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Option : *Applied Microbiology*

Theme

Viability of free and microencapsulated *Lactobacillus plantarum* during gastrointestinal transit simulated *in vitro*

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Summary

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List of abbreviations

AAD	Antibiotics associated diarrhea
CFU	Unity forming colony
FAO	Food and agriculture organization of united nations
GIT	Gastro intestinal tract
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IgA	Immunoglobulin A
LAB	Lactic acid bacteria
Lb. plantarum	*Lactobacillus plantarum*
ME	Microencapsulation
MRS	De Man, Rogosa and Sharpe
NEC	Necrozing entero-colitis
SGIT	Simulated gastro intestinal tract
USP Units	United states pharmacopeia units
WHO	World health organization

Introduction

Introduction

In recent years, people are increasingly interested in foods which both offer a balanced diet and promote human health. This is why the demand on functional foods is growing steadily (Sagalowicz and Leser, 2010). Probiotics is one of the main components of functional foods present generally in products in two forms: supplements and foods. They are also found in various foods and beverages ranging from fruit juices to breakfast cereals (Champagne *et al.*, 2011).

Nowadays, it is well known that probiotics are micro-organisms belonging most commonly to lactic acid bacteria like: *Lactobacillus*, *Bifidobacteruim* and some other species such as *Saccharomyces* and *Streptococcus* (Ranadheera *et al.*, 2010). In addition, several studies have shown that probiotics possess many beneficial effects including the prevention or treatment of intestinal infections, irritable bowel syndrome, the simulation of human system, the improvement in the digestibility of food products, the control of blood cholesterol levels and the prevention of atopic allergies (Nualkækul and Charalampopoulos, 2011).

However, in order to exert its beneficial effects, probiotics must be present at a minimum level of 10^6 CFU/g of food product or 10^7 CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU/g (Chávari *et al.*, 2010). Furthermore, they must also survive during their passage through digestive tract, resist gastric acid and bile salts to arrive viable and in sufficient amounts to intestine (Gbassi *et al.*, 2011).

For this purpose, microencapsulation appears to be an ideal tool for bacterial cell protection for which many studies proved the protective role against adverse conditions to which probiotic can be confronted (Brinques and Ayub., 2011).

In spite of its use which is limited to industrial scale, alginate remains one of the most used materials as recommended by the academic community for probiotic microcapsulation which is carried out mainly with two techniques that are: extrusion and emulsion (Farnworth and Champagne, 2010).

The aim of this work is to study of viability of free and microencapsulated probiotic *Lactobacillus plantarum* using sodium alginate (2%) as matrix for microencapsulation on cell viability in conditions simulating the gastrointestinal tract in presence of three beverages: green tea, black coffee and orange juice.

CHAPTER 1

Literature review

1 -The human gastrointestinal tract:

The human gastrointestinal tract (GIT) is a continuous tube that extends from the mouth to the anus through the thoracic and abdomino pelvic cavities. The GIT's organs include: the mouth, esophagus, stomach, small intestine and large intestine (Tortora and Derickson, 2012).

The function of the digestion of food occurs by mechanical process which includes: chewing, swallowing, peristalsis, defectation and chemical process consisting of enzymatic breakdown of food in the mouth, stomach and essentially in small intestine under the action of enzymes secreted by the liver and exocrine pancreas (**Figure 1**) (Reed and Wickan, 2009). However, digestion time may vary quite considerably and depends upon individuals characteristics such as: age, sex, health status and food properties like: total amount, composition, and particle size (Hur *et al.*, 2011).

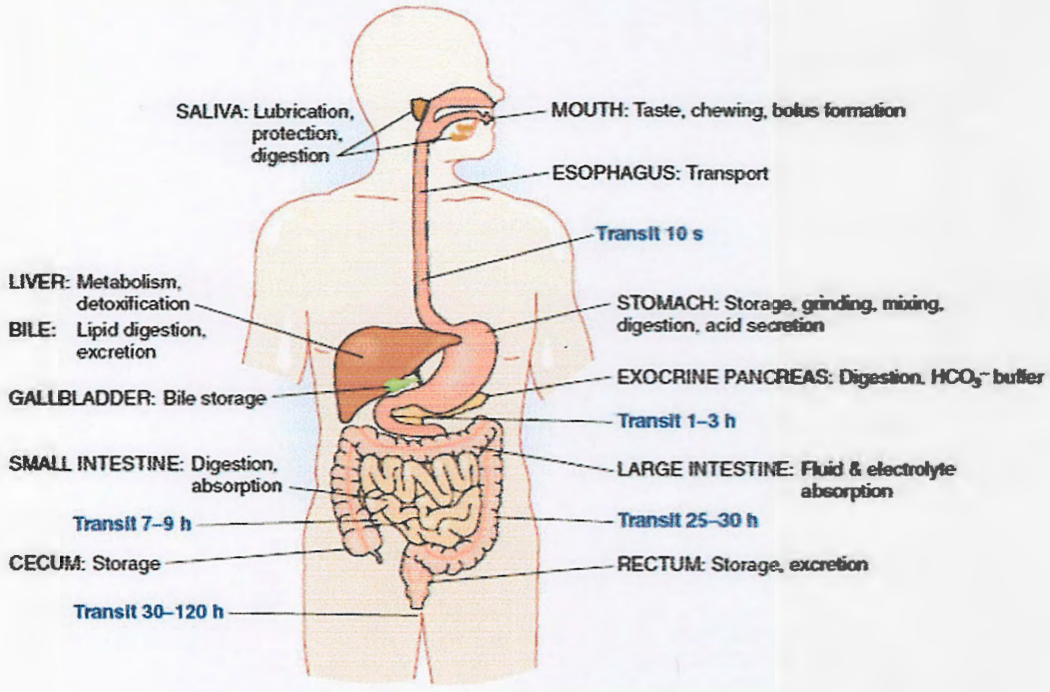

Fig. 1*: Gastro-intestinal tract organs and transit time* (Kibble and Halsey, 2009)

In the human body, the digestive tract harbours the largest and most complex microbial community. This human microbiota which is considered as a complex ecosystem may contain as many as 1000–1150 bacterial species and between 10^{13} and 10^{14} microorganisms with the largest density and diversity of bacteria being found in the distal small bowel and colon (Quigley, 2011). Moreover, it has been reported that human intestinal region contains 300 to 500 different species of bacteria which varies significantly between individuals (Prakash *et al.*, 2011).

2- Probiotics:

Probiotics is a greec word used initially as an antonym of antibiotic and meaning "for life" (Vasiljevic and Shah, 2008). The mid nineteenth century was marked by a renewed interest in microorganisms especially with the discovery of lactic acid bacteria (LAB). However, 150 years before this period, several discoveries based on the relationship between microorganisms and the human health has been realized like the discovery of bifidobacteria in the gut flora of breast fed infants by Henri Tissier (Smolyansky, 2010). In fact, one of the most important theories which derive the concept of probiotics is one that was proposed by Metchnikoff in 1908 which believed that the secret of the long and healthy life span of Bulgarian peasants may be due to the consumption of fermented milk products (Dicks and Botes, 2009).

Over the years, many definitions has been given to the probiotic term by several scientists such as: Kollat (1953), Vergin (1954), Sperit (1971) and fuller's (1992) till 2002 when the Food and Agriculture Organization of United Nations and the World Health Organization (FAO/WHO), suggested a definition which describes probiotic as live microorganisms that when administrated in adequate amount confer a health benefit on the host (Vasiljevic and Shah, 2008).

Many microorganisms are constituted principally by different species and strains of bacteria, yeast and even fungi have been selected for their probiotic potential (**Table1**) (Brunser and Gotteland, 2010). Furthermore, the most commonly microorganisms used in probiotics preparation include bacterial genera of Lactobacilli, Bifidobacteria and some non-pathogenic strains. But, more often than most probiotic culture include *Lactobacillus rhamnosus GG, Lactobacillus caseie shirota, Bifidobacterium animalis* and *Saccharomyces cerevisiae boulardii* have already shown their benefits on human health (Figueroa *et al.*, 2011).

Table 1: Some microorganisms used as probiotics (Brunser and Gotteland, 2010)

Bacteria	***Lactobacillus***	***Bifidobacterium***	***Streptococcus***	**Other**
	acidophilus	***bifidus***	***lactis***	***Leuconostoc***
	johnsonii	***infantis***	***cremoris***	***Pediococcus***
	plantarum	***longum***	***salivarius***	***Propioni bacterium***
	rhamnosus	***thermophilus***	***intermedius***	***Enterococcus***
	delbrueckii	***adolescentis***		***Escherichia coli***
	euteri	***catenulatus***		***Lactococcus***
	fermentum	***pseudocatenulatus***		***Aspergillus***
	brevis	***lactis***		***niger***
	lactis			***oryzae***
	cellobiosus			
	paracasei			
	helveticus			
Yeast and fungi	***Saccharomyces***			
	boulardii			

The selection of probiotic strains depends on various criteria which characterize microorganisms like: functional criteria, safety criteria, and technological criteria (Muller *et al.*, 2009). However, it has been suggested that in order to be considered as probiotic strain. microorganism must include the following properties (Collado *et al.*, 2010):

- Have a demonstrated beneficial effect on the host.
- Be non-pathogenic, non-toxic, and free of significant adverse side effects.
- Be able to survive through the gastrointestinal tract (*in vitro* and *in vivo*).
- Be present in the product in an adequate number of viable cells to confer the health benefit.
- Be compatible with product matrix, processing and storage conditions to maintain desired properties; and labeled accurately.

