001), vaccines (Vila et al., 2004) anticancer agents (Janes et al., 2001), insulin (Anal & Stevens, 2005), and nucleic acids (Mansouri *et al*., 2004; Mao, H-Q. *et al*., 2001) Chitosan considerably enhanced the absorption of peptides such as insulin and calcitonin across the nasal epithelium (Illum *et al*., 1994).

The methods proposed to prepare chitosan nanoparticles are based on the spontaneous formation of complexes between chitosan and polyanions (Calvo *et al*., 1997) or the gelation of a chitosan solution dispersed in an oil emulsion (Tokumitsu *et al*., 1998).

Chitosan nanoparticles obtained by the formation of a spontaneous complex between chitosan and polyanions such as tripolyphosphate (Calvo *et al*., 1997) have small diameters (200–500 nm) and show a quasi spherical shape under transmission electron microscopy. Chitosan nanoparticles produced by a promoting gelation in an emulsification-based method as show in Figure 2.3, results in a diameter of 400 nm. Compared with the previously described method, this technique has a major disadvantage of involving organic solvents during the isolation of the particles; they are difficult to remove and may cause toxicity (Vauthier & Couvreur, 2000).

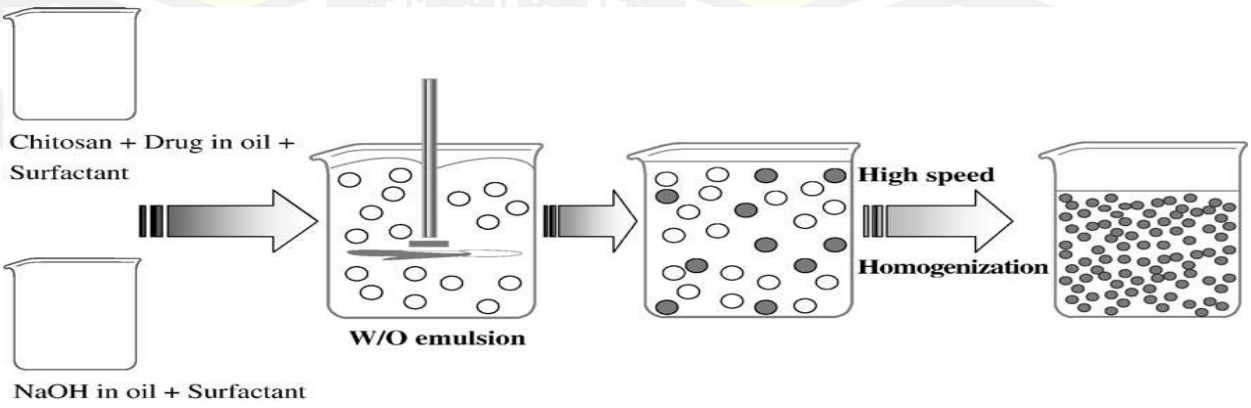


Figure 2.3 Chitosan nanoparticles preparation by the emulsification technique

2.3.3.4 Chitosan Succinate cross-linking with pyirdin

Chitosan- Succinate was developed as one potential solution for this pH-dependency problem (Taha and Aiedeh, 2000a). Also, used of ferric ion- cross-linking hydroxamated alginic acid in sustained drug delivery (Taha and Aiedeh, 2000b), combined with two recent reports indicating the use of ferric ion- cross-linking carboxymethylchitin and carboxymethylcellulose as drug releasing polymeric matrices (Mi *et al.*, 1997; Sungur, 1999), prompted us to develop, and evaluate the drug releasing properties of, polymeric beads based on iron cross-linking chemically modified chitosan.

Recent reports indicating the indefinite stability of iron (III)–amide complexes in various protic solvents including water (Marlin and Mascharak, 2000) suggested the possibility of producing pharmaceutically useful polymeric matrix based on chitosan-succinate cross-linking with ferric ion (CS-Fe), as shown in Figure 2.4.

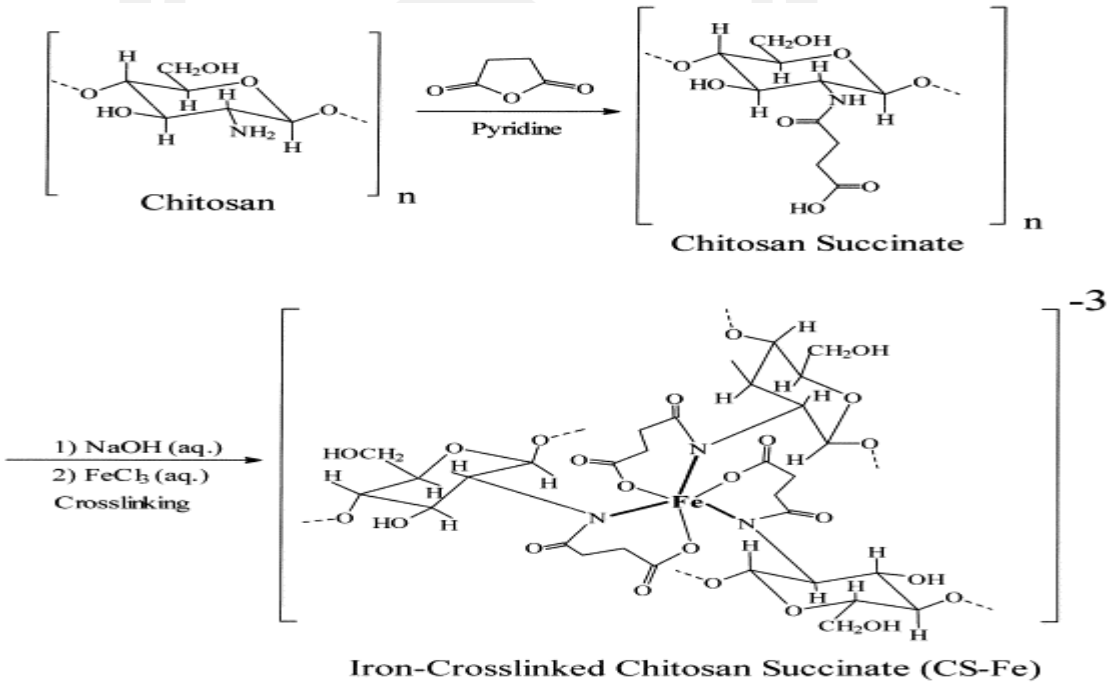


Figure 2.4 Chitosan-succinate cross-linking with ferric ion (CS-Fe)

On the other hand, hydroxamic acids are known to form particularly stable and relatively pH-independent complexes with ferric ions, i.e., within physiological limits (Feng *et al*., 1994; Lee and Hong, 1995). Such complexes were used for analytical (Connors, 1982a) and clinical purposes (Faa and Crisponi, 1999), as well as in the development of novel sustained release polymeric matrices (Taha and Aiedeh, 2000b).

2.3.3.4.1 The synthesis and characterization of HCS

The conjugation of CS to hydroxylamine was achieved by first activating the carboxylic acid moieties with DCCI, followed by quenching the reaction mixture with hydroxylamine hydrochloride. The added DCCI was theoretically calculated to activate only 60% of the available carboxylic acid groups (Taha and Aiedeh, 2000b). The protruded free succinic carboxylic acid groups are expected to possess superior reactivity towards DCCI. Hydroxylamine hydrochloride was added in 20-fold excess (compared to DCCI) to ensure the forwardness of the reaction, particularly under the satirically hindering environment of the polysaccharide.

2.3.3.5 Genipin as cross-linking of Chitosac Succinat and probiotics bacteria

Genipin is a new cross-linker extract from Gardenia jasminidides or Genipa americana. The fruits from these plants are eaten raw and extracts used for medicinal purposes and as food colourants (Butler et al., 2003). Genipin has been approved for use as an additive in pharmaceuticals and foods in countries such as Japan, Korea and Taiwan (Nickerson et al., 2006) but has yet to be approved in Canada and USA.
In processing when using the same concentration of gelatin, (Annan et al., 2007) found the size of uncoated gelatin microspheres cross-linked with 2.5 mmol l^{-1} genipin to be 41.2 ± 13.2 lm compared to 49.0 ± 12.7 lm obtained in the present study. A lower concentration of Genipin (1.25 mmol l^{-1}) was used in the study which may have increased swelling of microspheres due to the uptake of water. Cross-linking of gelatin microspheres controls water uptake and swelling (Ugwoke and Kinget, 1998), hence a higher cross-linker concentration will minimize changes in shape and size of microspheres.

These microspheres were physically stronger as indicated by their resistance to degradation in simulated gastro-intestinal juices and required longer homogenization times for complete disintegration. It is possible that entrapped cells were not released

or were injured during homogenization thus leading to the decrease in the apparent EY obtained for these microspheres.

Chandy and Sharma, (1990) were documented that, chitosan is a weak base and is insoluble in water and organic solvents, however, it is soluble in dilute acidic solution (pH < 6.5), which can convert the glucosamine units into a soluble form R–NH^{3+}. It gets precipitated in alkaline solution or with polyanions and forms gel at lower pH. It also acts as flocculant for the treatment of waste water (Demarger-Andre and Domard, 1994).

Membranes prepared from chitosan have shown greater permeability for acidic drugs than basic drugs (Sawayanagi *et al*., 1982b).

Reacting chitosan with controlled amounts of multivalent anion results in cross-linking between chitosan molecules. The cross-linking can be achieved in acidic, neutral or basic environments depending on the method applied. This crosslinking has been extensively used for the preparation of chitosan microspheres (Sinha et al., 2004).

Figure 2.6 show, different methods which have been used or the preparation of chitosan microspheres.

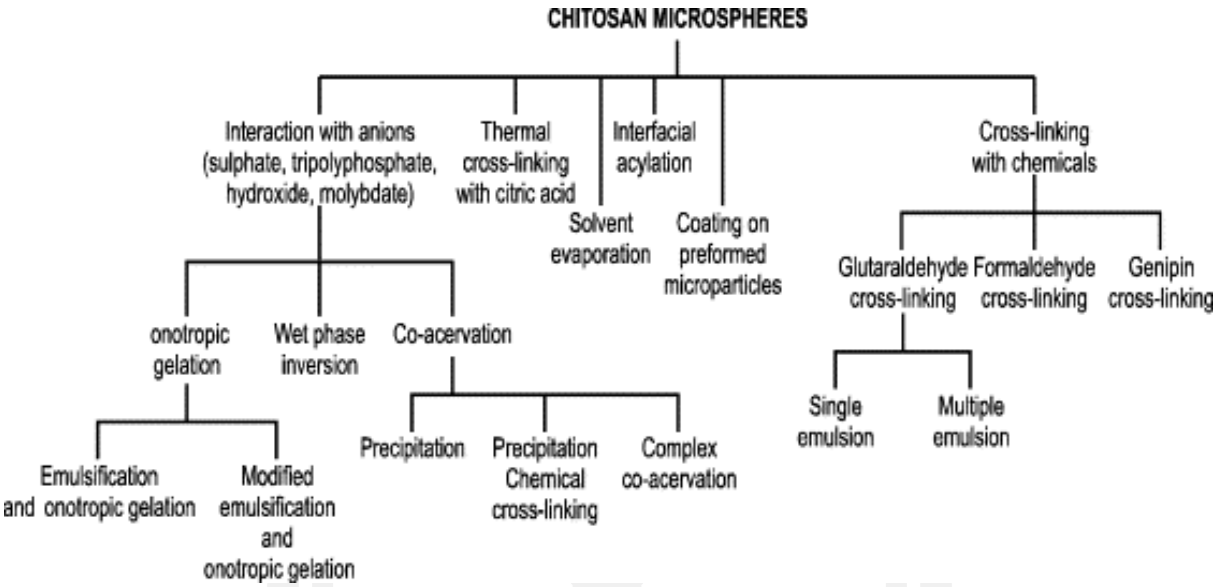


Figure 2.6 Methods for preparation of chitosan microspheres

Annan *et al*., (2007), found that the effect of different concentrations (0, 2.5, 5.0, 7.5 and 10.0) of genipin on the viability of free cells of Bifidobacteria did not change, indicating no significant ($p > 0.05$) effect of Genipin to the viable cells. Also, in the control experiments, genipin concentrations were replaced with ethanol equivalent to the amount that Genipin was dissolved in, and no significant difference ($p > 0.05$) in counts of bacteria was observed. However, Akao *et al*., (1994) been found not to be cytotoxic to human cells and is widely used in herbal medicine in Asia and in the fabrication of food dyes (Touyama *et al*., 1994).

2.3.4 Chitosan Cross-linking with other chemicals

There are varieties of Cross-linking agents used for preparation of chitosan microspheres like: glutaraldehyde, formaldehyde and Genipin.

2.3.4.1 Emulsion cross-linking method

In this process chitosan solution (in acetic acid) is added to liquid paraffin containing a surfactant resulting in formation of water in oil w/o emulsion. A cross-linking agent of varying amount is added depending upon the cross-linking density required (Thanoo *et al*., 1992; Jameela and Jayakrishnan, 1995; Akbuga and Bergisadi, 1996, 1999; Al-Helw *et al*., 1998; Jameela *et al*., 1998; Denkbas *et al*., 1999). The microspheres formed are filtered, washed with suitable solvents and dried.

The cross-linking with a naturally occurring agent, that the Genipin cross-linked chitosan microspheres had a superior biocompatibility and a slower degradation rate than the glutaraldehyde-cross-linked chitosan microspheres (Mi *et al*., 2002),. Therefore, Genipin-cross-linked chitosan microspheres can be a suitable polymeric carrier for long-acting injectable drug delivery.

There are many factors that can be affected on the entrapment efficiency of the drugs in the chitosan microspheres such as: nature of the drug, chitosan concentration, drug polymer ratio, stirring speed. Generally a low concentration of chitosan shows low encapsulation efficiency (Orienti *et al*., 1996). However, at higher concentrations, chitosan forms highly viscous solutions, which are difficult to process. But, many reports have shown that entrapment efficiency increases with an increase in chitosan concentration. This may be explained on the basis that an increase in viscosity of the chitosan solution with increase in concentration prevents drug crystals from leaving the droplet.

2.3.4.1.1 Parameters affecting the release characteristics of drugs from chitosan microspheres

Sinha *et al.*, (2004) were reported, that many factors can be affect on the drug release behavior from chitosan microspheres such as: concentration and molecular weight of the chitosan, the type and concentration of cross-linking agent, variables like stirring speed, type of oil, additives, cross-linking process used and drug chitosan ratio.

