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Microencapsulation of *Sacharomycess boulardii*

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Abstract :

Microencapsulation is defined as a technology of including sensitive ingredients (solid, liquid or gaseous) within several matrices since the ingredients are entrapped or completely surrounded by the protective matrices, in this research both matrix and core shell microencapsulation techniques were used to encapsulate *S. boulardii* , by using sodium alginate as a first layer of shell and chitosan as a second layer.

Survivability rate for microencapsulated *S. boulardii* in simulated gastrointestinal (GI) conditions were detected by using different simulated gastrointestinal solutions : simulated saliva juice, simulated gastric juice and stimulated pancreatic juice.

Also microencapsulated cells were tested under heat stress conditions , at different time temperature compilations: 60°C5min, 60°C10min,60°C15min, 70°C3min, 70°C8min, 70°C10min.

The tested microcapsules under heat stress showed *S. boulardii* cells are generally sensitive to heat, even after the microencapsulation technique with one layer and double layers. The use of sodium alginate as a first layer of shell and chitosan as a second layer does not provide sufficient protection for *S. boulardii* cells against thermal stress. In order to produce thermal resistance cells, other substances other than sodium alginate can be used. Microencapsulation provides protection for *S. boulardii* cells against gastrointestinal stress.

Content

List of Abbreviations

Broth trypton soya broth	TSB
Trypton soya agar	TSA
Malt extract broth	MEB
Malt extract Agar	MEA
Simulated saliva juice	SSJ
Simulated gastric juice	SGJ
Stimulated pancreatic juice	SPJ
Uncountable	Un
No growth	0
S. boulardii count in TSB	S.b
S. boulardii in alginate	S.b in AL
After microencapsulation	MC
Disaggregation of microcapsule	MC in SC

Microencapsulation of *Saccharomyces boulardii*

1.Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefits on the host (FAO/WHO2001). Most of the probiotic food products are categorized as functional foods and comprise between 60 and 70% of the whole functional food market (Tripathy&Giri, 2014).

One of probiotic species is *S. boulardii,* which generally reported as *Saccharomyces boulardii,* it is a variety of *Saccharomyces cerevisiae* and it should be reported as *Saccharomyces cerevisiae* var *.boulardii. S.boulardii* categorized as GRAS *S. boulardii* has a big impact on diarrhea treatment, able to prevent intestinal infections caused by the adherence or invasion of *C. difficile*, *Escherichia coli,* and *Candida albicans* to the epithelial layer of the gastrointestinal tract (Berg, et al 1193) (Czerucka et al ., 2000) (Czerucka, et al. 2002).

To consider strain as probiotic it must reach the intestine alive, and able to colonized there, from this point of view the use of microencapsulation for probiotic developed. Microencapsulation is defined as a technology of including sensitive ingredients (solid, liquid or gaseous) within several matrices since the ingredients are entrapped or completely surrounded by the protective matrices (Anal, A. K., & Singh, H. 2007).

1.1Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefits on the host (FAO/WHO2001). The first interest of relation between bacteria and health benefits was started by Abraham, when he suggests the relation between consumption of sour milk and longevity.

The interest comes back to the modern world in 1886, by Theodor Escherich, who linked between physiological digestion in infants with bacteria which lives normally in the intestine, another interest was in 1892 when Ludwig Doderlein assumed that the consumption of lactobacillus can reduce the incidence of vaginal infections.

Eli Metchnikoff who consider now as the father of probiotic word, reported in his book in 1907 that bacteria in sour milk which consumed by people who lived in Bulgaria ,has a good effect in increasing ages even with hard living conditions, he supposed that by modifying normal flora through using good bacteria in sour milk can prevent aging and promote health, to support his claim he drank sour milk every day and he dead at age 71 .

In 1917, in the first world war salmonellosis and shigellosis was widely spread between soldiers, while only few of soldiers didn't developed enterocolitis. Non– pathogenic *Escherichia coli* was isolated by Alfred Nissle from a soldier who didn't develop enterocolitis, later the strain named *E. coli Nissle,* in few years flowed the strain used as a salmonellosis and shigellosis treatment.