Many experimental studies have shown that probiotics differ greatly in their mechanisms of action not only between probiotic species but also between certain strains (O'Hara and shanahan, 2007). It has been reported that mechanisms of probiotic action are susceptible to be multifactorial processes and according to different levels of interaction between host and microbe, probiotics can exert their beneficial effects (**Figure 4**) (Collado *et al.*, 2010):

- Firstly, by the adhesion to mucosal and epithelial cells, the stimulation of mucus secretion, and the production of defensive molecules.
- Secondly, by the comprising of immune modulation and the regulation of immune responses.
- Thirdly, by the exclusion and inhibition of pathogens, the inhibition of replication of pathogen via the secretion of antimicrobial substances, and also, by nutrients competition with pathogens.

However, until now the exact modes of action of probiotic bacteria are not yet fully known (Collado *et al.*, 2010, Verna and Lucak, 2010).

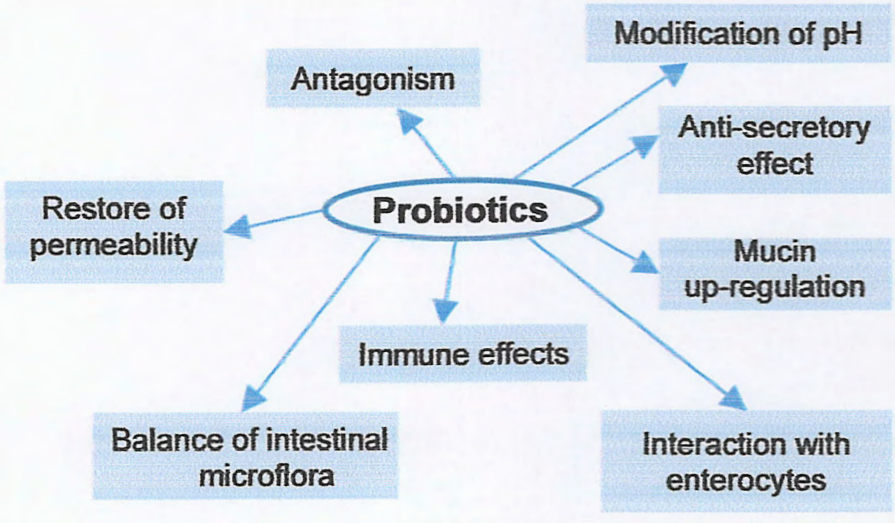

Fig. 4: *Mechanisms of probiotics action in the gut* (Salvatore and Vandenplas, 2010)

It is also important to note that in order to improve the beneficial effects of probiotics; it is possible to combine different compatible or synergetic strains at the same time. This combination has shown its best effect when it deals with different strains than only one kind of it (Collado *et al.,* 2010). In addition, probiotics can be also used in association with prebiotics which are defined as food ingredients that cannot be digested by the human digestive system but are metabolized by discrete enteric cells in order to colonize more species of the GIT. This interaction between probiotics and prebiotics is known as symbiotics (Rauch and Lynch, 2012).

Actually, the great interest in probiotics is not only due to their low cost production, safety, side effects free, but also to their beneficial effects on human health especially in the treatment of various diseases such as: immunological, digestive, and respiratory diseases (Prakash *et al.*, 2011). Also, several experimental observations have demonstrated the beneficial role of probiotics in a variety of gastrointestinal pancreatic and liver disorders **(Figure 5)** or even in systemic disorders as obesity and allergy (Quigley, 2011). For example, and according to many experimental trials, the administration of specific strains of *B. infantis* 35624 and *E. coli* DSM1752 can reduce significantly symptoms in individuals affected by IBS (Parkes, 2010). Furthermore, in the case of antibiotic associated diarrhea (AAD) which is, most commonly caused by *Clostridium difficle* or *Klebsiella oxytoca* , most studies carried out in adult have indicated that the administration of *S. boulardii* can neutralize the cytotoxin produced by *C. difficile*. (Brunser and Gotteland, 2010).

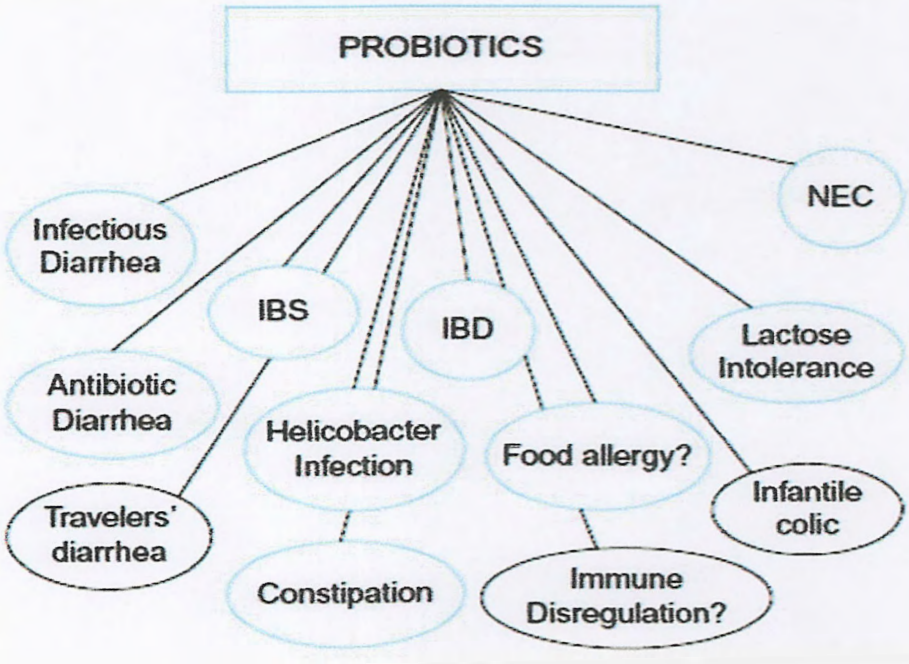

Fig. 5: *Spectrum of clinical application of probiotics in gastro-intestinal disorder in children* (Salvatore and Vandenplas, 2010)

NEC: Necrotizing Enterocolitis, **IBS:** Irritable Bowel Syndrome, **IBD:** Inflammatory bowel diseases

As a result of all these beneficial effects on human health, probiotic cultures are exploited largely by the dairy industry as a tool for the development of novel functional products, that we often find them in various food products such as: yoghurt, mayonnaise, edible spreads, meats and cheese with predominance of strains belonging to *Lactobacillus* ssp. and *Bifidobacterium* ssp. which are the most used in these applications (Vasiljevic and Shah, 2008).

On the other hand, production of probiotics is facing several problems including that of viability. Probiotics must remain viable with sufficient number during production processes, storage, and during transit in the GIT. It is within this context that scientists are trying to find new techniques that improve the viability of probiotics such as, the microencapsulation which has proved its effectiveness (Figueroa *et al.*, 2010, Prakash *et al.*, 2011).

3- Microencapsulation:

Microencapsulation can be defined as the technology of packaging solids, liquids or gaseous materials in miniature sealed capsules that can release their contents at controlled rates under the influence of specific conditions (Anal and Singh, 2007).The birth of this technique dated back to 1950s when Green and Schleicher produced microencapsulated dyes by complex coacervation of gelatin and arabic gum for the manufacture of carbonless copying paper (Dubey *et al.*, 2009). In addition, a microcapsule consists of a semipermeable, spherical, thin, and strong membrane surrounding a solid/liquid core, with a diameter varying from a few micrometers to 1mm (Anal and Singh, 2007). Microcapsules can be classified based on their seize into micro or nanocapsules and also according to their morphology into three basic categories as monocored microcapsules which have a single hollow chamber within the capsule, polycored microcapsules characterized by a number of different sized chambers within the shell, and matrix type microparticles which have the active ingredients integrated within the matrix of the shell material **(Figure 6)** (Dubey *et al.*, 2009).

Fig. 6: *Different types of microcapsules* (Dubey *et al.*, 2009)

3.1- Common food-grade materials used for microencapsulation:

Microencapsulation is conducted using a variety of materials including essentially biopolymers which are widely applied in food industry such as: carbohydrate polymers, polysaccharides, lipids, and proteins (Meiners, 2009).

- *Alginate:*

Alginate (**Figure 7**) is a naturally derived polysaccharide extracted from various species of algae, and composed of β-D-mannuronic and α-L-glucoronic acids. This biopolymer is widely used in cell microencapsulation. For example, calcium alginate is favored for probiotic encapsulation because of its simplicity, non-toxicity, and biocompatibility and, low cost despite some disadvantages like sensitivity to the acidic environment. On the other hand, alginate can also be used in association with other polymer compounds like: starch which has proved its effectiveness in probiotic microencapsulation (Burgain *et al.*, 2011).