2.3.4.1.2 Effect of molecular weight of chitosan

Drug release studies from chitosan microspheres have generally shown that the release of the drug decreases with an increase in molecular weight of chitosan. For example, Shiraishi *et al.*, (1993) found the effect of molecular weight of chitosan hydrolysate on the release and absorption rate of indomethacin from gel beads. The release rate of indomethacin was found to decrease with increasing molecular weight of chitosan.

Same results were found when oxytetracycline release from acylated chitosan microspheres was studied. It was observed that the release decreased with increasing

molecular weight of chitosan (Mi *et al*., 1997). Similarly, Al-Helw *et al*., (1998) reported that the release of phenobarbitone from crosslinked chitosan microspheres was slower from high molecular weight chitosan when compared to medium and low molecular weight chitosan. However, Genta *et al*., (1998) reported that the fastest ketoprofen dissolution profile from chitosan microspheres was obtained from medium molecular weight chitosanm due to swelling behavior of chitosan microspheres. An increase in molecular weight of chitosan leads to an increase in viscosity of the gel layer, which influences the diffusion of the drug as well as erosion of the microspheres.

2.3.4.1.3 Effect of concentration of chitosan

Nishioka *et al*., (1990) reported that the example rate of cisplatin release reduced with the increasing concentration of chitosan. Aiedeh *et al*., (1997) observed that the method of chitosan interfacial cross-linkage by ascor byl palmitate in water/oil dispersion was suitable to produce biodegradable system for insulin. The microcapsules obtained had release kinetics approaching zero order and a release rate, which could be increased by decreasing the chitosan content in the preparative solution.

2.3.4.2 Chitosan microspheres with Genipin as cross-linking

Touyama *et al*., (1994) and Fujikawa *et al*., (1987) have reported that Genipin can spontaneously react with amino acids or proteins to form dark blue pigments. In vitro evaluation of the cytotoxicity of genipin for biological tissue fixation has been investigated in a previous study (Sung *et al*., 2000). It was found that genipin is about 5000–10,000 times less cytotoxic than glutaraldehyde. In addition, it was reported that the genipin-fixed tissue had a comparable mechanical strength and resistance against in vitro enzymatic degradation as the glutaraldehyde fixed tissue (Sung *et al*., 1998). The results of these studies prompted us to evaluate the feasibility of using genipin to prepare biodegradable chitosan microspheres for long-acting drug delivery application.

Different ways are used to prepare micro/nanoparticles of chitosan for drug delivery such as emulsion cross-linking, coacervation/ precipitation, spray-drying

etcetera (Agnihotri, Mallikarjuna and Aminabhavi, 2004). (Yuan et al., 2007) mixed chitosan with bovine albumin and genipin to make cross-linked microspheres. The degree of cross-linking of the chitosan microspheres and their swelling ratio increased with increased cross-linking time or Genipin concentration. The chitosan microspheres cross-linked with Genipin released albumin more slowly than the plain ones. The drug release rates and the swelling ratio of chitosan microspheres were controlled by the degree of crosslinking. (Liang et al., 2003) and (Tannock et al., 1989) prepared chitosan microspheres by a water-in-oil dispersion method, using genipin as a cross-linker. The chitosan microspheres with indomethacin were cross-linked for various time periods and at different pH values.

Their results showed that the release of indomethacin from the chitosan microsphere prepared from a chitosan–indomethacin suspension at high pH exhibited increased dissolution rate compared with that of chitosan microspheres prepared from a chitosan– indomethacin suspension at low pH due to the difference in the ionization of indomethacin. The release of indomethacin from the microsphere was influenced by the degree of cross-linking and by the chitosan/indomethacin ratio.

Chitosan–Genipin microspheres were obtained by cross-linking in inverse emulsion, and were observed to swell significantly in water at pH values below 6.5 but to a smaller extent at pH values above 6.5.

Mexican and South American plants, as a ‘‘beautifully crystalline substance” were defined the chemical structure of genipin (Fig. 2.6).

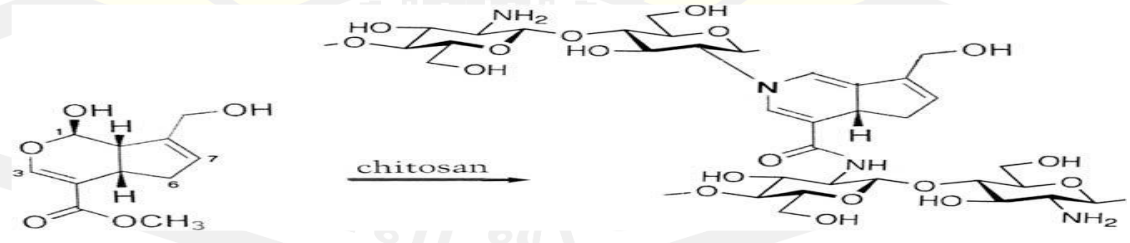


Figure 2.7 Genipin reacts with chitosan to yield two main cross-linking reactions. On the right, two chitosan chains (represented by their structural units) are cross-linked by one mole of genipin: the formula shows the two newly formed chemical groups, namely the monosubstituted amide and the tertiary amine. An additional reaction is the homopolymerization of genipin.

2.3.4.2.1 Reaction mechanism and products of chitosan cross-linking with Genipin

The cross-linking reaction mechanisms for chitosan with genipin are differs at different pH values. Under acidic and neutral conditions, a nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurs, followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehydo group. In other words, Genipin acts as a dialdehyde but its condensation products are much more stable compared to glutaraldehyde (Mi, Shyu, and Peng, 2005; Sung, Huang, Huang, and Tsai, 1999). In the product, short chains of condensed Genipin act as crosslinking bridges. However, under basic conditions, the ring-opening reaction of Genipin occurs via a nucleophilic attack by hydroxyl ions in aqueous solution to form intermediate aldehyde groups, which subsequently undergo aldol condensation. The terminal aldehyde group on the polymerized Genipin undergoes a Schiff reaction with the amino groups on chitosan to form cross-linked networks. Therefore, the pH condition plays an important role in influencing the cross-linking reactions.

Butler, Ng, and Pudney, (2003) found that the fastest reaction is a nucleophilic attack of an amino group of chitosan to carbon 3 of Genipin which results in the opening of the dihydropyran ring and the formation of a tertiary amine, for example: a Genipin derivative linked to a glucosamine unit. The subsequent slower reaction is a nucleophilic substitution of the ester group of Genipin to form an amide. In the same time, polymerization can take place between Genipin molecules already linked to amino groups of chitosan which could lead to the cross-linking of amino groups by short Genipin copolymers.

The dark-blue coloration due to the hydrogels exposed to air is associated with the oxygen radical-induced polymerization of Genipin as well as its reaction with amino groups.

The hydrogels were cross-linked, and the swelling behavior of the resulting hydrogels was studied in deionized water at 25, 35 and 45 ºC and in media of various pH values at 25 ºC. The swelling behavior of the gels was found to depend on temperature, pH value, and amount of poly (vinyl alcohol) present in the gel.

Nand, Rohindra and Khurma (2007) observed that the solutions with chitosan: poly(vinyl alcohol) (mass ratio 1 and constant genipin quantity), turned into hydrogels exhibiting a sharp viscosity rise after ca. 500 min at 25 ºC, whilst in the absence of poly(vinyl alcohol) the chitosan–Genipin hydrogel was formed after ca. 400 min. Thus poly (vinyl alcohol) delayed the crosslinking reaction. PVA-containing hydrogels prepared in Petri dishes were dried at 55 ºC to produce 0.2 mm thick blue films. Similar semi-interpenetrating polymeric networks were prepared with chitosan and PVP. Genipin was used in all cases at the 0.5 w/v concentration, which is largely lower than the chitosan concentration reported by (Khurma et al., 2005).

The shell-like cross-linked chitosan membranes were affected by the reaction of temperature but not by the reaction of time and Genipin concentration. Cross-linking at higher temperatures tended to form relatively thinner membranes. The construction of the genipin-cross-linked chitosan membranes could be varied by manipulation of the Genipin cross-linking conditions.

2.4 Principles of targeted release in the gastrointestinal tract

When proposing targeted release of bioactive food components in the gut, we have to discuss the processes that facilitate the dissolution of capsules in specific parts of the gastrointestinal tract.

The first is the strong peristaltic waves in the colon. In the upper part of the gastrointestinal tract the pressure is relatively low due to the presence of large amount of fluids in the stomach and the small intestine. By increasing the mechanical resistance of capsules as such that they can withstand the pressure in the stomach and small intestine but will break more distal when the pressure in the lumen is increasing, we can facilitate the release of bioactive molecules in the lower part of the gastrointestinal tract. The other mechanism is to use the pH variations and the time of transport in the gastrointestinal tract. By applying pH sensitive polymers that stay intact in the stomach but will be susceptible for digestive enzymes we can facilitate the release in specific parts of either the small intestine or large intestine. The third, and most promising method according to the present authors, is to take advantage of the precise, local activity of enzyme-systems produced by the microbiota. The bacterial populations in the gut and the associated enzyme activities are specific for

different parts of the gastrointestinal tract and allow for precise delivery of bioactive food components.

The microbiota produces a wide range of enzymes such as b-glucuronidase, b-xylosidase, a-arabinosidase, b-galactosidase, nitroreductase, azoreductase, deaminase, urea hydroxylase
(Kinget, Kalala, Vervoort and van den, 1998; Kosaraju, 2005; Shantha, Ravichandran and Rao, 1995). The variations in redox potential induced by the microbiota of the colon can be applied as a specific tool for solving capsules and delivery of bioactive food components.

2.4.1 Selecting the in *Vitro* Conditions for Cells Release

When probiotics are encapsulated, it is essential to check two conditions. First, ensure that the protective device of probiotics is reliable in media simulating the gastric fluid, and then ensure that the encapsulated probiotics are released in media simulating the intestinal fluid.
These models evaluate the tolerance of probiotics to acidic media, bile and enzymes. There are generally two types of experimental models, known under the names of “conventional model” and “dynamic model”.

The dynamic model differs from the conventional model because it is semi-automated. The conventional model simulates either the stomach or the gut. It consists of a single reactor (glass container) containing the simulated gastric fluid or the simulated intestinal fluid. The dynamic model consists of a series of reactors with respective volume for stomach and gut, in which the temperature was maintained at 37 °C and the pH was automatically controlled to maintain values of gastric and intestinal pH. All reactors were continuously stirred, and the sterile culture medium was fed to gastric reactor by a peristaltic pump which sequentially supplied the gut reactor. Flow rate was set to obtain the mean transit time throughout the model (Maestro et al., 2008); (Barmpalia-Davis et al., 2008).The in *vitro* conditions used for the simulation of the stomach are detailed in Table 2.3.

Table 2.3 In vitro conditions most often used to simulate the stomach

Gastric fluid	pH values	Pepsin content (g/L)	Exposure time (min)	References
NaCl (2 g/L)	1.55	0	180	[18]
	2 and 3	0	120	[19]
	1.55	0	120	[32]
	2	0	60	[48]
NaCl (5 g/L)	2	3	60	[49]
	2	3	180	[50]
	2 and 3	3	240	[51]
NaCl (8.5 g/L)	2.5	3	90	[52]
	2 and 3	3	90	[53]
	2	0	120	[40]
NaCl (9 g/L)	1.8	3	120	[20]
HCl (3.65 g/L)	1.1	0	120	[54]
	1.9	0.26	30	[55]
	2 and 3	0	120	[56]
MRS broth (55 g/L)	2	0	120	[57]
Peptone broth (7.5 g/L)	2 and 3	0.3	20	[58]
Cheese broth (8.5 g/L)	2.5 and 3	0.016	120	[59]
	2 and 3	0	180	[60]
Skimmed milk (12 g/L) glucose (2 g/L) yeast extracts (1 g/L) and cysteine (0.05 g/L)	2 and 3	0	60	[10]
	2 and 3	0	180	[41]
Glucose (3.50 g/L) NaCl (2.05 g/L) KCl (0.37 g/L) KH_2PO_4 (0.60 g/L) $CaCl_2$ (0.11 g/L) porcine bile (0.05 g/L) and lysosyme (0.10 g/L)	2	0.013	90	[61]

When reading Table 2.3, a preference for the NaCl medium was noted. Most authors have suggested this. However, concentrations of 2 and 5 g/L of NaCl used seem insufficient to maintain the isotonicity of the medium. The American society of microbiology (ASM) recommends saline solution at 9 g/L in the microbiological procedures such as microbial cells suspension or dilution, and tolerance tests to antimicrobial substances (Chapin & Lauderdale, 2003). NaCl provides an isotonic medium that maintains the integrity and the viability of the microbial cells. The ASM also reported that phosphate can be added to NaCl medium to buffer it. In this case, the concentration of NaCl should be reduced (8 to 8.5 g/L). Phosphate addition

provides a stable pH because of its buffering capacity, which helps to maintain cell viability.

Hovgaard *et al*., (1996) noted that regarding the gastric fluid pH, it should be noted that the values vary between 1 and 3. This pH range covers the values generally observed in human's stomach Pepsin was sometimes used as a model of gastric enzyme. However, (Hersey, 1994) documented that no information is yet available about the true concentration of this enzyme in the stomach. This reflects the fact that pepsin is secreted in the form of pepsinogen (inactive form) which is then activated in pepsin by the presence of acidic medium. Pepsin activity requires a pH under 5.6 (Hersey *et al*., 1994;Tobey *et al*., 2001). Any artificial gastric fluid must include this enzyme in its composition.

Finally, regarding the exposure time, several values were observed, ranging from 20 min to 240 min. However, clinical studies have shown that a period of 120 min was sufficient to ensure the gastric emptying of 90% of a liquid meal (Malmud *et al*., 1986) and 60% of a semi-solid meal (Malmud *et al.*, 1986 ; Singh *et al*., 2006). An exposure time of 120 min is reasonable for the stay of probiotics in an artificial gastric medium. After a stay of probiotics in the stomach, the gut is naturally the second favorite place, so tests are conducted in this part of the gastro-intestinal tract. The Table 2.4 explains the *in vitro* conditions used for the simulation of the gut.