Another beneficial bacteria was isolated in 1930 by Minoru Shirota the bacteria called *Lactobacillus casei* strain *shirota*, which is able to survive in gastrointestinal tract and able to colonized in the host intestine, it used to change gut microbiota profile.

In 1964, Probiotic term was used for the first time by Lilly and Stillwell the term used to identify substances secreted by one organism which stimulate the growth of another (Lilly and Stillwell 1965).

In 1989 Roy Fuller modify the definition of probiotic, by removing the word substance, which might include anti biotic, he defined probiotic as: "alive microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance," emphasized that probiotics must be viable organisms. (Fuller 1989). The most recent and used definition was proposed by WHO, their definition of probiotic is: "live microorganisms that when administered in adequate amounts confer a health benefit on the host".

To establish microorganisms as probiotics, it should be classified as generally recognized as safe (GRAS) and should be consumed in sufficient amount (106-107 CFU/g) (Lapsiri. et al. (2012). and be able to reach alive and colonized in the intestine. Probiotics must be identified by their genus, species, and strain level *(*Barry. Et al. 2015). Probiotics mostly bacteria includes *Bifidobacteria* eg *B. bifidum, B. lactis, B.longum, B. breve, B. infantis, B. thermophilum, and B. pseudolongum*, also lactic acid bacteria (LAB) like *L.acidophilus, L. acidophilus DDS-1, L. bulgaricus, L. rhamnosus GG, L. plantarum, L. reuteri, L. salivarius, L. casei, L .johnsonii, and L.gasseri. B (*Barry et al., 2015). but also, yeast can be classified as probiotic such as *sacharomyss* in particularly *S. boulardii.*

The interest of using probiotics derives from its benefits on health such as reducing diarrhea, enhance gut microbiota growth, reducing cholesterol serum, immune system stimulation, protect colon and gallbladder from cancer and antibiotics properties. Another important properties of probiotic that it should survive through gastro intestinal tract, and

be able to resist digestive enzymes, bile salts and low pH, so it can colonize in the host intestine.

Scientists suggests that probiotic act in different mechanisms in host, like: Protein inhibitory secretion, modification of the intestinal mucosa, direct adherence to the epithelial layer causing competition for colonization sites (Czerucka, D., and P. Rampal. 2002). (Fooks, L. J., and G. R. Gibson. 2002). Also, inhibition of toxins produced by pathogens and stimulation the secretion of immunoglobulin A.

From the importance of probiotic, the idea of using probiotic microorganisms to functionalize foods become very popular, the term functional food was first used in Japan.

food fictionalization is important tool to enhance, and maintain human health rather than medication use, where food can latter, or even prevent diseases.

Most of the probiotic food products are categorized as functional foods and comprise between 60 and 70% of the whole functional food market (Tripathy & Giri, 2014). This means that probioticts can found in both food that normally contains probiotic, or probiotic added during processing.

Traditionally fermented milk is widely used as probiotic food in the industry, dairy products including fermented, non-fermented milk and cheese consider the most probiotic products worldwide spreading, food technologist expand the use of probiotics in new different products, the idea of expansion to have more products that match with more people individualization, for example: vegans, people with lactose intolerance, peoples who dislike milk. This leads to increase the production of non-diary probiotic product, like fruit juices and chocolate-based product, fermented sausage, fruit and vegetable-based probiotic food, chocolate coated breakfast cereal, rice-based fermented beverage and kefir candies (Chen et al.2011).

One example of probiotic found in non-diary product is fruit juices, the main propose of functionalizing this product is to confirm that its closely related to the increasing demand of product with low cholesterol content and free from animal derivatives and milk allergens (Cespedes et al. 2013). fruit and vegetable beverages are consumed by a large percentage of the global population, the high content of vitamins, mineral salts, dietary fibers and anti-oxidants together with the absence of competing starter cultures render these products ideal matrices for the addition of probiotics (Antunes et al.,2013)

Another factor supports the addition of probiotic in fruit juices, that fruits have a wide

variety, which can used as probiotic functional drinks, to satisfy largest number of population .