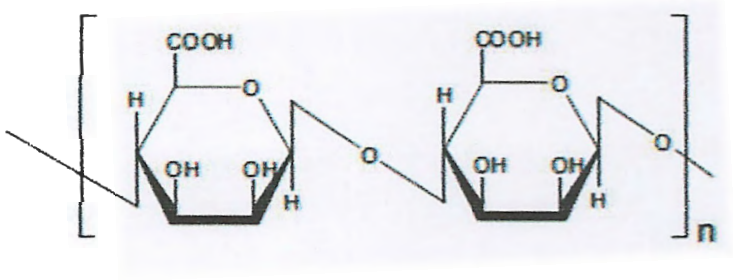

Fig. 7: *Molecular structure of alginate* (Vos *et al.*, 2010)

- *K-carrageenan:*

Carrageenan **(Figure 8)** is a natural polysaccharide that is extracted from marine macro-algae and is commonly used as a food additive. In this case, microencapsulation is realized by the addition of cell suspension to the heat sterilized carrageenan solution at 40 to 45°C and gelation occurs by cooling to room temperature. Finally, the formation of beads is carried out after dropping the mixture of polymer and cells into potassium chloride (KCl) solution (Anal and Singh, 2007).

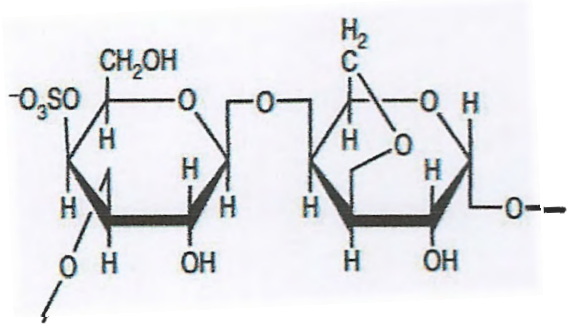

Fig. 8: *Molecular structure of K-carragennan* (Wandrey *et al.*, 2010)

- *Chitosan:*

Is a weak anionic polysaccharide consisting mainly of β (1, 4) linked glucosamine units. It is produced by de-acetylation of chitin extracted from crustacean shells (Meiners, 2009). In fact, chitosan can be used preferably as a coating material for the microencapsulation of probiotic cells. But, it may have an inhibitory action on lactic acid bacteria (Burgain *et al.*, 2011).

- *Starch:*

Starch is a polysaccharide consisting of the succession of glucose units connected together by glucosidic bonds. Starch contains mainly two molecules which are amylose and amylopectin. The ingestible form of starch which resists pancreatic enzymes offer an ideal surface for the adherence of the probiotic cells to the starch granules (Burgain *et al.*, 2011). Furthermore, different types of starch and modified starches have been used for microencapsulation of probiotics cells (Nazzaro *et al.*, 2011).

- *Cellulose acetate phthalate:*

This polymer contains inonizable phosphate groups which make it insoluble in acid media at pH lower than 5 and soluble at higher pH, for this reason it is extensively used as an enteric coating material for the production of core substances for intestinal targeted delivery systems (Anal and Singh, 2007).

- *Gellane gum and Xanthan gum:*

Gellane gum is a microbial polysaccharide formed by the succession of four units of monosaccharide molecules which are: glucose, glucuronic acid, glucose, and rhamnose. As a result, beads derived from the microencapsulation of probiotic cells of gellane gum combined with xanthan gum are more resistant to acidic conditions (Chen and Chen, 2007).

- *Gelatin:*

Gelatin is a high molecular mass polypeptide derived from connective animal tissue, such as bone and skin. This biopolymer can be employed in microencapsulation due to its amphoteric nature in association with anionic carbohydrates to form gum and gelatin coacervates (Meiners, 2009).

- *Milk proteins:*

Milk proteins offer various structural and physico-chemicals properties. One of these properties is their excellent gelation which can be applied in microencapsulation of probiotic cells (Burgain *et al.*, 2011).

- *Other polymers* :

Many acid resistant polymers are mainly used in pharmaceutical field for microencapsulation like: Xypropyl methylcellulose, methyl cellulose, polyvinyl acetate phthalate and ethylcelullose (Meiners, 2009).

3.2- Description of some techniques for microencapsulation:

A large number of techniques is available for microencapsulation of cells or other bioactive materials. In general, these techniques involve chemical processes such as: phase micro-separation, conservation, liposome encapsulation, molecular inclusion or physical processes such as: spray drying, spray chilling, and extrusion. Furthermore, it is possible that some steps are common to several processes (Meiners, 2009). In general, the choice of encapsulation method is related to the type of polymeric or monomeric material used (Dubey *et al.*, 2009).

- *Spray drying*: Spray drying is one of the most commonly used encapsulation methods. It is basically designed to evaporate water from the dry matter. The solution is injected in a hot air stream in a closed vessel and the solvent, which most of the time is water, is evaporated (Meiners, 2009). In general, Arabic gum and starches are the main matrices applied for this method because of their ability to form spherical microcapsules. Its negative effect on the survival of bacteria is due to high temperature which can be overcome by the addition of protectants to the media, spray drying offers many advantages like: rapidity, low cost and its appropriate industrial application (Burgain *et al.*, 2011).

- *Spray freeze drying*: Processing steps of this method are similar to the spray drying, except that in this case, probiotic cells are in a solution which is atomized into a cold vapor phase of cryogenic liquid nitrogen. Spray freeze drying, have also many advantages such as: providing controlled size and larger specific surface area (Burgain *et al.*, 2011).

- *Extrusion:* Microencapsulation by extrusion involves projecting an emulsion core and coating material through a nozzle at high pressure. It involves preparing a hydrocolloidal solution, adding the active substance and extrusing the suspension through a nozzle in the form of droplets into a hardening or setting bath. The most suitable matrices for the encapsulation of active substances with this process are carbohydrates **(Figure 9)** (Meiners, 2009).

- *Coacervation*: Is largely used not only for the preparation of gelatin and gelatin-acacia microcapsules but also for various products based on cellulose derivatives and synthetic polymers. This technique can be divided into simple coacervation which is marked by the use of a single polymer such as gelatin in aqueous or organic media, and complex coacervation which depends on two oppositely charged polymeric materials like gelatin and acacia. The principle of this technique is the dispersion of the hydrophobic core material in aqueous polymer solution (1-10%) at 40-50°C (Dubey *et al.*, 2009).

- *Emulsification:* Emulsification is a chemical technique which is based on the use of hydrocolloids like alginate, carrageenan and pectins for encapsulation. The principle of this process is founded on the relationship between the discontinuous and the continuous phases (Burgain *et al.*, 2011). Furthermore, various advantages are related to this technique like: ability to produce smaller microsphere (10 µm-1 mm) and the easiness to scale-up (**Figure 9**) (Zuidam and Shimoni, 2010).

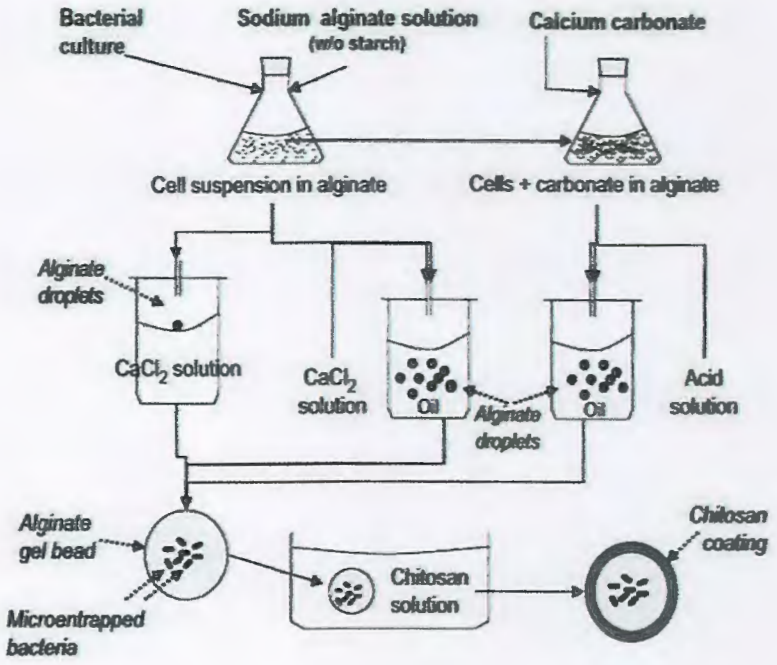

Fig. 9: *Three methodologies based on extrusion or emulsion to obtain alginate beads* (Farnworth and Champagne, 2010)

Nowadays, microencapsulation (ME) is extensively employed in various domains such as: food industry, pharmaceuticals and agriculture (Dubey *et al.*, 2009). In addition, application of ME is relatively dependent on the objectives of each field. In food industry, ME is most used in cheese production, frozen dairy desserts and yoghurt production with a major goal which is the protection of probiotic cells against unfavorable environmental conditions during storage and metabolic activity of the GIT (Chen and Chen, 2007). For example, the study of Chávarri *et al.*, 2010 has demonstrated that microencapsulation of *L. gasseri* and *B. bifidum* with alginate and chitosan coating offers an effective means of delivery of viable bacterial cells in appropriate levels to the colon and helps maintaining their survival during simulated gastic and intestinal juice. In another study, using several microencapsulated strains of *Lb. plantarum* in alginate-whey protein beads, indecated that encapsulation ensured the delivery of high amounts of viable bacteria in the jejunum (Gbassi *et al.*, 2011). On the other hand, pharmaceutical industry applied ME in drug production in order to control drug release in the GIT and essentially for masking the taste and the odor of many drugs (Bansode *et al.*, 2010).