Table 2.4 In vitro conditions most often used to simulate the gut.

Intestinal fluid	pH values	Bile (g/L)	Enzymes (g/L)		Exposure time (min)	References
			Pancreatin	Trypsin		
$NaHCO_3$ (25.2 g/L)	6.5	40	3.5	0.1	240	[47]
NaCl (5 g/L)	8	45	1	0	180	[50]
Na_2HPO_4 (2.84 g/L)	7.5	150	1.95	0	360	[55]
PBS* (1 mol/L)	8	1	1	0	180	[58]
PBS (np**)	7.4	2	1	0	180	[69]

* Phosphate Buffer Saline ** Unspecified. PBS defines a medium composed of various salts whose proportions vary from one author to another.

Bile and pancreatic enzymes are present in the lumen of the gut reported by (Cuillerier et al., 2002) and (Ouwehand & Vesterlund, 2003) so only studies involving the presence of bile and at least one pancreatic enzyme have been emphasized. Table 4 show, the sodium salts are only used as intestinal fluid at various concentrations. In reality, it consists mainly of NaCl in which other salts were added: NaCl (8.5 g/L),

K_2HPO_4 (1.1 g/L) and KH_2PO_4 (0.32 g/L) (Gerhardt, P., 1981). Sometimes it consists of NaCl (8 g/L), Na_2HPO_4 (1.44 g/L) and KH_2PO_4 (0.24 g/L) (Chapin & Lauderdale, 2003). One author used it incorrectly to refer to an aqueous solution containing only sodium chloride. In many cases, the composition of phosphate buffered saline (PBS) was not mentioned (FSA protocol., 2005; Duc *et al*., 2004). Moreover, it can be a medium in which the salt concentrations have been adjusted or supplemented by other salts as needed (NCCLS., 2000). The pH values used are between 6.5 and 8. These values reflect the pH usually met in the gut (Vandamme et al., 2002).

In conclusion, the concentrations of bile and enzymes, no published data allows specifying the exact levels, which may explain the variations observed from one author to another.

The studies summarized in Tables 3 and 4 clearly show a lack of standard protocol in establishing the *in vitro* conditions for simulating the stomach or the gut.

2.5 Cell load of the beads measurements

2.5.1 Scanning electron microscopy (SEM)

Sultana *et al*., (2000) found, the best way to reach a mean of the beads cell load (number of the cells in each bead) is by direct observation of the beads from different samples by Scanning Electron Microscopy SEM method.

Electron microscopy (SEM or TEM) is an effective technique to provide evidence of the presence of probiotics in capsules or beads and to assess the bacterial loading reported by (Gbassi et al., 2009).

The challenge of equipment refers to beads or capsules sizes, which are crucial and should be carefully controlled. Small capsules or beads under controlled conditions will not affect the texture of food products (Rokka & Rantamäki, 2010). Most of the procedures of probiotic encapsution technique reported involve emulsification technology and extrusion technology (also called ionotropic gelation).

2.5.2 Zeta potential

Tiede *et al*., (2008) documented that the Zeta potential measures the overall charge a particle acquires in a specific medium giving an indication of the stability of a system. If particle have a large Zeta potential (negative or positive), they will repel

each other, this means a higher stability than a neutral particles charge. The Zeta potential is a measure of the net charge, and there may be significant charge heterogeneities that can lead to aggregation, even though, the net Zeta potential suggests otherwise. (Geze et al., 2004), noted that many works were done on characterization of nanoparticles by DLS, cryo-transmission electron microscopy, and Zeta potential within the food field. (Mortazavian et al., 2007), found that the amphiphilic b-cyclodextrins (bCDa) were synthesized by statistically grafting hexanoyl carbon chains. The obtained derivative was used to prepare submicronic colloidal nanosphere suspensions using a nano-precipitation method. The fresh suspensions contained particles with diameters ranging from 60 to 100 nm.

The long-term stability of the aqueous nano-dispersions was investigated. An unexpected good physical stability of the suspensions after 3-year storage at room temperature was observed. This behavior appears to be related to the small size and structural organization of the nanoparticles.

2.6 Tomato Ketchup

Ketchup has moderate health benefits. Ketchup is a source of lycopene, an antioxidant which may help prevent some forms of cancer. This is particularly true of the organic brands of ketchup, which have three times as much lycopene. Ketchup, much like marinara sauce and other cooked tomato foods, yields higher levels of lycopene per serving because cooking increases lycopene bioavailability (Ishida & Chapman, 2004). US government standard regulations for 1901 ketchup state that ketchup includes: cooked and strained tomato sauce,vinegar, sugar or a similar sweetener, salt, onion or garlic flavors, various spices such as allspice, nutmeg, cinnamon, cloves, mace, ginger and cayenne pepper

Tomato ketchup is often used as a condiment with various dishes that are usually served hot, including chips/fries, hamburgers, sandwiches, hot dogs, eggs, and grilled or fried meat. Ketchup is sometimes used as a basis or ingredient for other sauces and dressings. Ketchup is also used as a flavoring for things such as potato chips, and this variety of chips is one of the most-popular flavors in Canada.

CHAPTER 3

METHODOLOGY

This research was an experimental research to determine survival of encapsulate (with Genipin and without Genipin) and free cells of probiotic bacteria Bb-12 by using Chitosan Succinate as nanoparticals in Vitro (Simulated Gastric Juice with 0.3% of pepsin, Simulated Gastric Juice without pepsin, Simulated Intestinal Juice, Simulated Colonic Juice) and in Tomato Ketchup during shelf life. Therefore, experimental plan design was followed by:

3.1 Experimental plan

3.2 Instruments and equipments

3.3 Materials and Chemicals

3.4 Methods

3.5 Statistical analysis

3.1 Experimental plan

This experiments were done in Sharjah Municipality Food Laboratory and United Arab Emirates University, and this research was divided into five experiments including (1) preparation of Chitosan Succinate conjugates, (2) determinate of particles size and charges by using Scanning Electron Microscope (SEM) and Zeta potential, (3) preparation of Bb-12 cells for encapsulated cross-linked Chitosan Succinate microspheres with and without Genipin as cross-linked,(4) survival of free and encapsulated Bb-12 cells. In vitro which based on: simulated gastric juice with 0.3% Pepsin and without Pepsin, simulated intestinal juice and in simulated colonic juice and (5) survival of free and encapsulated cells in Tomato ketchup.

The zeta potential of the chitosan succinate microspheres was determined in distilled water, using Malvern 3000 HS zetasizer (MalvernInstruments, Worcestershire, UK). The microspheres were dispersed in paraffin oil and particle size was analyzed after sonification of the sample.

The shape and surface characteristics of the microspheres were studied by SEM (Leo 435VP, Cambridge, UK).

3.2 Instruments and equipments

3.2.1.1 Scanning electron microscopy (SEM). The shape and surface characteristics of the microspheres were studied by SEM (Leo 435VP, Cambridge, UK).

3.2.2 Ultra Sonic

3.2.3 Centrifuge (SORVALL1 Evolution RC centrifuge)

3.2.4 pH meter

3.2.5 Zeta potation

3.2.6 Water bath

3.2.7 Incubator

3.2.8 GasPak Plus system (gas generator envelopes and anaerobic jars) from BBL (Fisher Scientific, Nepean, ON)

3.2.9 0.5 Mc Farland units to a bacterial concentration of 15×10^7 cfu/ml.

3.2.10 glass wears

3.2.11 Propeller stirrer

3.3 Materials and Chemicals

Materials and Chemicals were divided to:

3.3.1 Preparation of Chitosan Succinate conjugates

3.3.1.1 Low molecular weight chitosan (molecular mass of 70000)

3.3.1.2 Reagent grade succinic anhydride

3.3.1.3 Pyridine were all purchased from Fluka-Aldrich

3.3.1.4 HCl aqueous solution (0.37%, 50 ml)

3.3.1.5 NaOH solution (1.0 M)

3.3.1.6 NaCl aqueous solution

3.3.1.7 Acetone

3.3.1.8 Diethyl ether

3.3.2 Preparation of Bb-12 cells for encapsulated

3.3.2.1 Freeze-dried Bifidobacterium lactis (Bb-12, Chr. Hansen, Denmark)

3.3.2.2 MRS broth (Oxoid Inc., Nepean, ON, Canada)

3.3.2.3 L-cysteine (0.5 g L^{-1}, Sigma, Oakville, ON, Canada)

3.3.2.4 MRS agar (Oxoid)

3.3.2.5 Reingal Solution (RS) (Oxoid Inc., Nepean, ON, Canada)

3.3.3 Preparation cross-linked Chitosan Succinate microspheres with Bb-12

3.3.3.1 Genipin 2.5% was obtained from Challenge Bioproducts Co Ltd, CBC, Taiwan, ROC, deMan,

3.3.3.2 Chitosan succinate polymer was synthesized in house

3.3.3.3 Light liquid paraffin

3.3.3.3 Span®80

3.3.3.4 Deionized distilled water (D.H_2O).

3.3.4 In *vitro* release studies for free and encapsulated cells

3.3.4.1 Simulated Gastric Juice (SGJ)

3.3.4.1.1 With 0.3% Pepsin

3.3.4.1.1.1 NaCl

3.3.4.1.1.2 Pepsin

3.3.4.1.2 Without Pepsin

3.3.4.1.1.1 NaCl

3.3.4.1.3 Simulated Intestinal Juice (SIJ)

3.3.4.1.3.1 Pancreatin

3.3.4.1.3.2 Bile salts

3.3.4.1.3.3 NaCl

3.3.4.1.3.4 KCl

3.3.4.1.3.5 $CaCl_2$

3.3.4.1.3.6 $NaHCO_3$

3.3.4.1.4 Simulated Colonic Juice (SIJ)

3.3.4.1.3.4.1 0.1 M KH_2PO_4

3.4 Methods

The experimental methods in this research study will divided into six experiments following: preparation of Chitosan Succinate conjugates, preparation of cross-linked Chitosan Succinate microspheres with Bb-12, survival of Bb-12 in different conditions of gastric juice (In Vitro), particles size and charges determinate, preparation of cross-linked Chitosan Succinate microspheres with Bb-12, enumeration of free and encapsulated Bb-12 cells

3.4.1 Preparation of Chitosan Succinate conjugates, Dissolved 1.00 g of chitosan (corresponding to approximately 6.20 mmols glucosamine) in HCl aqueous solution (0.37%,50 ml) at room temperature, and succinic solution (6.25 mmol),which prepared by dissolved 0.63 g succinic in 5 ml pyridine was added dropwise with vigorous stirring. The reaction pH was maintained at 7.0 by the dropwise addition of 1.0 M NaOH solution. NaOH addition was continued till the pH was stabilized. After 40 min the reaction was terminated by the addition of NaCl aqueous solution (20%, 200 ml). The resulting precipitate was filtered, washed with acetone and diethyl ether, and desiccated to give chitosan succinate conjugates (Aiedeh, K and Taha, M, 1999)

3.4.2 Preparation of Bb-12 cells for encapsulated, Prior to encapsulation, 1 g of freeze-dried cells were rehydrated in 9 ml MRS broth (Oxoid Inc., Nepean, ON, Canada) with addition of filter sterilized L-cys (0.5 gL−1, Sigma, Oakville, ON, Canada) and incubated at 37 °C for 24 h under anaerobic conditions using the Gas Pak Plus system. Used 1 Mc Farland to obtain a predicted cell density of about 15 x 10^7 colony forming units per ml (cfu ml^{-1}) after harvesting and washing. Cells was harvested by centrifugation at 4000 rpm for 15 min at room temperature. The supernatant was discarded, and the pellet was re-suspended in distilled de-ionized water and centrifuged again. This procedure of washing was repeated thrice, and then the cells were used for encapsulation. The cell suspension was divided in two parts: one part was used for encapsulation and the second part was used as free cells. Cells

were enumerated by pour plating in MRS agar containing filter sterilized L-cys (0.5 gl^{-1}, MRS L-cys agar), and incubating an-aerobically at 37ºC for three days. Anaerobic conditions were achieved using the GasPak Plus system (gas generator envelopes and anaerobic jars).

Fresh cell suspensions were prepared for each experiment and numerated by pour plating in MRS L-cys agar. Plates were incubated under the same conditions as before (Chávarri *et al*., 2010).

3.4.3 Preparation of cross-linked Chitosan Succinate microspheres with Bb-

Two methods are used in this procedure: first, Preparation of Genipin cross-linked Chitosan Succinate microspheres with Bb-12 and the second, preparation Chitosan Succinate microspheres with Bb-12 without cross-linked.

3.4.3.1 Preparation of Genipin cross-linked Chitosan Succinate microspheres with Bb-12, microspheres were prepared from chitosan succinate by cross-linking with genipin on the surface of the emulsion droplets. 20 ml of chitosan succinate polymer (1% w/v) was dissolved in distilled water by Ultra Sonic, filtered to remove any un-dissolved particulate and autoclaved at 121ºC to 15 min. After cooling the aqueous phase was mixed with 2ml of washed cell suspension and taken by a syringe (No. 20) and extruded dropwise in 100 mL of the external oily phase (liquid paraffin) containing 0.2% Span180 and stirring was carried out using propeller stirrer (Remi, Bombay, India) at 1000 rpm. After 15 min, 2.0 mL of Genipin solution was added drop-by-drop to the emulsion and stirring was continued. The emulsion was left stirring overnight at a constant speed and room temperature (25ºC) to allow the completion of the genipin-chitosan siccinate cross-linking reaction. Then centrifuged (SORVALL1 Evolution RC centrifuge) at 2000 rpm for 15 min. The microspheres were harvested with two subsequent washings in a separatory funnel to remove the oil phase and excess crosslinking agent. Then, centrifuging (2000 rpm/10 min) to collect the microspheres.