Another example using probiotic is fermented meat products, probiotic usually used as starter culture, like using *lactobacillus* strain in fermented sausages, resulting in a final product with no draw backs on sensorial and technological properties as well as an increased functional value (Gao, Li, &Liu,2014).

Recently, research has supports the concept that not all probiotics are equally effective, but a consensus has not been uniformly reached as to which probiotic product should be used for specific disease conditions (Cruchet et al., .2015) (Szajewska et al., 2016).

2.1 *S. boulardii* as probiotic

One of probiotic species is *S.boulardii*, which generally reported as *Saccharomyces boulardii* is a variety of *Saccharomyces cerevisiae* and it should be reported as *Saccharomyces cerevisiae* var *.boulardii.* It is non-pathogenic vegetative cells of tested yeasts are cylindrical, of dimensions 2–3μm x 5–8μm. (McFarland 2010). It first isolated from Lychee fruit in Indocina in 1923 by French scientist Henry Boulard and marketed as freeze-dried form by Laboratoires Biocodex (Montrouge, France) in 1962 as probiotic.

Studies show that *S. boulardii* has a big impact on diarrhea treatment, Many in vitro and some in vivo studies suggested that *S. boulardii* is able to prevent intestinal infections caused by the adherence or invasion of *C. difficile*, *Escherichia coli*, and *Candida albicans* to the epithelial layer of the gastrointestinal tract (Berg, et al 1193) (Czerucka et al., 2000) (Czerucka et al., 2002).

S. boulardii also affect the immune system, through stimulation of immunoglobulin such as IgA and serum Ig G (Rodrigues et al. 2000; Qamar et al. 2001) as well as serum Ig M (Stier and Bischoff, 2016)

Many mechanisms of action have been suggested against the host as well as pathogenic microorganisms and include regulation of intestinal microbial homeostasis, interference with the ability of pathogens to colonize and infect the mucosa, modulation of local and systemic immune responses, stabilization of the gastrointestinal barrier function and induction of enzymatic activity favoring absorption and nutrition (Czerucka *et* al., 2007; Im and Pothoulakis, 2010; Pothoulakis, 2009).

In 2011 a study was applied to check the effect of using *S. boulardii* in treating acute diarrhea in infants, they demonstrated that oral treatment with *S. boulardii* diminished in approximately 50% of the patients with diarrhea since the second day after the beginning of the intervention when compared with a placebo group (Naflesia et al.2011). The timing of the first administration of the probiotic appears to be critical, because the earlier administration of *S. boulardii,* is the greater efficacy. (Vilarruel et al., 2007) showed that children given *S. boulardii* within 48 hours of the onset of diarrhea had significantly fewer number of stools than those who were administrated the product when the duration of diarrhea was more than 48 hours.

During last few years, researches suggests that probiotics can use as a treatment for irritable bowel syndrome (IBS), in 2011 double blind of *S. bouladii* versus placebo in the treatment of IBS patients, study was done it's conclude that the probiotic agent significantly improved the quality of life, but did not improve intestinal symptoms (Choi *et al*. 2011).

Another study investigates the effect of using *S. boulardii* on reducing Ulcerative colitis small pilot study of 25 adults with mild to moderate ulcerative colitis that were treated with a combination of mesalazine and *S. boulardii* for 4 weeks, most (68%) of the patients responded to the probiotic treatment (Guslandi *et al*. 2003). This pilot study had a promising result, but the implications were uncertain as patients were treated for only a short time, were not followed up for subsequent disease flare ups, and no control group was included.

Generally, there is no complication of using *S. boulardii* as probiotic, but there is only one consideration, that it can be opportunistic pathogen, and can cause fungemia. therefore, the use of it shouldn't be in immunocompromised patients.