In conclusion, ME is a promising technique for the preservation of probiotic cells during storage and under gastro-intestinal conditions (Figueroa *et al.*, 2011). Furthermore, the use of lipidic matrices for encapsulation confers strong gastro resistance to the cell and confers also a significant improvement in final product stability (Del Piano *et al.*, 2011).

CHAPTER 2

Materials and methods

Materials and methods

1-Materials:

1-1-Microorganism:

The study was conducted with a *Lactobacillus plantarum* G1 strain isolated from local chicken crop ISA15 at the Laboratory of Microbiology of the University of Jijel/ Jijel/ Algeria. The strain was preserved in three forms: lyophilized, glycerol stocks, and conservation agar stocks.

1-2- Products and reagents:

- The Man Rogosa Sharpe (MRS) broth and agar (Institut PASTEUR D'ALGER).
- Sodium Alginate of food grade (Louis François).
- Hydrochloric acid (HCl) 5N.
- Sodium hydroxide (NaOH) 5N.
- Phosphate buffer (pH 7.0) 1M.
- Calcium chloride (CaCl 0.05M)
- Tween 80.
- Ethanol solution (80 %).
- Normal saline solution.
- Distilled water.
- Bile salts (Institut PASTEUR D'ALGER).
- Glycerol (Institut PASTEUR D'ALGER).
- Agar-Agar (Institut PASTEUR D'ALGER).
- Gram stain reagents.

1-3-Equipment:

- Colony counter (Funke Gerber).
- Shaking water bath (FALC).
- Vortex (MinishakerIKA).
- Centrifuge (HETTICH ZENTRIFUGEN).
- pH meter (HANNA Instrument).
- Incubators (Memmert).
- Oven (Controls).
- Heat magnetic stirrer (Bunsen).
- Balance (Scout Pro).
- Spectrophotometer (Amersham Biosciences).
- Filter paper (Wathman).
- Water bath (Gerhardt Bonn).
- Analytical balance (Kern ALS 220.4N).
- Precision optical microscope (Paralux).
- Autoclave (Slli AVX electronic).
- Syringes (2.5ml).
- Vernier caliper (Ningbo Richland).

1-4- Enzyme:

In this study and to simulate the intestinal conditions, we have used the pancreatin enzyme of pharmaceutical grade from "*Swiss herbal*®: Pancreatin Enzyme (Porcine pancreas) 125mg, Amylase (*Aspergillus oryzae*) 12500 USP units, Protease (*Aspergillus oryzae*) 12500 USP units, Lipase (*Aspergillus niger*) 1000 USP units ".

1-5- Beverages:

In order to conduct this experiment, three beverages and distilled water were used which were:

- Black coffee ("*NESCAFÉ Gold*®").
- Green tea ("*EsSahraa*®").
- Orange juice ("*Rouiba*® " : water, orange juice based concentrate, sugar, pulp of orange, citric acid, thickening agents: pectin, aroma, coloring agent: beta carotene, vitamins (C, E, B2, provitamin A, B6, B1) content of fruits 12% minimum.

All beverages were autoclaved for 15 minutes, after adjusting pH to 2.0 with HCl 5N.

2- Methods:

2-1-Strain activation:

Lb. plantarum was activated by overnight incubation in Man Rogosa Sharpe (MRS) broth at 37°C. The purity of the strain was checked by streaking on MRS agar. Single colony was used and stained by Gram stain.

2-2-Preparation of cells for microencapsulation:

Flasks (250 ml) containing 100 ml of the MRS broth, were inoculated with 2 ml of an overnight MRS broth culture and incubated at 37°C for 24 hours in a shaking water bath at180 rpm.

2-3- Microencapsulation of *Lb. plantarum*:

The method described by Sheu *et al.* (1993) with some modifications, was used for microencapsulation of *Lb. plantarum* cells. Cells in MRS broth were harvested by centrifugation at 2000 g for 10 min at 4°C. The cell pellet was washed twice with normal saline, and was finally re-suspended in 10 ml of the same solution, to reach a final optical density of 1.6 at 600 nm. The cells were microencapsulated by mixing 5 ml of the bacterial suspension with 45 ml of sterile (2 %) sodium alginate solution prepared by dissolving 1 g of sodium alginate in 45 ml distilled water. The mixture was homogenized by a magnetic stirrer and transferred to sterile syringes (2.5 ml) injected and allowed to drop into 500 ml of sterile cold $CaCl_2$ (0.05M) supplemented with 0.1 % tween 80.

The beads were stabilized for 30 min by homogenization, after that they were washed three times with normal saline and collected by filtration through Wattman filter paper. Finally, the beads were stored in sodium chloride solution at 4°C for 8 days (Todorov *et al.*, 2012).

2-4-Viability of free and microencapsulated *Lactobacillus plantarum* under *in vitro* simulated human gastrointestinal tract conditions:

In order to study the viability of free and microencapsulated *Lb. plantarum* in simulated human gastro-intestinal tract and to evaluate the effect of some commonly consumed beverages on this lactic acid probiotic bacteria, four beverages were prepared for this study which were: black coffee solution "*NESCAFÉ Gold*®", green tea "*EsSahraa*®", orange juice "*Rouiba*®" and distilled water.

The tests were performed using sterile Erlenmeyer (250 ml), free and microencapsulated cells were exposed to simulated gastro-intestinal tract *in vitro* **(Figure 10).**

At the beginning, either free or microencapsulated *Lb. plantarum* cells were treated in simulated gastric conditions by the inoculation of strains in 50 ml of sterile acidified beverage (pH 2); the Erlenmeyers were incubated for 90 min at 37±1°C in a shaking water bath at 50 rpm. Subsequently, the strains were incubated for 150 min under intestinal conditions by adding a mixture of 1 % pancreatin, 0.3% bile salts, 0.5%NaCl, (pH 6.8) in sterile conditions to the beverages. The same temperature and peristaltic movements were kept. Samples of 1 ml (for free cells) or 5 beads (for microencapsulated cells) were taken after 0, 90, and 240 minutes for the determination of total viable counts (Pacheco and Toro, 2010).

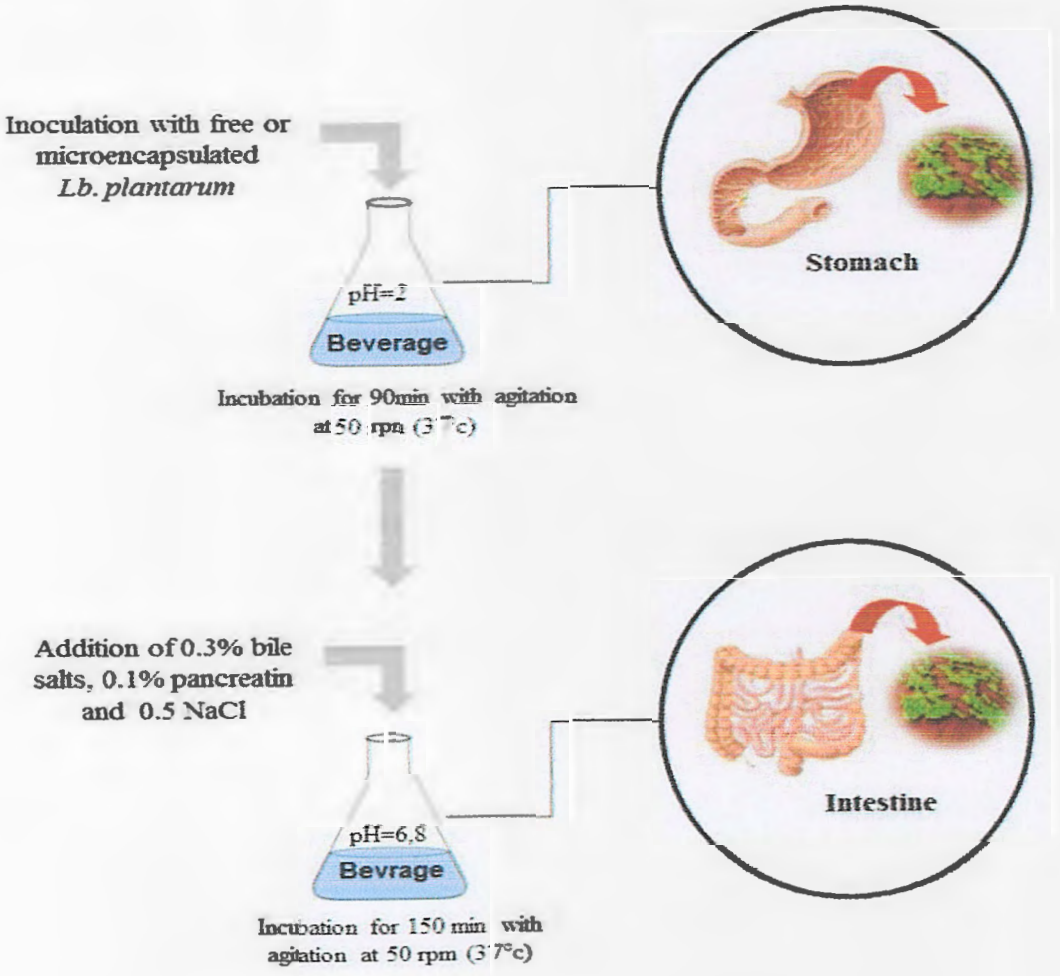

Fig. 10: *Schematic representation of the laboratory designed simulated GIT*

2-5-Solubilization of sodium alginate beads:

The microencapsulated cells on sodium alginate (2%) were released after dissolving of five beads in 2 ml of phosphate buffer 1M (pH 7.0), followed by shaking for 15 minutes using a vortex.