The microspheres were kept in the washing mixture and stored at 4ºC until ready for use. A genipin stock solution was prepared in 60% (v/v) ethanol and appropriate volumes were added to the chitosan succinate solution to attain concentrations of 2.5 mM Genipin (Annan *et al*., 2007)

3.4.3.2 Preparation of without cross-linked Chitosan Succinate microspheres with Bb-12. microspheres were prepared from chitosan succinate by without crosslinking of ginipin on the surface of the emulsion droplets. 20 ml of chitosan succinate polymer (1% w/v) was dissolved in distilled water by Ultra Sonic, filtered to remove any undissolved particulate and autoclaved at 121ºC to 15 min. After cooling the aqueous phase was mixed with 2 ml of wished cell suspension and taken by a syringe (No. 20) and extruded dropwise in 100 mL of the external oily phase (liquid paraffin) containing 0.2% Span180 and stirring was carried out using propeller stirrer (Remi, Bombay, India) at 1000 rpm. The emulsion was left stirring overnight at a constant speed and room temperature (25ºC) to allow the completion of the genipin-chitosan siccinate cross-linking reaction. Then centrifuged at 2000 rpm for 15 min. The microspheres were harvested with two subsequent washings in a separatory funnel to remove the oil phase. Then, centrifuging (2000 rpm/10 min) to collect the microspheres.

The microspheres were kept in the washing mixture and stored at 4ºC until ready for use.

3.4.4 Particles size and charges determinate by

3.4.4.1 Scanning Electron Microscopy (SEM), the shape and surface characteristics of the microspheres were studied by SEM (Leo 435VP, Cambridge, UK). The samples for SEM analysis were mounted on metal grids using double sided adhesive tape and coated with gold under vacuum before observation (Priya *et al.,* 2011).

3.4.4. 2 Zeta potential, the charge on the bacterial surface after the adsorption of consecutive after coating was monitored using a ζ-sizer (Malvern Zetasizer, Nano-ZS 90). About 10^3 cells/mL were suspended in doubly distilled deionized water, and the ζ-potential was calculated from the mobility measurements (Priya *et al.,* 2011).

3.5.5 Survival of Bb-12 in different conditions of gastric juice (In *vitro*)

3.4.5.1 Survival of free and encapsulated cells In *vitro*:

3.4.5.1.1 Simulated Gastric Juice (SGJ)

3.4.5.1.1.1 With 0.3% Pepsin, simulated gastric juice (SGJ) consisted of 9 g/L of sodium chloride (autoclaved) containing 3.0 g/L of pepsin with pH adjusted to 2.0 with hydrochloric acid (Altman, 1961) 1.0 ml of encapsulated Bb-

12 were mixed in 10 mL of SGJ and incubated for 5, 30, 60 and 120 min at 37 °C with constant agitation at 50 rpm. 1.0 mL of free cell suspensions (10^9) of Bb-12 placed separately in test tubes containing 10 mL simulated gastric pH solution, were incubated at 37 ° C (Lee and Heo, 2000). 1.0 ml added in the duplicate plates, then the plates were incubated anaerobically for three days at 37 ºC and the encapsulated bacteria enumerated as cfu ml^{-1}. 1.0 ml added in the duplicate plates of MRS agar with 0.5 % L-cys, then the plates were incubated anaerobically for three days at 37 ºC and the encapsulated bacteria enumerated as cfu ml^{-1} (Priya *et al*., 2011).

3.4.5.1.1.2 Without Pepsine, simulated gastric juice (SGJ) consisted of 9 g/L of sodium chloride with pH adjusted to 2.0 with hydrochloric acid (Altman, 1961). 1.0 ml of encapsulated Bb-12 were mixed in 10 mL of SGJ and incubated for 5, 30, 60, 120 min and 24 hours at 37 °C with constant agitation at 50 rpm.

1.0 mL of free cell suspensions of Bb-12 ($15x10^7$) placed separately in test tubes containing 10 mL simulated gastric pH solution, were incubated at 37 ° C (Lee & Heo, 2000). 1.0 ml added in the duplicate plates of MRS ager with 0.5 % L-cys, then the plates were incubated anaerobically for three days at 37 ºC and the encapsulated bacteria enumerated as cfu ml^{-1}(Priya *et al*., 2011)

3.4.5.1.2 Simulated Intestinal Juice (SIJ),simulated intestinal Juice (SIJ) was made by dissolving pancreatin (1 g/L) and bile salts (3 g/L) in intestinal solution autoclaved, whose composition is as follows: 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$, 1.386 g/L $NaHCO_3$.4 The pH of the solution was 8. Then, 1.0 ml of encapsulated were transferred into 10 mL. The encapsulated cells were mixed, incubated at 37 °C and sampled 5, 30, 60, 90, 120 min and 24 hours. 1.0 ml added in the duplicate plates of MRS ager with 0.5 % L-cys, then the plates were incubated anaerobically for three days at 37 ºC and the encapsulated bacteria enumerated as cfu ml^{-1} (Priya *et al*., 2011).

3.4.5.1.3 Simulated Colonic Juice (SIJ)**,**

to study survival of encapsulated Bb-12 at simulated colonic Juice, 1.0 ml of encapsulated Bb-12 cells were transferred into 10 mL simulated colonic pH solution (0.1 m KH2PO4, pH 7.4±0.2), mixed gently and incubated at 37 °C, at 0, 30, 60,90,120 min, 1 and 2 days time intervals, aliquots (1mL) were taken and viable

counts were enumerated in MRS agar with 0.5 % CyHCL, then the plates were incubated anaerobically for three days at 37 ºC.

3.4.5.1.4 Survival of free and encapsulated cells In Tomato Ketchup, one brand of Tomato Ketchup was selected from the local market and was free of any preservative which was tested by using HPLC.

15×10^7 cfu/ml of free and encapsulated cells of Bb-12 were added in 5 ml of tomato ketchup which was divided in 5 parts to study the survival of free and encapsulated cells during the shelf life 0, 30,60, 90, 120 min, 1, 2, 3 and after 7 days which has been store at room temperature (25 °C).

3.4.6 Enumeration of free and encapsulated Bb-12 cells, the homogenized samples were diluted to appropriate concentrations and pour plated in MRS L-cys agar. The plates were incubated anaerobically for three days at 37 ºC and the encapsulated bacteria enumerated as cfu ml^{-1}. The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as

$$EY = \frac{N}{N_0} \times 100$$

Where N is the number of viable entrapped cells released from the microspheres, and N_0 is the number of free cells added to the biopolymer mix during the production of the microspheres.

3.5 Sampling and methods

The number of samples of each method was replicated 5 times and the period of experiments took six months, starting from first of Jun 2013 till the end of December 2013.

3.6 Media and reagents

All media and reagents required for the enumeration and identification of BBb-12, Chitosan Succinat, SGJ, SIJ and SCJ were prepared and stored as manufacture requirements.

All items were Sterilized by the Autoclave

3.7. Statistical analysis

Results are presented as mean ± standard deviation (SD) of replicated determinations. Data were subjected to one-way analysis of variance (ANOVA) and multiple comparisons were performed by Duncan's test. Statistical significance was set at $p>0.05$. All analyses were performed using SPSS version 17.0 for Windows (SPSS, Chicago, Illinois, USA).

CHAPTER 4

RESULTS AND DISCUSSIONS

The results of data analysis and describing were sequent expressed followed

4.1 Symbols used for data resulting expression

4.2 Sequence of resulting expression

4.3 Results and discussions

4.1 Symbols used for data resulting expression

In this study, expression of data analysis results was conducted as various symbols

SD = Standard deviation

X = Means

P = Probability

4.2 Sequence of resulting expression

The results were sequent expressed following as

4.2.1 Experiment 1 was to prepared of Chitosan Succinate conjugates

4.2.2 Experiment 2 was to prepared of Bb-12 cells for microspheres

4.2.2.1Preparation of Genipin cross-linked Chitosan Succinate microspheres

4.2.2.2 Preparation of Chitosan Succinate microspheres without Genipin Experiment 4 was to determinate Particles size and charges

4.2.2.3 Scanning Electron Microscopy (SEM), the shape and surface characteristics of the microspheres

4.2.2.4 Zeta potential, the charge Chitosan Succinate

4.3 Experiment 3 was to determinate survival of Bb-12(free, encapsulated with genipin and encapsulated without genipin cells) in different conditions of gastric gut (In Vitro):

4.3.1 Rengal Solutions as control

4.3.2 In simulation of gastric juice with 0.3% pepsin

4.3.3 In simulation of gastric juice without pepsin

4.3.4 Simulated Intestinal Juice

4.3.5 Simulated Colonic Juice

4.4 Experiment 4 was to determinate survival of Bb-12 survival of free and encapsulated cells In Tomato Ketchup

4.3 Results and discussions of experiment 1

4.3.1 Description of experimental procedures:

4.3.1.1 Preparation of Chitosan Succinate conjugates

The characteristic and the results of absorption peaks of chitosan succinate microspheres by used IR spectra was 1495 cm^{-1} (Figure 4.1).

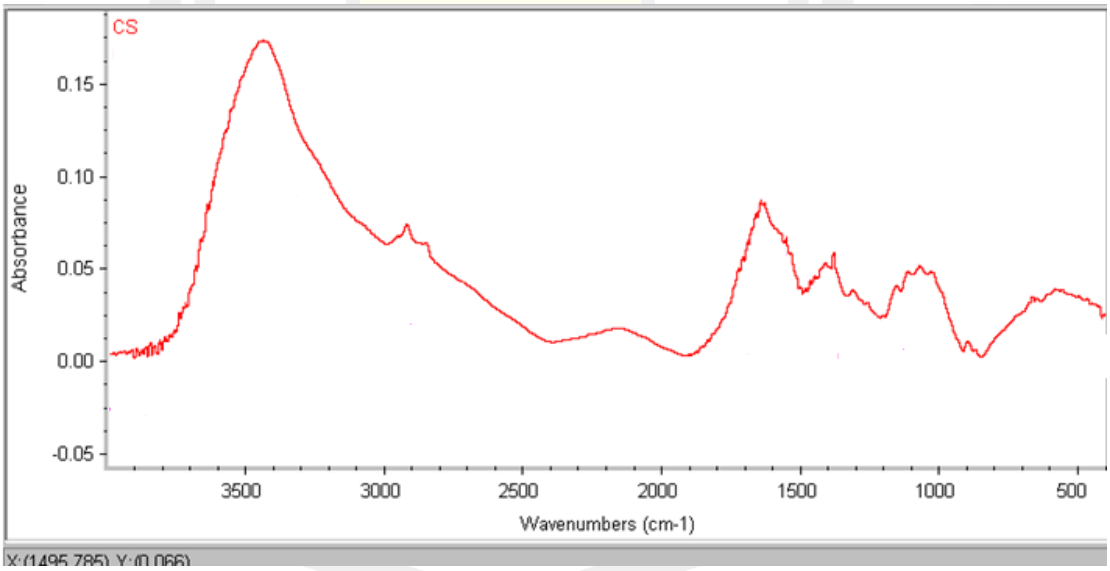


Figure 4.1 FT-IR spectra of chitosan succinate microspheres

The similar was found by (Ubaidulla et al., 2007), were study the characteristic of absorption peaks of chitosan and chitosan succinate microspheres by IR spectra and the results of absorption was 1662, 1598, and 1380 cm^{-1} indicating

amide I, NH_2 bending, and amide III, respectively and microspheres contain carboxylic moieties, and confirm that are linked to the chitosan backbone chain. Moreover, by IR spectrum, (Zhang et al., 2004), documented, that characteristic peak of chitosan in the chitosan succinate microspheres was 1712 cm^{-1}, due to C—O stretching vibrations of carboxylic moieties. Also, Aiedeh., (1999) , used the IR spectra of the prepared chitosan conjugates and found amide of carbonyl (a) stretching between 1669 and 1564 cm–1, and carboxylic carbonyl stretching was 1719 cm–1, and amide links with succinate (b) by formula (Figure 4.2), due to: C-O stretching viboration of carboxylic, mean carbonyle amid of chitosan link with carboxylic amid of succinate. However, the carboxylic acidic chitosan are stable in low pH conditions and degradation in neutral and alkaline conditions.

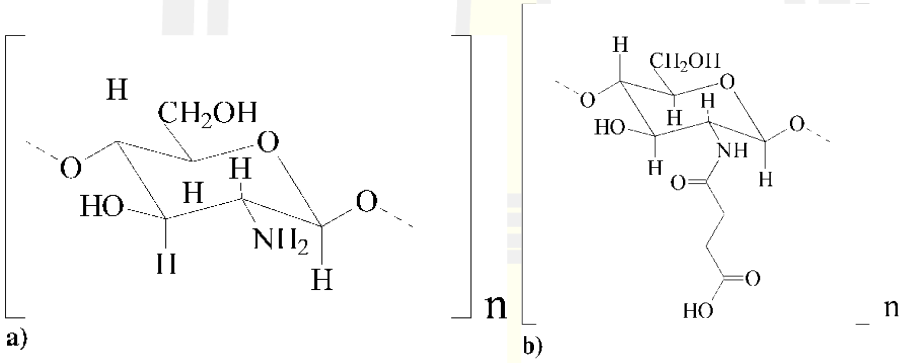


Figure 4.2 a) Structure of chitosan; b) structure of chitosan succinate conjugate

(Bieck, 1993), explain that, the excellent nucleophilic advantage compared to the surrounding hydroxyl groups can be caused selective acylation of the amino groups probably. The recent studies in biotechnology caused producing and development new peptide and protein products and used as a good host to deliver the materials to the colon as a part for drug releasing and observation. Moreover, the large intestines are the good part for peptide transported due to the low activity of enzymes and long time of incubation which is documented by Mackay, 1993. So, there are many studies used it to avoid materials of low pH sensitive (Ashford, 1993), as well as bacterially-degradable hydrogels (Schacht et al., 1996).

Aiedeh and Taha, (1999) , study the Carboxylic acid in Chitosan are degradation with the neutral or slightly alkaline pH conditions and stable in low ph

conditions. The same authors study the amino groups within the chitosan backbone were partially substituted with carboxylic acid residues.

4.3.1.1 Zeta potential, the charge and size of Chitosan Succinate

This study the shows, a moderate stability behavior of the colloid because the degree of electrostatic repulsion of particle in the microspheres was -33 mV by using Zeta potential measurement, and chitosan succinate microspheres had a net negative charge, indicate of reacted amine group, beside that the size of chitosan succinate microspheres was chitosan succinate microspheres 482.8 nm (Figure 4.3, 4.4).