3.1 Microencapsulation technique to increase probiotic viability

As mentioned before to consider strain as probiotic it must reach the intestine alive, and able to colonized there, from this point of view the use of microencapsulation for probiotic developed. Microencapsulation increase probiotic viability, protect it during gastro-intestinal passage, low pH, also protect it from food-related stressful agents and hurdles such as: preservatives, natural antimicrobial agents, oxygen, low water activity and other competitive bacteria.

Microencapsulation is defined as a technology of including sensitive ingredients (solid, liquid or gaseous) within several matrices since the ingredients are entrapped or completely surrounded by the protective matrices (Anal, A. K., & Singh, H. 2007).

Bioactive compounds usually called core, active agents or internal phase, while the substance used in encapsulation called external phase, coating membrane, shell or wall material, the shell material should be chosen carefully to be appropriate of microencapsulation technique and suitable for the core, the importance of the shell comes from that it will provide protection against surrounding conditions.

Shell materials have certain specification such as being categorized as GRAS, low coast and provide protection, generally there is specific substances used for this purpose including starches, inulin, pectin and most carbohydrates alginate, (De Vos et al., 2010). Proteins (Gluten, casein, whey protein, albumin) and the use of lipids such as (waxes, paraffin, diglycerides, monoglycerides, fats, stearic acid, and oils).

The general objective of encapsulation is to protect bioactive substances such as probiotics, vitamins, essential oil, anti oxidants and so on, from certain environmental condition, then release it in the appropriate position, to ensure food functionalization, the most common use of encapsulation in the industry includes: shelf life extending, flavors masking, reactions control.

The specific objective of probiotic microencapsulation, to protect it during food processing, hurdle technology, storage conditions and during food consumption such as low pH, gastric juices and bile salts.

The basics of encapsulation technology to package bioactive substances in different sizes, mili, micro or nano scale, by creating shell and make it more resistance to environmental condition, then release it in specific human body organ.

In the case of probiotic microencapsulation, coating material designed to protect probiotic from acidic conditions which found during gastrointestinal tract, this mechanism ensure achievement to colon and enhance probiotic viability and colonization.

It’s important to have inconsideration the place of realizing of bioactive compounds, in the case of probiotic encapsulation probitic should released after gastrointestinal passage, specifically in colon, the release mechanism from microcapsule depends on shell material chemistry, in general there are three common mechanisms used in releasing, which are: Thermal, physical and the dissolution methods.

Thermal mechanism based on melting temperature of shell material, which easily dissolved when it reaches specific temperature, for example during cooking. While physical mechanism based on microcapsules break down caused by physical or mechanical factor, like chewing. Otherwise dissolution method used when the products contains even a small amount of water, which can be used to assure the release of an ingredient trapped in a water-soluble coating membrane (Lakkis, 2007).

There are many methods used for probiotic microencapsulation, each strain of probiotic has its own proprieties and specifications, so it's important to choose the most suitable method, which helps to have higher quality and functionality in product where probiotic is used .

The most popular methods used for probiotics are: Spray drying Its application in *Lactobacillus* spp. (Desmond et al., 2002) and *Bifidobacterium* (O'Riordan et al., 2001), this method is common in the industry, in 2006 microencapsulation experiment applied on *B. infantis* by using spray drying method, microcapsule diameter was very small (15 to 20 μm) the result shown Microencapsulation significantly protected the bacteria at room temperature and in a simulated stomach and small intestine conditions, when compared with free cells (Crittenden et al., 2006).

Another experiment applied on *S. boulardii* by using spray drying method the result showed that the spray drying process. *S. boulardii* microcapsules produced with either gum Arabic or gelatin by spray drying at 125C can replace free cells in food formulation or a curing agent (Arslan et al 2015).

The second method is Lyophilization Encapsulated, probiotics by lyophilization have better storage stability, especially at low temperatures and inert atmosphere (nitrogen or vacuum) (Zuidam and Nedovic, 2010). The main disadvantage of this method that its conceder an expensive method, in 2013 an expermint applied on *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Enterococcus durans*, *Enterococcus faecalis,* to check its viability during storage after *Lyophilization.* Encapsulation results shown that an increase on LAB survival during storage (Carvalho et al., 2003).