2-6-Characterization of beads:

Beads diameter and weight were measured by the use of vernier caliper and analytical balance, respectively.

2-7-Determination of total viable counts:

Total viable counts of *Lb. plantarum* were determined by a pour plate method using MRS agar after serial dilutions in normal saline solution. Plates were incubated at 37°C for 48 hours, the results were expressed in percentage of viability (Pacheco and Toro., 2010). Based on the following formula:

$$\% \text{ Viability}_t = \frac{\log CFU_t}{\log CFU_{t0}} \times 100$$

Where: CFU_t: final viable count in time.

CFU_{t0}: initial viable counts.

CHAPTER 3

Results and discussion

Results and discussion

1-Simulation of the GIT conditions *in vitro*:

In order to simulate the gastro-intestinal tract *in vitro*, we have designed a model according to tools found in our laboratory which is presented in **figure 10**. The model consists of Erlenmeyer flasks (250 ml) which were incubated in shaked water bath at 37°C (the same temperature of the human body) at 50 rpm (to simulate peristalsis movement of stomach and intestine) (Pacheco and Toro, 2010). Several factors, such as ingested food characteristics, enzyme activity, stress and digestion time, have significant influence on the results of *in vitro* digestion methods. Therefore, *in vivo* conditions can never be completely simulated under *in vitro* conditions (Hur *et al.*, 2011).

Digestions were done according to a fast transit protocol, with a delivery time of about half of that found on literature based on *in vivo* data reported by Kibble and Halsey, (2009). In our study, a digestion time of 4 h was used according to many *in vitro* models including one that was proposed by Pacheco and Toro (2010).

In order to survive in the human gastrointestinal tract conditions lactic acid bacteria must be active and not below 10^7 Colony Forming Units (CFU) per gram of product (Pacheco and Toro, 2010). Results found in our study indicated that free and microencapsulated *Lb. plantarum* grew well in black coffee solution, green tea, commercial orange juice, as well as in distilled water.

2-Characterization of sodium alginate (2%) beads:

The initial beads resulting from the microencapsulation of *Lb. plantarum* cells within sodium alginate (2%) were spherical, relatively uniform in size (2.5 mm), with a weight of approximately (0.0133 g) (**Table 2**). After their exposure to the simulated gastro-intestinal tract conditions, the beads incubated in sample water remained stable. However, the size and the weight of beads incubated in coffee and tea have decreased to (2 mm) and (0.0060 g), respectively. Furthermore, the diameter of beads incubated in orange juice have increased to (2.7 mm) and (0.0157 g) (**figure 11**).

Table 2: Characterization *of Lb. plantarum* beads in sodium alginate matrix

matrix	size of diameter (mm)	form	weight (g)	number of cells/ bead	number of beads/ ml of gel
Sodium alginate (2%)	2.5	spherical	0.0133	1.06×10^{13}	60

The decrease in size and weight of the beads which are incubated in coffee and tea can be probably due to disintegration in the presence of excess monovalent ions, and harsh chemical environment. Especially when we know that alginate may undergo a reduction in its molecular weight when it is exposed to low pH (Krasalkoopt *et al.*, 2004). Moreover, the increase in volume of beads incubated in orange juice can be explained by the formation of hydrophobic alginate in acidic condition and thus less swollen state, whereas in low pH the beads may swell and have a large volume and thus making the structure more hydrophilic, thus particles are most stable in acidic environment (Sohail *et al.*, 2012).

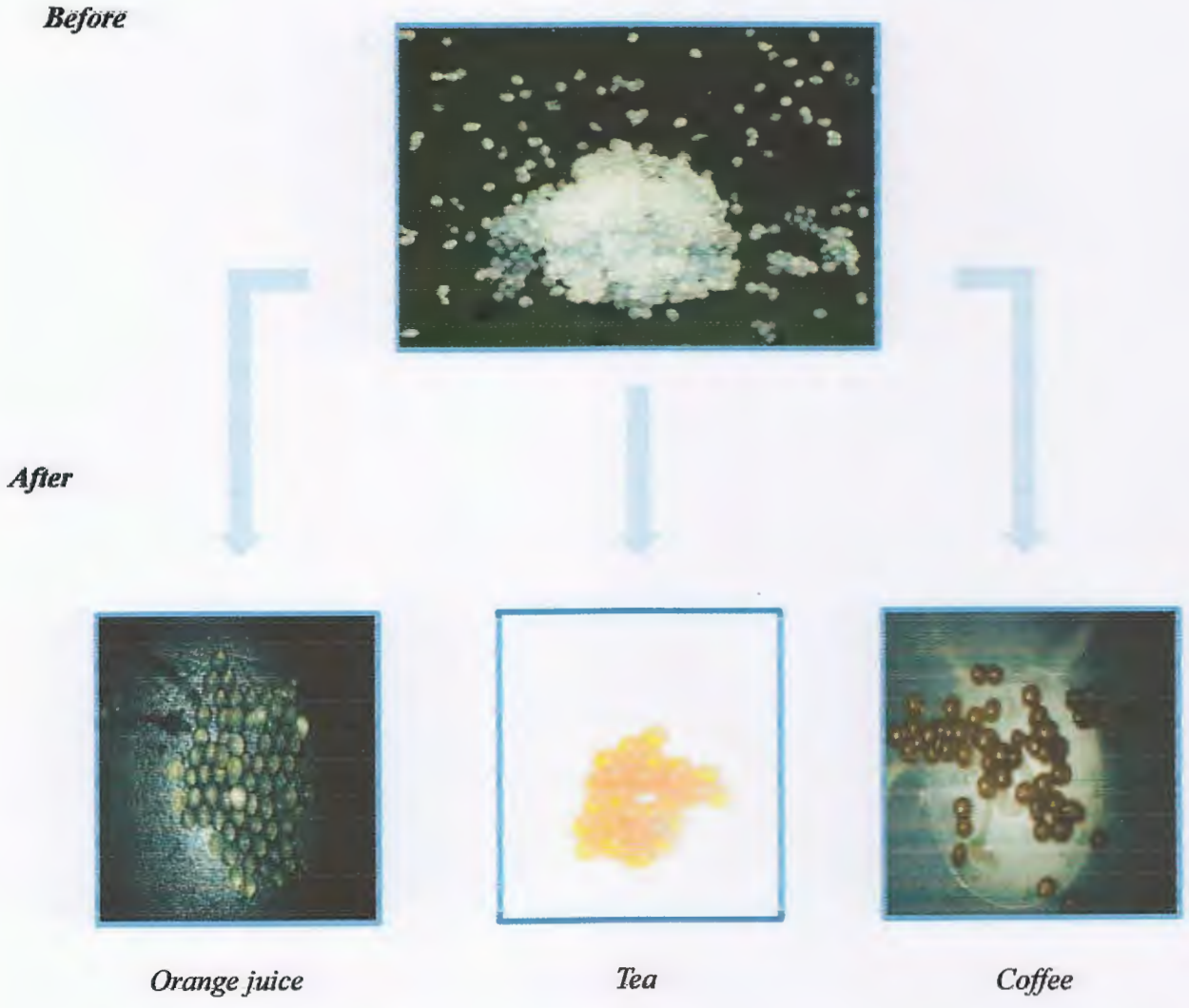

Fig. 11: *Aspect of beads enveloping L. plantarum cells before and after incubation in different beverages*

3-Viability of free and microencapsulated *Lb. plantarum* in distilled water:

Counts of free and microencapsulated *Lb. plantarum* in distilled water after exposure to conditions simulating the transit through the GIT are shown in **figure 12.** After 90 minutes in the solution simulating the stomach (pH 2.0), 67% (2. 4×10^{10} CFU/ml) for microencapsulated cells kept their viability, however, the count of free cells after the same treatment were 2% lower than the microencapsulated cells count indicating that the microencapsulated cells remained inside the capsules and were not affected by the low pH. When both free and microencapsulated cells were exposed to intestine fluid (digestive enzymes and bile salts), 50% (11×10^{6} CFU/ml) of free cells and 66% (1, 92×10^{10}CFU/ml) of microencapsulated cells survived in these conditions, this means that viability of non-encapsulated cells was 16% lower than microencapsulated ones. This result indicates that the number of free cells is lower than that recorded for that microencapsulated. The same results were found by Todorov and coworkers (2012), they have indicated that the protection given by sodium alginate coats is more evident in conditions simulating intestine than in conditions simulating the stomach conditions. They also mentioned that microencapsulated *Bifidobacterium bifidum* and *Lactobacillus acidophilus* viability decreased after exposure to 2% (w/v) and 4% (w/v) bile salts (Todorov *et al.*, 2012).