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -33.7	Peak 1:	-27.8	71.5	3.44
Zeta Deviation (mV): 11.2	Peak 2:	-56.7	15.1	2.91
Conductivity (mS/cm): 0.265	Peak 3:	-40.8	13.4	4.09

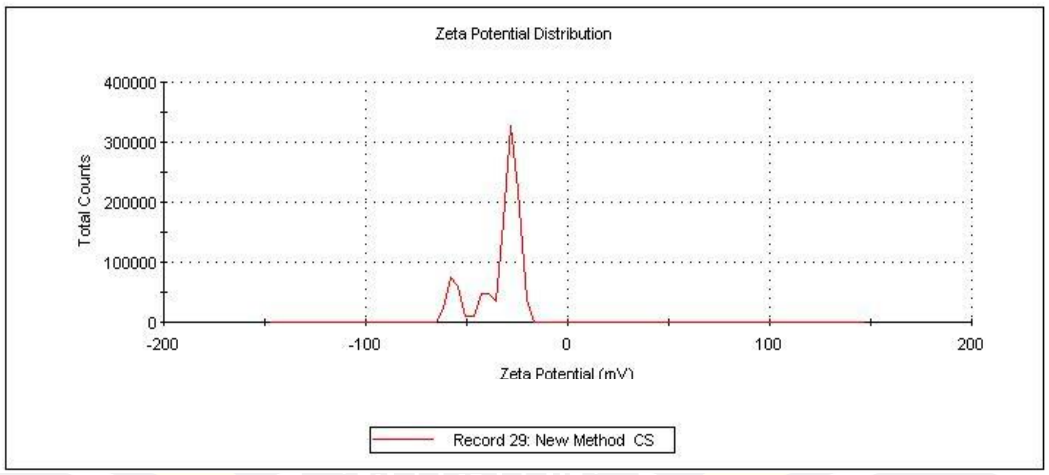


Figure 4.3 Zeta potential of Chitosan succinate microspheres

			Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm):	482.8	Peak 1:	253.6	1.8	55.71
PdI:	0.480	Peak 2:	19.65	28.7	4.403
Intercept:	0.610	Peak 3:	8.899	69.5	1.697

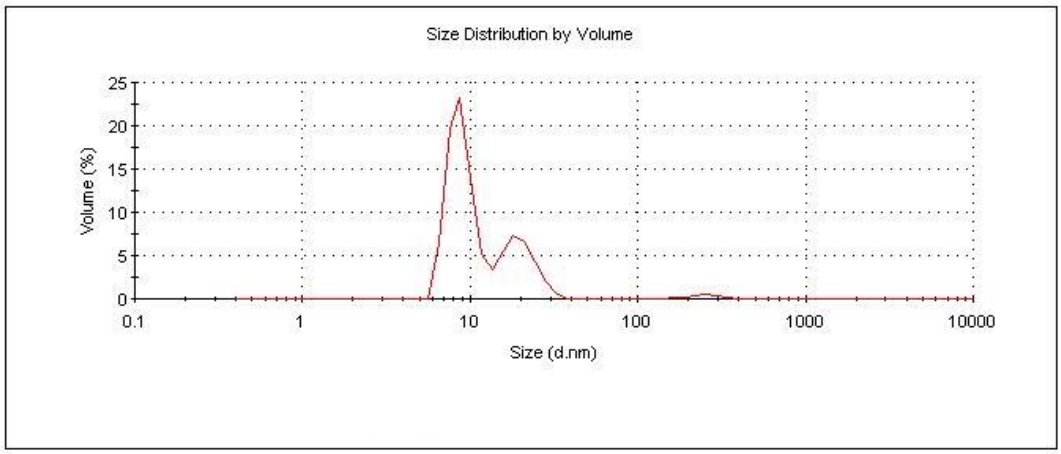


Figure 4.4 Size of Chitosan succinate microspheres

However, the study which done by (Ubaidulla et al., 2007) shows, a high reproducibility of particle size distribution which is unimodal with narrow size distributions and the mean of particle size in the microspheres was 49±2 mm by used Zeta potential measurement, and chitosan succinate microspheres had a net positive charge and the average of zeta potential which was +10±1.5 mV. Moreover, the positive zeta potential in chitosan succinate microspheres due to present of the unreacted amino groups. While, colloids with high zeta potential (negative or positive) are electrically stabilized (Greenwood & Kendall, 1999).

4.3.2 Results and discussions of experiment 2

4.3.2.1 Scanning Electron Microscopy (SEM), the shape and surface characteristics of the microspheres

Figure 4.5 shows Bb-12 encapsulated with CS with Genipin and encapsulation of the beads was determined by Scanning Electron Microscope.

4.3.2.2 Zeta potential, the diameter of Bb-12 cells in Chitosan Succinate microspheres

Also in this study the size of free, encapsulation of the beads with Genipin and encapsulation of the beads without Genipin were determined by Zeta potential measurement (Figure 4.5).

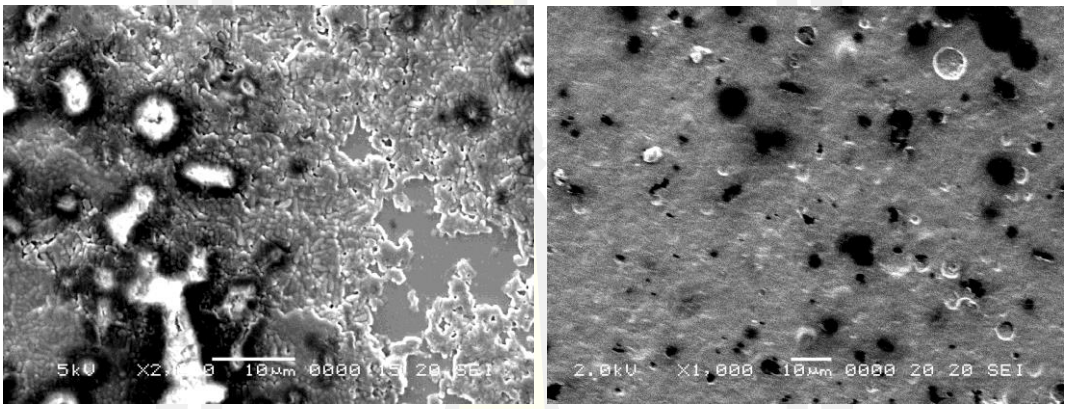


Figure 4.5 (a) NEC with Genipin (b) NEC without Genipin

Figure4.6, show, the mechanisms of cross-linking reaction between chitosan and genipin are differs at different pH conditions. Under acidic and neutral conditions, a nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurs, followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehydo group. However, under basic conditions, the ring-opening reaction of Genipin occurs via a nucleophilic attack by hydroxyl ions in aqueous solution to form intermediate aldehyde groups, which subsequently undergo aldol condensation.

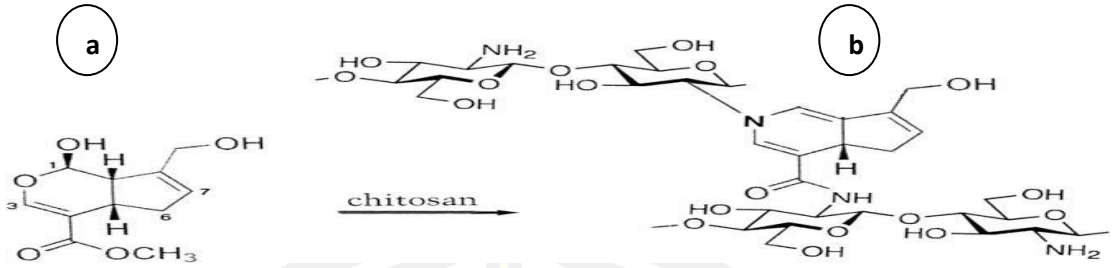


Figure 4.6 Genipin reacts with chitosan to yield two main cross-linking reactions. (a) genipin, (b) two chitosan chains are cross-linked with genipin : the formula shows the two newly formed chemical groups, namely the monosubstituted amide and the tertiary amine.

In other words, Genipin acts as a dialdehyde but its condensation products are much more stable compared to glutaraldehyde (Groboillot et al., 1993); Sung, Huang, (Sung et al., 1999). In the product, short chains of condensed Genipin act as crosslinking bridges. However, under basic conditions, the ring-opening reaction of Genipin occurs via a nucleophilic attack by hydroxyl ions in aqueous solution to form intermediate aldehyde groups, which subsequently undergo aldol condensation. The terminal aldehyde group on the polymerized Genipin undergoes a Schiff reaction with the amino groups on chitosan to form cross-linked networks. Therefore, the pH condition plays an important role in influencing the cross-linking reactions.

(Butler et al., 2003) found that the fastest reaction is a nucleophilic attack of an amino group of chitosan to carbon 3 of Genipin which results in the opening of the dihydropyran ring and the formation of a tertiary amine, for example: a Genipin derivative linked to a glucosamine unit. The subsequent slower reaction is a nucleophilic substitution of the ester group of Genipin to form an amide. In the same time, polymerization can take place between Genipin molecules already linked to amino groups of chitosan which could lead to the cross-linking of amino groups by short Genipin copolymers.

The different between NEC with Genipin and without genipin, however is shown in (Figure 4.7) shows. NEC with genipin had blue color due to reaction between ginipin and amino acids or proteins of chitosan succenite.

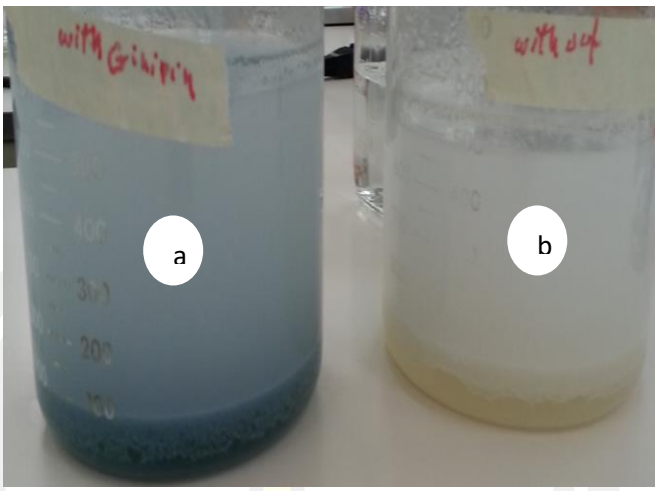


Figure 4.7 (a) NEC with Genipin (b) NEC without Genipin

(Butler et al., 2003) found that this dark-blue coloration due to the hydrogels exposed to air is associated with the oxygen radical-induced polymerization of Genipin as well as its reaction with amino groups. Moreover, (TOUYAMA et al., 1994) and (Fujikawa et al., 1987) have reported that Genipin can spontaneously react with amino acids or proteins to form dark blue color.

The width diameter of free cells was 2.144 µm (2144 nm), while the width diameter encapsulation of the beads with Genipin 2.925 µm (2925 nm) and width diameter encapsulation of the beads without Genipin were determined by Zeta potential 2.212 µm (2212nm) in figure 4.8, 4.9 and 4.10 respectively, however the size of Chitosan Succinate microspheres was 482.8 nm in figure 4.4. So the width diameter encapsulation of the beads with Genipin bigger than free cells and width diameter encapsulation of the beads without Genipin

		Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm): 2144	Peak 1:	1481	100.0	229.6
PdI: 0.246	Peak 2:	0.000	0.0	0.000
Intercept: 0.955	Peak 3:	0.000	0.0	0.000

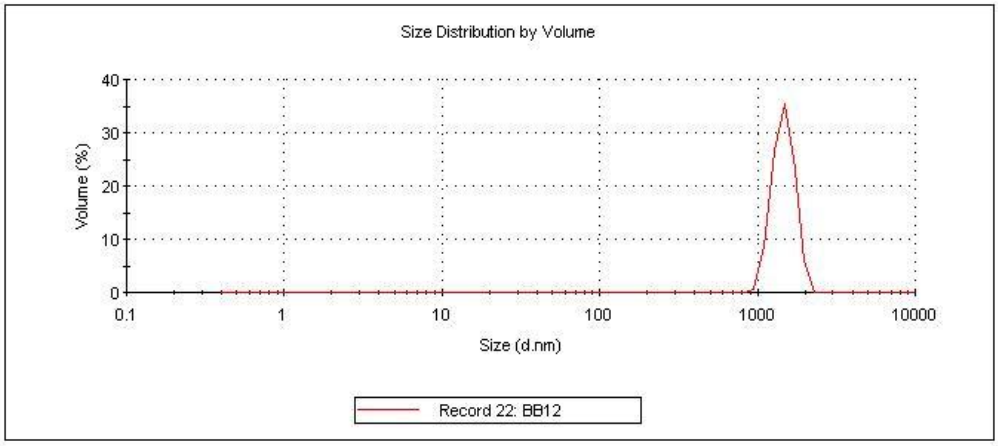


Figure 4.8 ZP of Free Cells

		Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm): 2925	Peak 1:	1499	100.0	224.5
PdI: 0.430	Peak 2:	0.000	0.0	0.000
Intercept: 0.933	Peak 3:	0.000	0.0	0.000

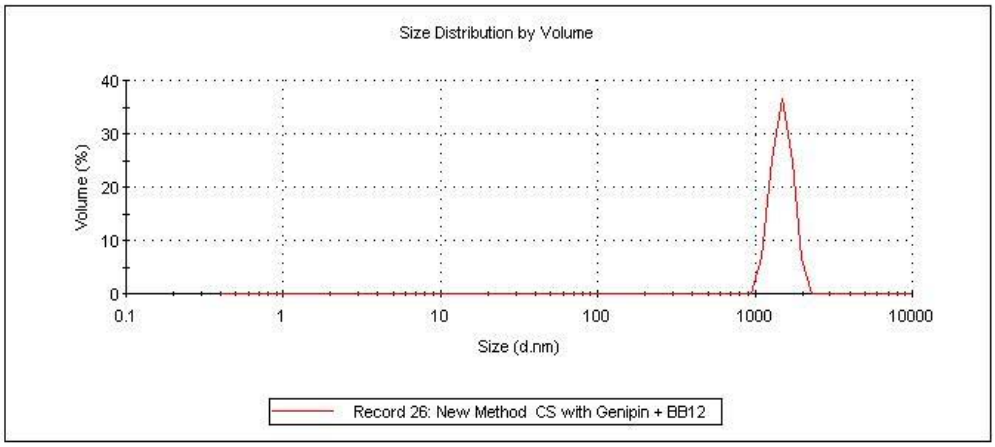


Figure 4.9: ZP of NEC with Genipin

		Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm): 2212	Peak 1:	1526	100.0	270.1
PdI: 0.002	Peak 2:	0.000	0.0	0.000
Intercept: 0.952	Peak 3:	0.000	0.0	0.000

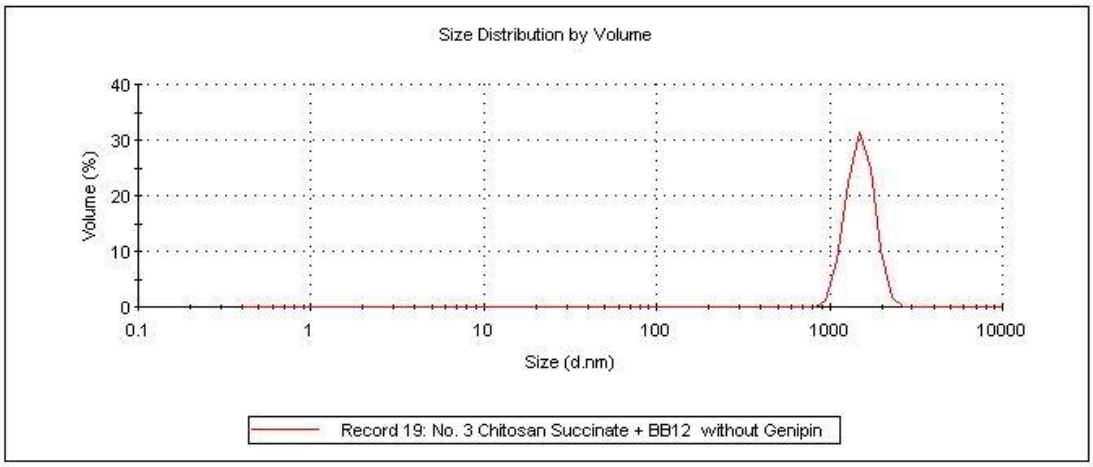


Figure 4.10: ZP of NEC without Genipin

When compared with other studies, (Annan et al., 2007) and (Annan et al., 2008) reported that, the size of uncoated gelatin microspheres cross-linked with 1.25 mmol l^{-1} genipin was 49.0 ± 12.7 µm while it is bigger than the size of uncoated gelatin microspheres cross-linked with 2.5 mmol l^{-1} Genipin was 41.2 ± 13.2 µm. Also (Annan et al., 2007), observed that swelling of microspheres was increased under 1.25 mmol l^{-1} of concentration of Genipin by the uptake of water. Furthermore, Ugwoke and Kinget, (1998), found the water uptake and swelling can be avoiding by cross-linking of gelatin microspheres, Also, higher concentration of can be cross-linker reduce changes in size and shape of microspheres.

Moreover, (Sabikhi et al., 2010) under Microscopic study,the diameter size of individual beads were from 100 to 300 µm and the beads were porous so the cells was entrapped in the matrix. (Gouin, 2004), reported, the solvents of environmental can be entry and exit smoothly by this pours.

(Giulio et al., 2005), in new study documented, by a novel rennet enzyme gelation technique to microencapsulation of probiotic strains, shows high survivability of probiotic cells at low pH, high encapsulation efficiency and small size of capsule approximately ~60 µm. (Heidebach et al., 2009b), noted that, the size of microspheres

by different encapsulation technique , such as: emulsification, extrusion, spray drying and immobilization were range between 30 μm and 3 mm and immobilization techniques are the best.

4.3.2 Results and discussions of experiment 3

4.3.3.1 In Ringers Solution (RS)

4.3.3.5 Ringers Solution as control

Ringers Solution was used in this study to see the count of cells to eliminate the effect of GI in NEC because the pH of RS is neutral.

Table 4.1 shows the initial time of free cells was adjusted with suitable dilution to 1.5×10^7 cfu/ml, while it was 5.77 log cfu/ml, then the survival rate of free cells was decreased to 4.13 log cfu/ml after 90 min and reduced to 1.00 log cfu/ml after 120 min.

After 120 min of incubation in RS, the survival rate of NEC with and without Genipin was increased to reach 5.85 log cfu/ml. Then, in 24h the cell population was slightly decreased to 4.71 and 5.66 log cfu/ml respectively.

Table 4.1 Number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in Ringers solution (RS) (pH 6.0)

Treatment	Ringers solution (RS)		
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	5.77±0.30	5.43±0.05	5.42±0.06
30 min	5.56±0.13	5.39±0.06	5.43±0.78

60 min	5.29±0.63	5.73±0.07	5.70±0.08
90 min	4.13±0.09	5.79±0.03	5.79±0.05
120 min	1.00±0.00	5.85±0.03	5.85±0.03
24 h	1.00 ±0.00	4.71±0.03	5.66±0.19

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

Table 4.2 Survival rates (%) of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in Ringers solution (RS) (pH 6.0)

	Treatment	Ringers solution (RS)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	100.05±0.51	100.00±0.95	100.00±1.27
30 min	96.42±2.31	99.43±1.13	101.79±1.48
60 min	91.70±1.10	105.60±1.38	106.86±1.63
90 min	71.58±1.53	106.74±0.64	108.59±0.94
120 min	17.33±0.00	107.88±0.58	109.71±0.60
24 h	17.33 ±0.00	86.91±0.60	107.57±3.61

[ae]The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

The percentage of survival rates of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in Ringers solution (RS) are shown in (Table 4.2). In general, the highest survival rate reductions ($p< 0.05$) in free cells were found at 120 min of incubation in RS. However, the reduction in survival rate of NEC with Genipin was less than 0.5% after 30 min of incubation than the initial time, and during incubation time the survival rate was increased by 7% after 120 min.
Since, the highest survival rate of f NEC without Genipin was 100% in initial time then increased about 9% after 120 min of incubated in RS

4.3.3.2 In simulation of gastric juice with 0.3% pepsin

Table 4.3, shows the initial time of free cells was 5.36 log cfu/ml to not match with the initial time of cell number in the NEC with and without Genipin 4.22 and 4.53 log cfu/ml respectively. Moreover, the initial cell population of free cells was 5.36 log cfu/ml then it was decreased to1.00 log cfu/ml after 30 min of exposure with GJ with 0.3% pepsin.

Besides that, NEC with Genipin was slightly different than free cells in the initial time 4.22 log cfu/ml. However, after 30 min of incubation in GJ with 0.3% pepsin, the survival of NEC with Genipin was increased to 4.30 log cfu/ml and after 90 min cells count was reached to 5.39 lgo cfu/ml, and reduced to 5.31 log cfu/ml after 120 min.

However, the survival of NEC without Genipin was increased to 1.73 log cfu/ml and after 90 min cells count was reached to 2.51 cfu/mL then, the cells count was slightly decreased after 120 min of exposure with SGJ with pepsin 0.3%.

While, NEC without Genipin in initial time was 4.53 log cfu/ml and gradually increased to reach 6.33 log cfu/ml after 120 min of exposure with GJ with 3.0% pepsin. Furthermore, the reductions of free cells, NEC with Genipin and NEC without Genipin in SGJ with pH 2 and contain 0.3% of pepsin were significant ($P<0.05$), and NEC with Genipin was protect cells of SGJ with 0.3% of pepsin.

Table 4.4: Number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices with 0.3% of pepsin (SGJ) (pH 2.0)

	Treatment	Simulated gastric Juices with 0.3% of pepsin (SGJ)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	5.36±0.13	4.22±0.10	4.53±0.30
30 min	1.00±0.00	4.30±0.75	5.70±0.13
60 min	1.00±0.00	5.21±0.17	5.60±0.37
90 min	1.00±0.00	5.39± 0.06	6.20±0.13
120 min	1.00±0.00	5.31±0.09	6.33±0.06
24 h	1.00±0.00	4.66±0.29	1.00±0.00

[ae]The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

The percentage of survival rates of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices with 0.3% of pepsin (SGJ) are shown in (Table 4.4). In general, the highest survival rate reductions ($p< 0.05$) in free cells were found at 120 min of incubation in simulated gastric Juices with 0.3% of pepsin (SGJ). However, the highest survival rate of NEC with Genipin was more than 27% after 90 min of incubation than the initial time.Since, the highest survival rate of f NEC without Genipin was 100% in initial time then increased about 39% after 120 min of incubated in simulated gastric Juices with 0.3% of pepsin (SGJ).

Table 4.5 Survival rates (%) of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices with 0.3% of pepsin (SGJ) (pH 2.0)

Treatment	Simulated gastric Juices with 0.3% of pepsin (SGJ)		
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	100.00±0.05	100.00±2.48	100.00±6.46
30 min	21.37±14.85	101.47±1.79	125.82±3.32
60 min	18.66±0.00	123.20±4.23	123.±7.90
90 min	19.57±5.00	127.55± 1.55	136.59±3.05
120 min	19.35±3.78	125.62±2.33	139.59±1.43
24 h	19.21±3.04	110.28±6.95	22.03±0.00

[ae]The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

(Chávarri et al., 2010), Found number of survival of *B. bifidum* capsule was decreased in the initial time but after 90 min was slightly increased than the survival was decreased, and they explain that, the strain were sensitive in acid condition (Ding & Shah, 2009) which is the same we found it in the present study.

Researcher found that, the main reason of cells encapsulation is to protect these cells from gastric tract enzymes and low pH. However, the pH of gastric juice is from 1.5 to 3 and contains pepsin, a proteolytic enzyme which is active only below pH 4. And in pH lower than 2 and contain pepsin the bacterial cells of E. coli was significant death, but the pH in the stomach variable depend of food intake which is it increases between 3 and 3.5 (Angel et al., 2011).

While, (Annan et al., 2008), they documented that, the survival NEC which coated of Genipin cross-linking in gelatin microspheres in the simulated gastric juice with pepsin 0.3% and without pepsin enzyme, using pepsin and Genipin concentrations of 2.5 mM were able to maintain the stability of gelatin microspheres in simulated gastric juice and the survival of coated cells of *Bifidobacterium lactis* was 10.48 ± 0.09^{a} log cfu ml^{-1}, when comparing with Genipin uncross-linked gelatin microspheres the survival of coated cells of *Bifidobacterium lactis* was 10.59 ± 0.18^{a}. This results were match with present study, the survival number of NEC with Genipin in initial time was $1.731\pm4.10\times10^{4}$e and then increased while the survival number of NEC without Genipin in initial time was$4.21\pm2.20\times10^{4}$d then increased, that mean, of NEC with genipin had good protection activity than of NEC without Genipin during exposure in SJC with 0.3% of pepsin.

Also (Annan et al., 2008), found that the time of releasing were decreased when increasing the concentrations of pepsin enzyme in the simulated gastric juice, in differant concentrations of genipin. As well as (Narayani & Rao, 1995), used radiographical studies and observed that the uncoated gelatin capsules released in the stomach after 15 min of intake.

Other authors reported that, cleavage sites in the gelatin with cross-linking where increased the resistance against of the enzyme-substrate (Liang et al., 2003).

According to (Vandelli et al., 2001), explained that the bulky heterocyclic structure of genipin may produce a higher stereohindrance for the penetration of enzymes. However, they observed that without used proteolytic enzymes, the swelling capability of microspheres and water penetration and increased time of degradation.

4.3.3.2 In simulation of gastric juice without pepsin

The number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices without pepsin (SGJ) are shown in (Table 4.6). The initial time of free cells was adjusted with suitable dilution to 15×10^{7} cfu/ml, and it was 5.72 log cfu/ml, then the survival rate of free cells was decreased to 2.39 log cfu/ml after 90 min.

The initial time of the survival rate of NEC with Genipin was 3.50 log cfu/ml, however, after 90 min of exposure with SGJ without pepsin was decreased to 1.00 log cfu/ml. Moreover, the survival rate of NEC without Genipin was slightly fluctuating 4.28, 4.27, 4.38 and log cfu/ml respectively in 0, 30, 60 and 90 min.

So, the reductions of free cells, NEC with Genipin and NEC without Genipin in SGJ with pH 2 were significant ($P<0.05$).

Table 4.6 Number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices without pepsin (SGJ) (pH 2.0)

	Treatment	Simulated gastric Juices without pepsin (SGJ)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	5.72±0.03	3.50±0.03	4.28±0.32
30 min	4.86±0.04	3.29±0.10	4.27±0.19
60 min	3.69±0.15	3.41±0.19	4.38±0.18
90 min	3.43±0.14	1.00±0.00	1.00±0.00
120 min	2.39±0.37	1.00±0.00	1.00±0.00
24 h	0.96±0.18	1.00±0.00	1.00±0.00

[ae]The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

Beside that (Table 4.7) shows the percentage of survival rates of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices without pepsin (SGJ). In general, the highest survival rate reductions ($p < 0.05$) in free cells were found at 120 min of incubation in simulated gastric Juices without pepsin (SGJ). However, the highest survival rate of NEC with Genipin was more than 71.4% after 90 min of incubation than the initial time. However, the highest survival rate of f NEC without Genipin was 100% in initial time then reduce about 76.6% after 90 min of incubated in simulated gastric Juices without pepsin (SGJ).

Table 4.7 Survival rates (%) of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated gastric Juices without pepsin (SGJ) (pH 2.0)

	Treatment	Simulated gastric Juices without pepsin (SGJ)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	100.00±0.50	100.00±0.97	100.00±7.70
30 min	85.61±2.81	94.06±5.14	99.86±4.58
60 min	66.00±5.38	97.69±5.50	102.46±4.23
90 min	60.98±5.10	28.57±0.00	23.36±0.00
120 min	44.65±10.59	28.57±0.00	23.36±0.00
24 h	22.21±13.68	28.57±0.00	23.36±0.00

[ae]The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

According to (Nag et al., 2011), the inhibition of survival of coated cells in GJ without pepsin of about <10 cfu/mL after 60 min of incubation, and found that the survival of encapsulated cells in gastric juice without pepsin was reduced to 3.1 log cfu after 120 min of incubation as long as decreasing rate of free cells, also (Heidebach et al., 2009b) found the samilr result, after 90 min of incubation at pH 2.5 the survival was decreased to 3.0 log cfu.