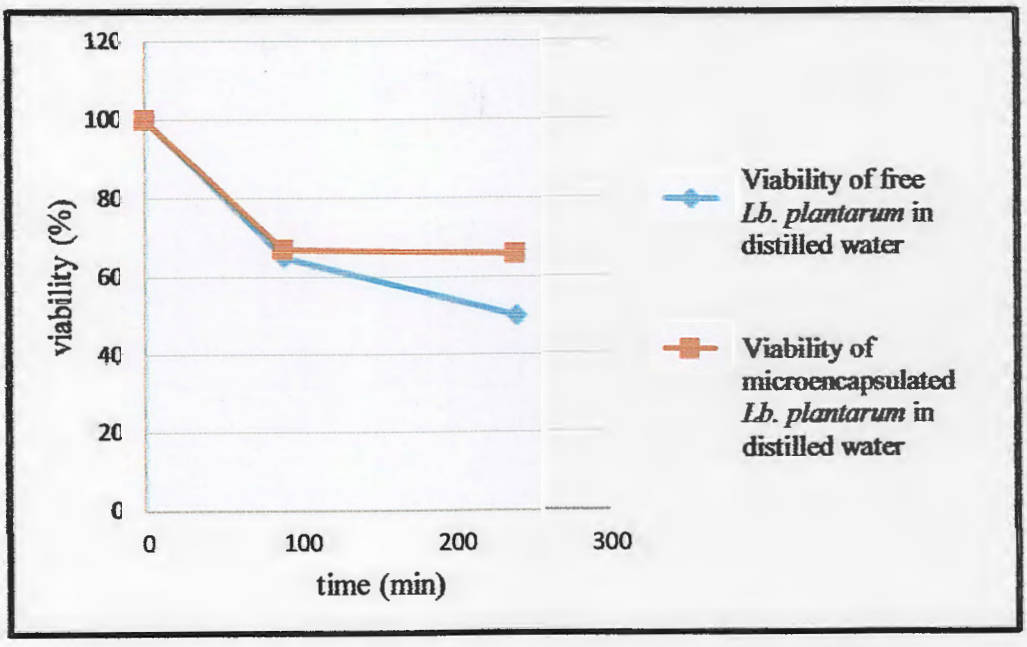

Fig. 12*: Viability of free and microencapsulated Lb. plantarum in blank sample (distilled water) in GIT-like conditions*

Chandramouli *et al.* (2004) reported that when microencapsulated *Lb. acidophilus* CSCC 2400 and *Lb. acidophilus* CSCC 2409 were subjected to low pH and high bile salts concentration (1%) under optimal microencapsulation conditions there was a significant increase in viable cells counts compared to the free cells under similar conditions. Mandel *et al.* (2006) have studied the viability of microencapsulated *Lactobacillus casei* NCDC-298 in different alginate concentrations (2%, 3% or 4%) at low pH (1.5) and high bile salt concentration (1% or 2%) they found that survival of microencapsulated *L. casei* was better compared to free cells.

4-Viability of free and microencapsulated *Lb. plantarum* in green tea and in black coffee:

Survival of free and microencapsulated *Lb. plantarum* in the presence of green tea, acid bile salts and pancreatic enzymes are represented in **figure 13.**

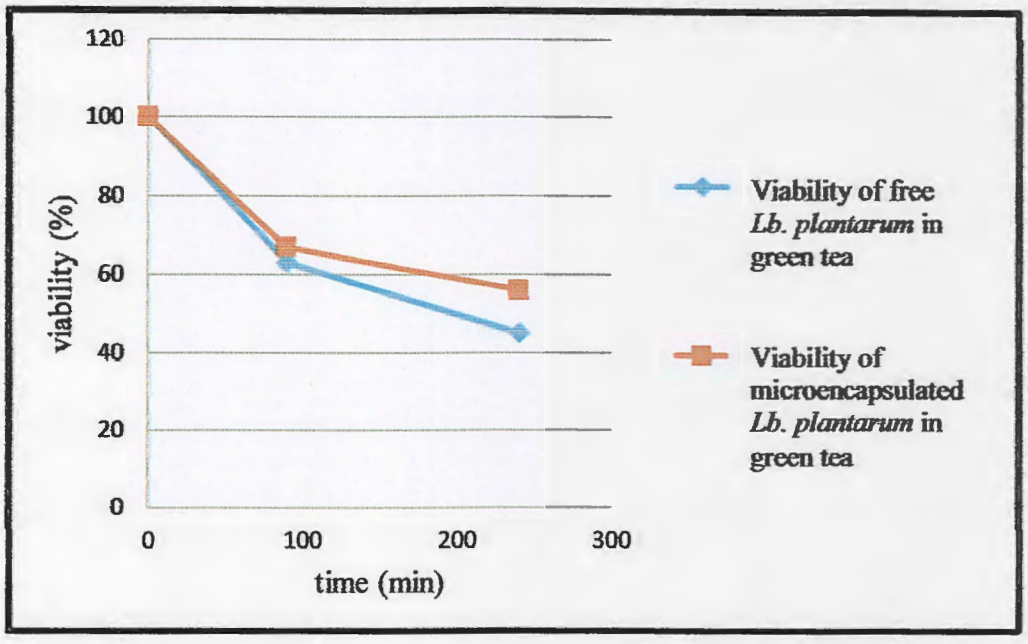

Fig.13: *Viability of free and microencapsulated Lb. plantarum in green tea solution incubated in GIT- like conditions*

The number of free cells decreased from 7.87×10^{14} CFU/ml to 2.5×10^{9} CFU/ml (63%) after treatment in gastric conditions, however, after incubation in simulated intestinal fluid it reached 5×10^{6} CFU/ml, which represents 45% of the initial count. On the other hand, the microencapsulated cells decreased to reach 6×10^{10} CFU/ml after incubation in acidic conditions, and 12×10^{8} CFU/ml after being incubated in intestinal conditions, this is to say that 56% of cells survived after the whole treatment.

This indicated that, the viability of *Lb. plantarum* cells was improved using microencapsulation by approximately 4% and 11% in acidic and intestinal simulated conditions, respectively.

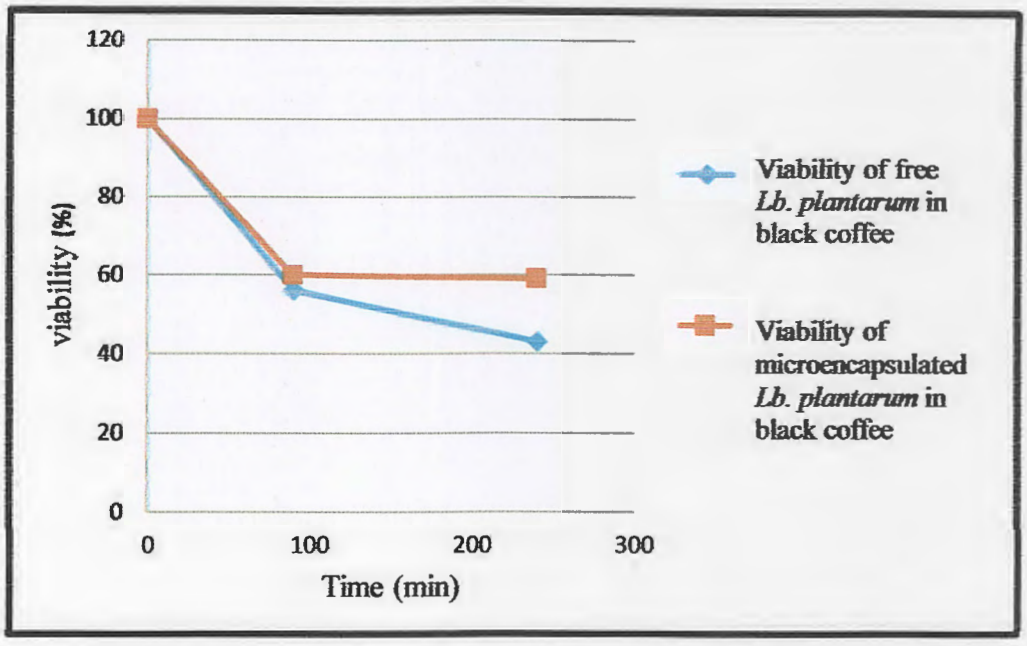

Fig.14: *Viability of free and microencapsulated Lb. plantarum in black coffee solution incubated in GIT-like conditions*

In order to study the effect of the ingestion of black coffee on the viability of *Lb. plantarum* cells, the same experiment described for green tea was carried out. Results shown in **figure 14** indicated that, the number of free *Lb. plantarum* cells decreased from 7.87×10^{14} CFU/ml to 2×10^{8} CFU/ml after incubation in acidic conditions, while it reached 3×10^{6} CFU/ml after incubation in intestine like conditions, that is to say only 43% of free cells survived the SGIT conditions. However, better results were recorded with microencapsulated cells, since slightly more than 59% survived the intestine like environment (3×10^{9} CFU/ml).