When used rennet in gelling technique, the survival of encapsulated cells *of L. paracasei* and *B. lactis* were higher than fee cells by only 0.8 and 2.8 log cfu respectively which is observed by (Heidebach et al., 2009a) and match with present study. (Guérin et al., 2003); (Kos et al., 2000); (Ainsley Reid et al., 2005), They observed, in any type of encapsulation technique, that the rate of survival cells in an encapsulated environment was best by absence of any contact with acidic condition, which is common for any kind of encapsulation technique; Also, the nature pH of milk protein can be work as protection conditions. According to (Nag et al., 2011): the best survival of encapsulated cells in low pH of gastric juice by used caseinate gels the synergistic effect of gellan gum.

On the other side (Both et al., 2010) they found that, there were no survivals of *Lactobacillus acidophilus* La-5 after 30 min of exposure in gastric juice at pH 2. Also, in present study there were no survivals of free BB-12. And, the reductions of free cells, NEC with Genipin and NEC without Genipin in SGJ with pH 2 were not significant ($P<0.05$).

Moreover, (Ivanovska et al., 2012) ducumented that, the spray-drying technique and alginate with chitosan as coating material, by added calcium chloride for polyelectrolyte complexation, the survival of microencapsulation of *L. casei* was significant protection of cells from high pH and antimicrobial effects of bile salts.

Also, when used extrusion technique of chitosan-alginate to encapsulated *L. acidophilus* and *L. casei* which protect cells during simulated gastric and intestinal juices to the colon (Chávarri et al., 2010), many studies showed protective action of alginate coatings in decreased of survival of probiotics cells in the gesteric conditions (Akhiar & Aqilah, 2010); (Mandal et al., 2006); (Chandramouli et al., 2004). Other researcher found that the Chitosan-alginate microparticles can be protect the survival

of probiotic cells in simulated intestinal conditions by their ion-exchange reaction resulting in formation of insoluble complex between chitosan and bile salts which probably limits the diffusion of bile salts into the particles.

Also (Nag et al., 2011) found that, after 120 min of incubation, the reduction in free cells was only about 4.6 log cfu in gastric juice with pepsin compared with 6.1 log cfu in gastric juice without pepsin. Moreover, Saarela *et al*., (2005) found the same results; the survival rate of free cells in SGj with pepsin was lower than survival rate in gastric juice without pepsin which might be the action of pepsin and presence of other unknown compounds in commercial pepsin which is extracts from porcine gastric mucosa.

(Ivanovska et al., 2012) The results indicated that microencapsulation of *L. casei* by spray-drying method using alginate as encapsulating material, chitosan as additional coating agent and calcium chloride for polyelectrolyte complexation, offered significant protection of probiotic cells from high acidity and antimicrobial effects of bile salts. This result is consistent with that of Krasaekoopt *et al*., who found that bacterial cells of *L. acidophilus* and *L. casei* encapsulated in chitosan-alginate beads using extrusion technique survived better in low pH conditions.

(Mandal et al., 2006), reported that, the survival of free cells and coated cells were decreased and not significant in distilled water (pH 6.5) after 3 hours of incubation time. While, the reductions of free cells and coated cell with 2% of alginate in pH 1.5 conditions were significant ($P<0.05$). However, after one and three hours the Free cells were decreased to 4.24 and 3.38 log cfu/mL under exposure conditions pH 1.5. Also the same researcher found that, the survival of cells in 2% alginate beads after 1 h of exposure was significantly higher than free cells, While the rate of viability was lower as compared with 3% and 4% alginate beads ($P<0.05$). As well as, at 3 h incubation time, the best viability of cell was documented in 4% alginate beads, followed by 3% and 2%. So, the viability of encapsulated *L. casei* NCDC-298 cells enhancement with increasing alginate concentration (Mandal et al., 2006). Also Chandramouli, Kailasapathy, Peiris, and Jones (2004) found that survival of *L. acidophilus* increase at pH 2.0 when encapsulated in alginate. Moreover, Guerin, Vuillemard, & Subirade, 2003; Picot & Lacroix, 2004; Wenrong & Griffiths,

2000) reported that the viability of immobilized *Bifidobactera* was Higher at acidic pH of Gastric intestinal tract.

Microencapsulated cells of *L. acidophilus* in alginate beads improved the survival after consecutive incubation in simulated gastric and intestinal juices which is oserved by (Krasaekoopt et al., 2004). According to (Lee et al., 2004); (Le‐Tien et al., 2004), the high survival of lactobacilli immobilized in alginate beads were incubated in simulated gastric juice.

(Mandal et al., 2006) found that there results are in similar of other workers (Hansen et al., 2002); (Sultana et al., 2000); (Favaro-Trindade & Grosso, 2002) who documented that microencapsulation in alginate bead method did not effectively protect the probiotic cells from low pH.

Acceding to (Pitino et al., 2010) that liquids remains in the stomach only about 20 min and food remains between 2 and 4 h. (Annan et al., 2008), reported that the encapsulation of *Bifidobacteria* in uncoated gelatin microspheres only not significant improved the survival over that of free cells, furthermore, the degradation of gelatin only microspheres were structurally unstable in simulation of gastric juice in percent of pepsin and completely disintegrated after 45 min of exposure. Also the same author found that the size 1–2 mm of thick alginate coating improved pepsin from release the gelatin microspheres and the cells in intact gelatin microspheres was significantly ($P < 0.05$) enhanced viability of the *bifidobacteria* during 2 hour of exposing time, indicating the protective buffering effect of intact gelatin microspheres (Annan *et al*., 2008). However, (Guérin et al., 2003) found that the pH of whey proteins can be protect survival rates for *bifidobacteria* encapsulated in a mixed gel of alginate, pectin and whey proteins than free cells when incubated in the SGJ at pH 2.5. Also (Narayani & Rao, 1995) noted that gelatin capsules loaded with a cancer drug and coated with 20% alginate remained intact for up to 8 h in SGJ however uncoated microspheres drug disintegrated after 15 min, their microspheres were, cross-linked with the cytotoxic gluteraldehyde. (Annan et al., 2008), observed that the reduction rate of survival cells was 3.45 log units for free of *B. adolescentis* cells, as well as (Charteris et al., 1998) and (Hansen et al., 2002) reported the similar results by of *B.*

adolescentis was decresed to 3 log cfu ml^{-1} after 2 to 3 h of incubated in SGJ and pH 2.0.

4.3.3.3 Simulated Intestinal Juice (SIJ)

The number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated intestinal juice (SGJ) are shown in (Table 4.8). The initial time of free cells was adjusted with suitable dilution to 15×10^{7} cfu/ml, and it was 5.80 log cfu/ml, then the survival rate of free cells was decreased to 3.62 log cfu/ml after 120 min.

The initial time of the survival rate of NEC with Genipin was 5.50 log cfu/ml, however, the survival rate was slightly fluctuating 5.69, 5.79, 5.84 and 5.81 log cfu/ml respectively in 0, 30, 60, 90 and 120 min. Moreover, the survival rate of NEC without Genipin 7.25 log cfu/ml in 0 min and reduce to 6.36 log cfu/ml.

So, the reductions of free cells, NEC with Genipin and NEC without Genipin in SGJ with pH 2 were significant ($P<0.05$).

Table 4.8 Number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated intestinal Juices (SGI) (pH 8.0)

	Treatment	Simulated Intestinal Juices (SIJ)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	5.80±0.00	5.50±0.09	7.25±0.07
30 min	5.54±0.39	5.69±0.07	6.36±0.10
60 min	5.37±0.61	5.79±0.07	6.53±0.04
90 min	3.70±0.30	5.84±0.05	6.43±0.11
120 min	3.62±0.07	5.81±0.16	6.36±0.10
24 h	2.80 ±0.80	5.66±0.07	5.31±0.08

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

Furthermore, (Table 4.9) shows the percentage of survival rates of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated intestinal Juices (SIJ). In general, in initial time the survival rate was 100% then it reduce ($p< 0.05$) in free cells were found at 120 min of incubation in simulated gastric Intestinal (SIJ). However, the highest survival rate of NEC with Genipin was more than 102% after 120 min of incubation than the initial time. Besides that, the highest survival rate of f NEC without Genipin was 100% in initial time then reached to about 153% after 60 min of incubated in (SIJ).

Table 4.10: Survival rates (%) of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated intestinal Juices (SGI) (pH 8.0)

	Treatment	Simulated intestinal Juices (SIJ)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	100.00±0.50	100.00±1.71	100.00±1.80
30 min	95.50±0.67	100.91±1.31	149.37±2.50
60 min	92.59±1.05	100.65±1.33	153.48±1.12
90 min	63.77±0.59	102.46±0.98	151.10±2.62
120 min	62.47±1.33	102.05±2.89	149.37±2.50
24 h	48.40 ±0.35	100.45±1.23	124.80±2.10

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

(Song et al., 2003), reported that, the survival of encapsulated *L. casei* with alginate and chitosan in bile salts solutions was higher than free cells. Also, some researcher found that the effect of emulsion technique of encapsulation on the viability rate of *L. casei* under strange gastrointestinal conditions (Park *et al*., 2003). While, the absence of cells on the surface of the Ca-alginate microcapsules with microporous glass membrane emulsification method does not ensure effective cell release in the colon (Ivanovska et al., 2012). (Ivanovska et al., 2012), observed that, the survival of the encapsulated cells in simulated gastric and bile juices was higher than free *L. casei* preservation. Besides that, this result was match with present study

and the survivals of free BB-12 was reductions while NEC with Genipin and NEC without Genipin in SGI were significant ($P<0.05$).

Besides that, Releasing the *L. casei* cells in neutral pH of bile juice can be effect on the destabilization of the gel network and the properties of the bile salts could possibly cause emulsification of the entrapped or surface oil to some bile juice (Ding & Shah, 2009). (Nag et al., 2011), observed that the encapsulated cells was higher than free cells in a very high bile tolerance. However, different researchers have observed a deadly effect of bile salts on probiotic bacteria (Ding & Shah, 2009), 2009; (Guérin et al., 2003). But (Guérin et al., 2003) Guerin *et al*., (2003) and Trindade and Grosso (2000) reported that the opposite results; free cells and encapsulated bifidobacteria cells were higher survival after 3 h of exposure of bile salts.

The study was done by (Mandal et al., 2006), they observed that water had no effect on the viability of free cells in distilled and it was no significant difference of the cells survival, however the survival rate of free cells was reduced from 9.45 to 7.29 log cfu mL^{-1} and 9.34 to 5.60 log cfu/mlafter 12 h of exposure by 1% and 2% bile salt, respectively. Moreover, concentration of alginate concentration enhancement the viability of coated cells from 2 to 4% improved the viability of cells at 1% and 2% bile salt (Mandal *et al*., 2006). Mandal *et al*., 2006 reported that, the viability of encapsulation with alginate was higher than free *L. casei* NCDC-298 during incubation time in bile salt. The similar results were noted with other researchers of survival of encapsulated cells bile salt conditions (Krasaekoopt et al., 2004); Lee and Heo, 2000). According to (Chandramouli et al., 2004) observed that the viability of *L. acidophilus* encapsulation in alginate was significantly increased the in 1% bile salt. while, (Guérin et al., 2003) found that the death-rate of bifidobacteria, immobilized in polysaccharide–protein gel beads was increased at to 2% and 4% bile salt. Moreover, Trindade and Grosso (2000) documented that the effect of protecting from 2% and 4% bile salt of immobilization of *B. bifidum* and *L. acidophilus* in Ca-alginate beads was not significant.

(Priya et al., 2011) used layers of PEs technique to coated *L. acidophilus* and they observed that good result of the advantage of encapsulating bacteria within layer.

While, the number of free cells was decreased from 10.4 log cfu/500 mg to 4 log cfu/500 mg from an initial time and the rate of cells survival was decreased in strong conditions of gastric juice during 120 min when compared with cells coated with (CHI/CMC) 1.5 layers. However, survival rate of coated cells was decreased from 9.4 log cfu/500 mg to 8.2 log cfu/500 mg from initial count during gastric juice conditions which is reported by (Priya et al., 2011).

(Priya et al., 2011), found that, the survival rate of coated *L. acidophilus* by layers technique was 1 log cfu/500 mg during exposure to SIF for 120 min after exposure to SGF (for 120 min) than uncoated bacterial cells.

However, under the strong conditions of gastric juice and intestinal juice, the encapsulated cells were reduced about 6.3 log cfu/500 mg of minim required for therapeutic effects (Priya *et al*., 2011). But, the loss of survival cells in strong conditions of gastric juice and intestinal juice are greater than the losses that occurred in gastric fluid which is reported by Priya *et al*., (2011) . as well as, this reduction due to action of the enzymatic degradation of polyelectrolyte layers by pepsin present in gastric fluid (Priy *et al*., 2011). (Zhu et al., 2010) and (Wang et al., 2007), found that the Chitosan, stable at low pH, but might be degraded by enzymatically action by many nonspecific enzymes such as pepsin. The degradation rate depends on the degree of deacetylation, molecular weight and the concentration of chitosan (Zhua *et al*., 2010 and (Wang et al., 2007) Thus, (Zhu et al., 2010) and (Wang et al., 2007) documented that the degradation of protective coating makes the cells more exposure to harmful intestinal conditions. Moreover, (Zhu et al., 2010) and (Wang et al., 2007) noted that the osmolar stress created by the polyelectrolyte layer degradation becomes larger and effected on the nano-layers and caused, exposing the cells to strong conditions in gastric tract. Moreover, at high concentration of pH, the layers start losing their solidity (because of repulsion of deprotonated carboxyl groups and lack of other stabilizing counter effects) and get degradation, which is also a cause for increased death rate of bacteria in intestinal solution (Zhu et al., 2010) and (Wang et al., 2007).

(Amor et al., 2002); (Chung et al., 1999); (LANKAPUTHRA & Shah, 1997); (Picot & Lacroix, 2004), reported that the many strains of *Bifidobacterium* spp have

sensitivity to bile concentrations in the human gastro-intestinal tract such as the survival rate of *B. adolescentis* was reduced in SIJ especially during the initial time of exposures with SIJ and may be sensitivity of *B. adolescentis* to bile and for the entrapped cells and physical effect of the microsphere matrix. (Clark et al., 1993) found, viable cell counts of *B. adolescentis* was reduced 5 log after 12 h of incubation at 37 °C At bile concentrations of 2%. Also, (Hansen et al., 2002) Truelstrup *et al*., (2002) observed that survival rate of *B. adolescentis* was decreased by about 2 log cfu ml^{-1} after 2 h of exposure in 0.5% (w/v) bile at 37 °C. (Amor et al., 2002) reported the survival of *B. lactis* and *B. adolescentis* during incubation in bile salt stress and observed *B. adolescentis* to be more sensitively to the damaging effects of bile salts. Same authors found that sub-lethal injuries affected by bile salts may result in a viable but non-culturable fraction of the population. Moreover, (Annan et al., 2008) observed the survival rate of free cells in simulated intestinal juice increased from 5.48 log cfu/ ml after 60 min to 6.40 log cfu/ml after 120 min and remained stable up to 240 min, while, this increase was due to cell recovery and not growth as there were no nutrients available in the SIJ. Picot and Lacroix, (2004) observed similar with previous study of the subsequent recovery of temporarily damaged *bifidobacteria* cells after incubation time in the low pH and bile salt stresses.

Both Picot & Lacroix were explain that survival numbers of cells in alginate-coated gelatin microspheres after incubation in SGJ contributed was higher than cells survival numbers of cells incubated into simulated intestinal juice and showed that the protecting effect of microencapsulation matrix was of the coated cells with levels of viable of 7.6 and 7.4 log cfu/ml as different to levels of 6.4–6.7 log cfu/ml reported for free cells and cells coated in gelatin-only microspheres after four h in simulated intestinal juice (Picot and Lacroix, 2004). Furthermore (Guérin et al., 2003) reported that an first time of immobilized *B. bifidum* count of 1010 cells g^{-1} in a mixed alginate, pectin and whey protein matrix can be reach the small intestine in numbers of 7.5 log cells g^{-1} and increased provide the host with a beneficial health effect.

4.3.3.4 Simulated Colonic Juice

The rate of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated colonic juice (SCJ) are shown in (Table 4.11). The initial time of free cells was adjusted with suitable dilution to 15×10^7 cfu/ml, and it was 5.84 log cfu/ml, then the survival rate of free cells was reduced to 4.20 log cfu/ml after 120 min. Moreover, the survival of NEC with Genipin was increased to reached 6.65 log cfu/ml after 60 min of exposure with SCJ. But, the survival rate of NEC without Genipin 4.60 log cfu/ml in 0 min and incresed to 5.58 log cfu/ml after 120 min of exposure.

Table 4.11: Number of surviving cells (log CFU/ml) for free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in Simulated intestinal Colon (SIC) (pH 7.4)

	Treatment	Simulated intestinal Colon (SIC)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	5.84±0.12	5.78±0.04	4.60±0.16
30 min	5.73±0.17	6.63±0.05	4.70±0.05
60 min	5.77±0.11	6.59±0.03	4.73±0.04
90 min	4.50±0.12	6.52±0.06	5.58±0.72
120 min	4.20±0.12	6.48±0.16	5.48±0.09
24 h	2.43 ±0.18	4.48±0.05	5.14±0.48

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

The percentage of survival rates of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in simulated intestinal Juices (SIJ) was shows in (Table 4.12). In initial time the survival rate was 100% then it reduce ($p < 0.05$) in free cells were found at 120 min of incubation in simulated gastric Intestinal (SIJ) to 76.8%. Furthermore, the highest survival rate of NEC with Genipin was more than 114% after 60 min of incubation than the initial time. However, the highest survival rate of f NEC without Genipin was 125% after 60 min of incubated in (SCJ).

Table 4.12: Survival rates (%) of free, NEC with Genipin and NEC without Genipin BB-12 during sequential incubation (37 °C) in Simulated intestinal Colon (SIC) (pH 7.4)

	Treatment	Simulated intestinal Colon (SIC)	
Time	Free Cells	NEC with Genipin	NEC without Genipin
0 min	100.04±2.06	100.00±0.70	100.00±3.58
30 min	98.10±2.98	114.63±0.92	102.47±1.18
60 min	98.91±1.92	114.01±0.60	103.07±0.94
90 min	96.01±2.06	112.74±1.15	121.62±1.58
120 min	76.88±2.18	111.98±2.90	125.66±2.05
24 h	41.60 ±3.10	77.38±0.98	119.41±2.09

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

(Sabikhi et al., 2010), explain, under simulated conditions of colonic pH (7.4± 0.2) the viability of rate of release of coated cells of *L. acidophilus* LA 1 was 7.47 in the first time then this rate was gradually increased by increased the time, and and during incubated at conditions of simulated colonic pH and stable at about 2.5 h then the viability of rate of release of coated cells of *L. acidophilus* LA 1 was decreased. The number of coated cells were 7.45 log (almost same as first time) after 2.5-h exposure, that mean all the coated cells were released at this time, which is about the same time as that taken for intestinal pass of microflora (Sabikhi et al., 2010). (Mandal et al., 2006) study that, the release of cells increase with increased incubation time, while after 60 min there was no significant change (P>0.05) even with high concentration of alginate. (Picot & Lacroix, 2004), noted that, the release of survival cells increase from whey-protein-based microcapsules in simulated intestinal conditions. (Sabikhi et al., 2010), explain the decrease of survival cells numbers after a specified time may be caused by lack of nutrients for the growth in the medium.

Manda *et al*., 2006, and Suita-Cruz and Goulet, (2001) reported, the coated cells must be released in colon to growth and colonization of probiotics; otherwise the coated cells in the beads will be pass out from the body without any beneficial effect.

The released cell numbers were between 3.40 and 3.70 log cfu/g during incubated in the simulated colonic pH from an first number~9.40 log cfu/g (Mandal *et al*., 2006).

4.3.3.6 Summary of experiment 3

In vitro release chitosan succinate microspheres with genipin cross-link were had protected effect of the degradation from gastric enzymes than NEC without genipin. The encapsulated BB-12 was higher cells survival in simulated intestinal juice in pH 8 and simulated colon juice in pH 7.4, while the encapsulated BB-12 had low number of survival was released in simulated gastric juice (pH 2.0).

Chitosan succinate microsphere showed pH can be effect of release matrix form of microsphere. chitosan succinate microsphere cross-link with Genipin had significant high level of survival was observed under pH 7.4 (Colon), besides, that is

it resisted in pH 2 (Stomach) condition than chitosan succinate microsphere no cross-link with Genipin

The stability of chitosan succinate cross-linked with Genipin observed a high stability in the acidic environment of simulated gastric juice with absent of pepsin enzyme, besides that, the simulated gastric juice with pepsin enzyme had reduced stability significantly. However, the stability of chitosan succinate without cross-linked with Genipin observed that no significantly, effect of simulated gastric juice with or without pepsin enzyme. Genipin under concentration 2.5 mM cross-link might be enhance the resistance of chitosan succinate to degradation by pepsin enzyme for controlled release of cells of probiotic.

4.3.4 Results and discussions of experiment 4

4.3.4.1 Survival of free and encapsulated cells In Tomato Ketchup

Table 4.6 shows the survival rate of both free and NEC with Genipin in Tomato Ketchup during storage time. The initial Survival rate viable cell of free cells and NE with Genipin cells were 5.54 and 5.540 log cfu/ml, respectively. However, the survival rate of viable cell of NEC was found to be lower than that of free cells; the NEC which leaked during storage grew in the medium as free cells. The viable cell numbers of NEC was reached 5.85 log cfu/m after 1 day and was decreased after 3 and 7 days to 4.48 and 1.00 log cfu/ml respectively.

But, the survival rate of viable free calls was increased gradually 5.85 log cfu/ml after 60 min, which is decreased to 5.68, 5.56, 4.46 and 1.00 log cfu/ml respectively, after 90 min, 120 min, 1 day, 3 days and 7 days. While, the reductions of free cells after 90 and in NEC with genipin in Ketchup after two days were significant ($P<0.05$) due to present of some toxic compound of BB-12 such as low pH and osmotic.

Sugar content was analyzed by HPLC: 7.93% of fructose, 10.93% of glucose and 7.14% of sucrose, and the total sugar was 26.0%. Besides that, the acidity of tomato ketchup was 1.60 % as acetic acid. And the pH was measured directly using a pH meter.

Table 4.13: Number of surviving cells (log CFU/mL) for free and NEC with Genipin during sequential incubation (37 °C) in Tomato Ketchup (pH 5.0)

Time	Treatment	Survival in Ketchup
Time	Free Cells	NEC with Genipin
0 min	5.54±0.04	5.40±0.06
30 min	5.64±0.02	5.50±0.06
60 min	5.80±0.02	5.71±0.03
90 min	5.68±0.09	5.78±0.05
120 min	5.56±0.10	5.78±0.10
1days	4.46±0.07	5.85±0.02
2 days	1.00±0.00	5.85±0.03
3 days	1.00±0.00	4.48±0.24
7 days	1.00±0.00	1.00±0.00

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

The percentage of survival rates of free; NEC with Genipin BB-12 during sequential incubation (37 °C) in Tomato Ketchup was shows in (Table 4.14). In initial time the survival rate was 100% then it reduce ($p< 0.05$) in free cells were found at two days of incubation in Tomato Ketchup to 18%. And, the highest survival rate of NEC with Genipin was more than 108% after two days of incubation than the initial time.

Table 4.14: Survival rate (%) of free and NEC with Genipin during sequential incubation (37 °C) in Tomato Ketchup (pH 5.0)

Treatment	Survival in Ketchup	
Time	Free Cells	NEC with Genipin
0 min	100.02±0.72	100.00±1.23
30 min	101.86±0.50	101.90±1.11
60 min	104.69±0.44	105.80±0.68
90 min	102.55±1.67	107.13±0.91
120 min	100.51±1.80	107.14±1.84
1days	100.52±1.38	108.37±0.54
2 days	18.05±0.00	108.25±0.57
3 days	18.05±0.00	83.35±5.21
7 days	18.05±0.00	18.52±0.00

The experimental values (means and standard deviations for n = 30) different litters in the same column are significantly different ($p < 0.05$).

According to (Lebeau et al., 1998), in immobilized cell fermentation technique, occurred some of cells release from the entrapped gel beads due to the growth of bacteria in the immobilized gel beads; these cells grew in the first condition with no cells. However, (King et al., 2007), documented that higher survival cell are observed during immobilized cell fermentation than free cell fermentation and

reported that, the cells numbers increased in microsphere after 6 h of beginning ion of the fermentation of immobilized cells and reached to 10^7 cfu/ml after 4 weeks. Also, (King et al., 2007), found, at the end of shelf life survival free cells was approximately 10^9 cfu/ml, and the number of cells in the gel beads of the immobilized cell fermentation was higher than 10^{10} cfu/ml-gel. Pilkington *et al*., (1999) explain that, immobilized cells fermentation technique can be protect cells from oxygen and products high concentrations of substrates [Williams D, Munnecke DM. (1981), Champagne *et al*., (1994)., Talwalkar A, Kailasapathy K. (2003)] and bad conditions, like: low pH, some compounds having toxic (Al-Jasass et al., 2010). Also the osmotic can be effect of the survival of cells which comferm by (Jay et al., 2005).

Beside that (Popa & Ustunol, 2011) recorded that the number of *B. bifidium* was increased to 0.28 log in the sample contain sucrose after 24 h of incubation however, in the control sample (without sugar) the numbers of cells was increased 0.36 log and it is indicator of the inhibitory activity of sucrose on the survival of the *B. lactis*.

(Al-Otaibi et al., 2013), documented that the date syrup have inhibitory activity on the survival of *Lactobacillus acidophilus* and *Bifidobacterium lactis* at high concentrations. Moreover, at the end of incubation time survival cells were maintained high enough (more than 10^6 cfu/ml) which is important to enhance the health benefits of these probiotic bacteria.

These results agree with those of Chick *et al*., (2001) who did not find significant difference between the numbers of *B. lactis* at initial time and after 24 h of incubation using fructose, sucrose, honey and also without any sweetener.

4.3.3.2. Summary of experiment 4

Survival rate of NEC of bacteria were maintained high enough for two days of incubation period time about more than 10^5 cfu/ml which is necessary to achieve the health benefits of probiotic bacteria in human.

1. **Statistical analysis**

2.8. Statistical analysis

Results are presented as mean ± standard deviation (SD) of replicated determinations. Data were subjected to one-way analysis of variance (ANOVA) and multiple comparisons were performed by Duncan's test. Statistical significance was set at $p>0.05$. All analyses were performed using SPSS version 17.0 for Windows (SPSS, Chicago, Illinois, USA).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

In this study was to encapsulate of probiotics bacteria with nano-particles to enhance their survival during shelf life of delivery food and to protect them from the physical and chemical rigors of digestive tract besides that to evaluative the survival in simulated gastrointestinal conditions

The objectives of present study included as follows:

1. To enhance survival of encapsulate probiotics bacteria with nano-particles with and without cross-linked, to protect them from the physical and chemical rigors of digestive tract and to evaluative the survival in simulated gastrointestinal conditions.

2. To enhance survival of encapsulate probiotics bacteria with nano-particles with and without cross-linked, during shelf life of delivery food

Conclusions

The findings of the present thesis can be concluded as follows:

1. Chitosan succinate microsphere showed pH can be effect of release matrix form of microsphere. chitosan succinate microsphere cross-link with Genipin had significant high level of survival was observed under pH 7.4 (Colon), besides, that is it resisted in pH 2 (Stomach) condition than chitosan succinate microsphere no cross-link with Genipin. The stability of chitosan succinate cross-linked with Genipin observed a high stability in the acidic environment of simulated gastric juice with absent of pepsin enzyme, besides that, the simulated gastric juice with pepsin enzyme had reduced stability significantly. However, The stability of chitosan succinate without cross-linked with Genipin observed that no significantly effect of simulated gastric juice with or without pepsin enzyme

genipin under concentration 2.5 mM cross-link might be enhance the resistance of chitosan succinate to degradation by pepsin enzyme for controlled release of cells of probiotic. According to these results the new carrier system described herein can be a promising tool probiotic delivery from oral to colon.

2. Results showed that the long time of incubation have inhibitory effects on the survival of NEC and free *Bifidobacterium lactis* (BB-12). This conclusion , the survival NEC of bacteria were maintained high enough for two days of incubation period time about more than 10^5 cfu/ml which is necessary to achieve the health benefits of probiotic bacteria in human.

Recommendations

These finding warrant further research towards the understanding the mechanisms effect of nanotechnology activities to protect benefit bacteria from rigors of digestive gut and food functional properties to be achieve the health benefits

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