Comparison of the results presented above, indicated that cell viability in water was higher than those in green tea and black coffee, while cell viability in the GIT like conditions supplemented with black coffee was the lowest for free cells. Some authors mentioned that the matrix food might protect the lactic acid bacteria, such as the *Bifidobacterium* from the acid pH present in the human stomach, as a result probiotic delivery will be enhanced (Pacheco and Toro, 2010), in contrast our results on green tea and black coffee effect, reflected a negative action on cell viability. In order to explain the loss of cell viability of *Lb. plantarum*, the composition of the two beverages as well as the effect of these constituents on the studied strain should be known.

The most important constituents of green tea include multiple catechin components like: epigallocatchin-3-gallate (EGCC) (50-80%), epicatchins-3-gallate (ECG), epicatchin (EC) and epigallocatechin (EGC) (Coyle *et al.*, 2008).

Furthermore, coffee includes: flavonoids (catechins and antrocyanins), caffeic acid, ferulic acid, nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid and caffeine. It is important to note that some of these constituents are known as polyphenol compounds which are associated generally to antioxidative and anti-inflammatory activities (Esquivel and Jiménez, 2012) (**Figure 15**).

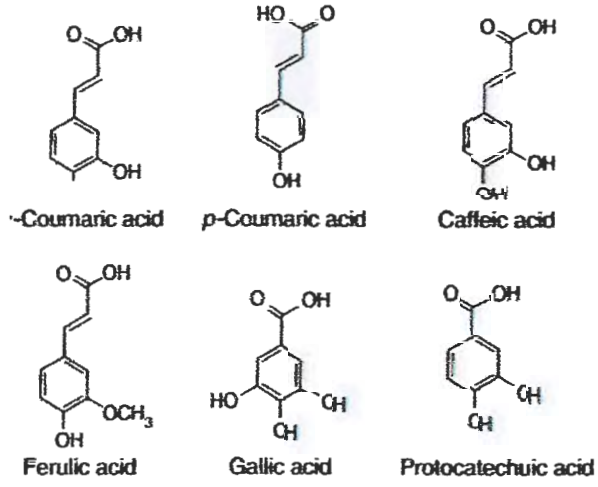

Fig. 15: *Structure of some phenolic acids* (Rodriguez *et al.*, 2009)

Despite that a large number of LAB species does not grow well in raw vegetable material when phenolic compounds are abundant. *Lb. plantarum* is the most frequently commercial starter used in the fermentation of vegetables. On the other hand, a few numbers of studies were devoted to the study of the influence of phenolic compounds on the viability of *Lb. plantarum*. For example, it was reported that growth of *Lb. plantarum* was significantly reduced in presence of 1 g/l p-coumaric acid (phenolic compound) and the inhibitory activity increased in presence of NaCl (Rodriguez *et al.*, 2009).

The same researchers reported that when *Lb. plantarum* cells are exposed to ripe olives, the inhibitory effect of diffused phenolic compounds on these cells was significant only when it is associated with NaCl, besides; the bactericidal effect of phenolic compounds is related to the alteration of cell wall and cytoplasmic membrane, which will possibly lead to destruction of cell envelope. Rozès and Peres (1998) studied the effects of phenolic compounds on the fatty acid composition of *Lb. plantarum* membranes. They described that increasing amounts of caffeic and ferulic acids induced a gradual increase in the amounts of myristic, palmitoleic, stearic and 9, 10-methylenehexadecanoic acid with a concomitant decrease of lactobacilli acid. On the other hand, the addition of tannins induced an increase in the lactobacillic acid level at the expense of vaccenic acid content. Their results suggested that, in the presence of acidic phenols, the fatty acid composition is altered in terms of what occurs in response to low temperature or high alcohol concentration.

Overall, the decrease in viability of cells during the gastric conditions may be due to certain compounds of tea and coffee like: polyphenol which possess antimicrobial activity and also to cells stress under hard acidity. Furthermore, the loss of viability during intestinal conditions can be explained probably by the polyphenol compounds and NaCl combination effect.

5-Viability of free and microencapsulated *Lb. plantarum* in orange juice:

The previous experiment was carried out to study the effect of the ingestion of orange juice on the viability of *Lb. plantarum.* Results showing the *in vitro* viability of free and microencapsulated cells of *Lb. plantarum* when simulated to gastrointestinal conditions in orange juice are presented in **figure 16.**

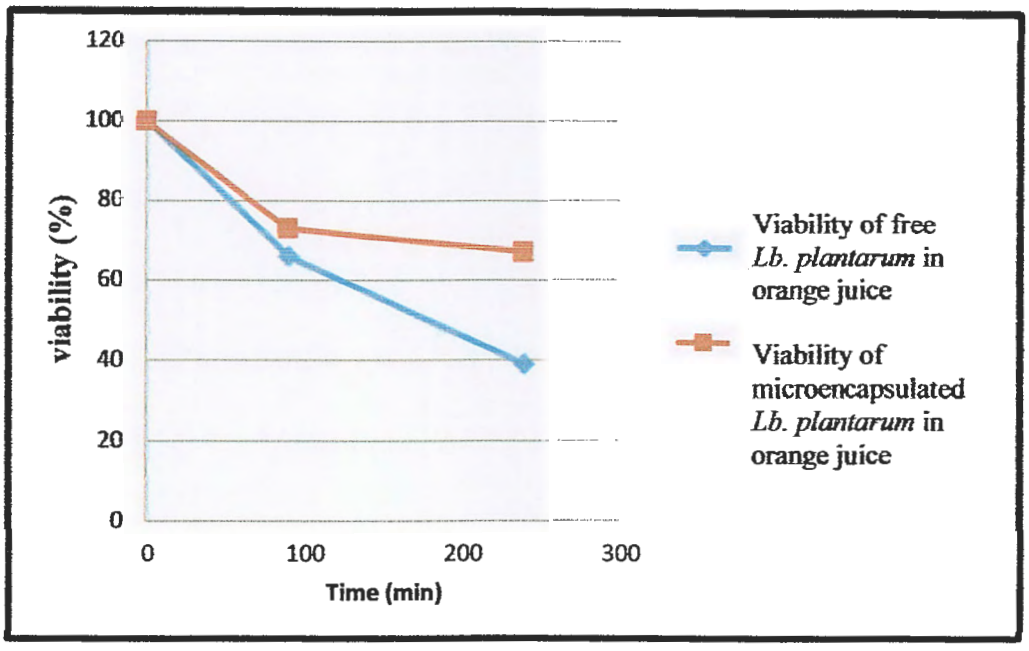

Fig. 16: *Viability of free and microencapsulated Lb. plantarum in orange juice incubated in GIT-like conditions*

The initial number of about 1.15×10^{14} CFU/ml for free *Lb. plantarum* cells was reduced to 18×10^{8} CFU/ml after treatment in acidic conditions and it was decreased more to reach 3×10^{5} CFU/ml after treatment in intestinal conditions which means that 39% of free cells survived the simulated gastrointestinal tract conditions. Results obtained in the case of microencapsulated cells were more interesting because 73% of cells resist gastric conditions which correspond to 3×10^{11} CFU/ml. In addition, the loss of viability during intestinal conditions was not important especially because the number of microencapsulated cells decreased from 3×10^{11} CFU/ml to 2.5×10^{10} CFU/ml that is to say that only 33% of cells has lost their viability.

In reality, orange juice is a source of bioactive compounds consisting of: vitamin C, sugar (sucrose, glucose, and fructose), organic acids (citric acids and manolic acids), dietary fibers, carotenoids (α and β carotene) and phenolic compounds which include phenolic acids (hydroxycinnanmic acid and its derivatives such as: ferulic acids and caffeic acid) and flavanones(Moreno *et al.*, 2005, Kelebek *et al.*, 2009, Sohail *et al.*, 2012, Nualkuekol *et al.*, 2012).

Furthermore, there are very little studies regarding the effect of ingestion of orange juice on viability of probiotics in human GIT. However, several researches have shown that the main factor influencing the survival of probiotics in fruit juice is the pH as well as the level of organic acids, dietary fibers, proteins, total phenol and oxygen (Nualkaekul *et al.*, 2012). For example, a study on *Bifidobacterium longum* in various solutions of fruit juices have demonstrated that the high levels of pH, citric acid, proteins and dietary fibres enhanced the cell survival (Nualkaekul

et al., 2011), other studies have demonstrated that the presence of glucose may improve the survival of Lactobacilli in acidic conditions (Champagne *et al.*, 2008).

On the other hand, literatures have shown that a low pH during bacterial growth induces an acid tolerance which may protect probiotic bacteria not only from acid pH but also from other stresses such as heat, osmotic or oxidative stress (Champagne *et al.*, 2008). Moreover, it has been suggested that citric acid have a significant positive effect on viability since it is probably metabolized by the cells (Nualkaekul and Charalampopoulos, 2011).

Based on the results of the experiment, the loss of viability for free *Lb. plantarum* cells during gastric conditions are more important than the microencapsulated one which is probably due to death of cells under hard acidity (pH < 2). Nualkaekul *et al.*, (2011), reported that when the cells are present in an environment of low external pH, the energy consumption which is required for maintenance of the intracellular pH is increased. As results, other crucial cellular functions are depressed of ATP and the cells cannot survive.

Furthermore, the action of bile salts on viability of free and microencapsulated cells when *Lb. plantarum* is ingested with orange juice is less harmful than gastric conditions. The lower decrease may be explained by the effect of phenolic compounds of orange juice in presence of NaCl on survival of cells.

Conclusion

Conclusion

In vitro simulation model designed in this study made possible the study of the effects of ingested black coffee, green tea and orange juice on the viability of free and microencapsulated cells. The results of this study demonstrated that:

- An improvement of about 28% was obtained in the viability of *Lb. plantarum* when exposed to GIT-like conditions in the presence of orange juice.

- When green tea was used, an improvement of about 16% was obtained in the viability of *Lb. plantarum* when exposed to GIT-like conditions.

- As well, and under the same conditions we have noticed an improvement of about 11% in the viability of *Lb. plantarum* in the presence of black coffee.

It is possible to suggest that *Lactobacillus plantarum* survives in high numbers and reach the colon when it is consumed together with certain beverages like those evaluated in this study. The microencapsulation of *Lactobacillus plantarm* with alginate offers an ideal tool not only for the protection of cells during simulated gastric and intestinal juice, but also, for the delivery of viable cells in levels appropriate to the colon.

Future research regarding the study of the effect of other beverages and foods on the viability of probiotics will make it possible to establish recommendation to consume the functional food appropriately; so that the consumer can take the whole advantages of the beneficial effects of probiotics.

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Annex

1-Gram stain steps and reagents:

- Application of the primary stain **Crystal Violet** to a heat-fixed smear of bacterial culture (20 secondes)
- Addition of **Gram's Iodine** (1 minute)
- Decolorization with 95% **ethyl alcohol** (10-20 secondes)
- Counterstain with **fuschin** (20 secondes).

2-Compounds of MRS broth:

Table 1: The Man Rogosa Sharp broth compounds for 1 litter

Compounds	Quantities
Glucose	20g
Beef extract	8g
Yeast extract	4g
Sodium acetate	5g
Bipotassic phosphate	2g
Ammonium citrate	2g
Magnesium sulfate heptahydrate	0.2g
Manganese sulfate tetrahydrate	0.05g
Tween 80	1ml
Distilled water	1000ml

pH adjusted to 6.2 at 25°C

3-Compounds of MRS agar:

The Man Rogosa Sharpe agar was typically contains the same compounds of MRS broth supplemented with **15g** of agar-agar.

4-Phosphate buffer pH 7 (1M for 100 ml):

Table 2: Phosphate buffer compounds for 1 litter

Compounds	K_2HPO_4	KH_2PO_4
Quantities (ml)	61.5	38.5

5- Calcium chloride ($CaCl_2$) 0.05 M for 1 litter: 5.55 g of $CaCl_2$ in1 litter of distilled water.

Table 3: Characterization of beads after incubation in SGIT in presence of different beverages

Beverage	Diameter (mm)	Weight (g)
Black coffee solution	2	0.0060
Green tea solution	2	0.0060
Orange juice	2.7	0.0157
Distilled water	2.5	0.0133
Initial characteristics	2.5	0.0133

Table 4*: Viability of free and microencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with distilled water (blank).*

Time (minute)	0	90	240
Number of free *Lb. plantarum* in distilled water(blank) (CFU/ml)	$1{,}15\times10^{14}$	15×10^{8}	11×10^{6}
Number of microencapsulated *Lb. plantarum* in distilled water (CFU/ml)	$4{,}3\times10^{15}$	$2{,}4\times10^{10}$	$1{,}92\times10^{10}$

Table 5: ***Percentage of viability of free and micoencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with distilled water.***

Time (minute)	0	90	240
Viability of free *Lb. plantarum* in distilled water (%)	100	65,22	50,07
Viability of microeencapsulated *Lb. plantarum* in distilled water (blank) (%)	100	66,41	65,77

Table 6: ***Viability of free and microencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with green tea solution.***

Time (minute)	0	90	240
Number of free *Lb. plantarum* in green tea solution(CFU/ml)	7.87×10^{14}	2.5×10^{9}	5×10^{6}
Number of microencapsulated *Lb. plantarum* in green tea solution(CFU/ml)	1.5×10^{16}	6×10^{10}	12×10^{8}

Table 7: ***Percentage of viability of free and micoencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with green tea solution.***

Time (minute)	0	90	240
Viability of free *Lb. plantarum* in green tea solution (%)	100	63,44	45,20
Viability of microencapsulated *Lb. plantarum* in green tea solution (%)	100	66,56	56,11

Table 8: ***Viability of free and microencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with black coffee solution***

Time (minute)	0	90	240
Number of free *Lb. plantarum* in black coffee solution (CFU/ml)	7.87×10^{14}	2×10^{8}	3×10^{6}
Number of microencapsulated *Lb. plantarum* in black coffee solution (CFU/ml)	1.5×10^{16}	6×10^{9}	3×10^{9}

Table 9: ***Percentage of viability of free and microencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with black coffee solution***

Time (minute)	0	90	240
Viability of free *Lb. plantarum* in black coffee solution (%)	100	55,74	43.24
Viability of microencapsulated *Lb. plantarum* in black coffee solution (%)	100	60.38	59

Table 10: ***Viability of free and microencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with orange juice.***

Time (minute)	0	90	240
Number of free *Lb. plantarum* in orange juice (CFU/ml)	$1,15\times10^{14}$	18×10^{8}	3×10^{5}
Number of microencapsulated *Lb. plantarum* in orange juice (CFU/ml)	$4,3\times10^{15}$	3×10^{11}	$2,5\times10^{10}$

Table 11: ***Percentage of viability of free and micoencapsulated Lactobacillus plantarum in simulated gastro-intestinal tract in vitro with orange juice.***

Time (minute)	0	90	240
Viability of free *L. plantarum* in orange juice (%)	100	65,78	38,90
Viability of microencapsulated *Lb. plantarum* in orange juice (%)	100	73,38	66,53

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Theme

Viability of free and microencapsulated *Lactobacillus plantarum* during gastro-intestinal transit simulated *in vitro*

Abstract :

Microencapsulated cells of *Lactobacillus plantarum* G1 were prepared by extrusion technique with sodium alginate 2%. The effect of ingestion of three beverages: black coffee, green tea and commercial orange juice on the viability of free and microencapsulated *Lactobacillus plantarum* cells in simulated gastric conditions (pH 2.0) and intestinal conditions (0.3% bile salts, 1% pancreatic enzyme, 0.5% NaCl) was then examined. Free and microencapsulated *Lactobacillus plantarum* showed a good resistance during simulated gastric environment, intestinal conditions and also to stress of certain compounds of beverage. Furthermore, the microencapsulation with alginate as a simple technique has proven its effectiveness by improving bacterial survival in simulated gastric and intestinal environment.

Key word: Gastrointestinal tract - probiotic - microencapsulation - *Lactobacillus plantarum.*

Résumé :

Des cellules microencapsulées de *Lactobacillus plantarum* G1 étaient préparées par la technique d'extrusion dans du gel d'alginate de sodium á 2%. L'effet de l'ingestion de trois boissons : le café noire, le thé vert, et le jus d'orange commercial sur la viabilité des cellules libres et microencapsulées de *Lactobacillus plantarum* dans des conditions similaire à celles gastriques (pH 2.0) et intestinales (0.3% sels biliaires, 1% enzyme pancréatique, 0.5% NaCl) a été examiné. Les cellules libres et microencapsulées de *Lactobacillus plantarum* montraient une bonne résistance durant l'incubation dans l'environnement similaire aux conditions gastriques et celles de l'intestin aussi bien qu'au stress de certains composants des boissons. De plus, la microencapsulation avec l'alginate comme une simple technique a prouvé son efficacité en améliorant la survie des bactéries dans les conditions du tube digestive simulées *in vitro*.

Mots clé : Tractus gastro-intestinal - probiotique - microencapsulation - *Lb. plantarum.*

الملخص:

تم كبسلة خلايا *Lb. plantarum G1* بتقنية extrusion وذلك بواسطة هلام ألجينات الصوديوم 2% بغرض دراسة