Extrusion is the oldest and most common technique to produce capsules with hydrocolloids (e.g., alginate and carrageenan, the main advantages of this method that its effective and increase cells viability, while the disadvantage of it that it produces very low microcapsule.

In one of experiments to check the survival of probiotic microorganisms *L. acidophilus* 547, *B. bifidum* ATCC, and *L. casei* were encapsulated with Sodium alginate (20 $g.L^{-1}$)

and chitosan (4 $g.L^{-1}$) microcapsules added to pasteurized milk, and storage for 4 weeks at 4°C. The results showed that the survival of the encapsulated probiotic bacteria was greater vs. free cells in approximately 1 log cycle. During storage, the number of probiotic bacteria, with the exception of *Bbifidum*. The *B. bifidum* count fell below 107 $CFU.g^{-1}$ after two weeks of storage. The UHT treatment in yogurt did not alter the probiotic bacteria viability when compared with conventional thermal treatment (Krasaekoopt et al., 2006).

Spray cooling or freezing (spray freeze-drying) this method is a combination between spray draying and freeze-drying, the size of microcapsules which produced by this method is more controlled, while the disadvantage its high coast.

Scope of the thesis

To investigate the ability of *S. boulardii* cells to be more resistance under heat stress in microencapsulated form.

2.Materials and methods

1.2 Isolation of *Saccharomyces cerevisiae* var. *boulardii* and standard growth

The strain which used for the analysis was *Saccharomyces cerevisiae* var. *boulardii,* under the name (CODEX 5 BILION *Saccharomyces boulardii* fig 1*)* in the form of capsule for sale in pharmacies, which was cultivated, subsequently in (TSB) broth trypton soya broth

(OXOID Ltd., Basingstoke, Hampshire, England) at 28°C. After checking the purity of the strain, the sample was stored in trypton soya agar (TSA Agar) (Oxoid), for all the experiments described below.

Figure 1: *S.boulardii* used for experiment

One pill of probiotic opened and diluted in 9 ml of sterile ringer solution, then in a series of gradually less potent dilutions was obtained to 10^{-7}. an aliquot of 1 ml of appropriate decimal dilutions were spread on tryptone soya extract agar. All plates incubated at 28°C for 48 hr. The cells were observed by microscope, then 1 colony from plate which has dilution factor -7 streaked on TSA agar at 28°C for 2 days.

2.2 Optimization of *S. boulardii* growth conditions

The aim of this experiment is to determine the best media and temperature for the growth of *S. boulardii* using two different media: Trypton soy extract (TSB) and Malt extract (MEB) (oxidized) at incubation temperature at 28 °C and 37 °C as shown in Table 1

Table 1 Type of broth, agar and temperature used in incubation of *S. boulardii*, eg : TSB-TSA28 *S. boulardii* inculcated on TSB and then spread on TSA at 28°C and so on.

Broth	**Temp**	**Agar**	**Temp**	**Name**
TSB	28°C	TSA	28°C	TSB28–TSA28
TSB	28°C	MEA	28°C	TSB28–MEA28
MEB	28°C	TSA	28°C	MEB28–TSA28
MEB	28°C	MEA	28°C	MEB28–MEA28

TSB	37°C	TSA	37°C	TSB37–TSA37
TSB	37°C	MEA	37°C	TSB37–MEA37
MEB	37°C	TSA	37°C	MEB37–TSA37
MEB	37°C	MEA	37°C	MEB37–MEA37

3.2 Microencapsulation of *S. boulardii*

The microencapsulation of yeast cells was performed using the B-395 Pro encapsulator (fig 2).

Cells of defined volume (100ml) of *S. boulardii* culture were collected, in the initial stationary phase which was incubated at 28°C for overnight culture, the sample centrifuged at 6500 rpm for 15 minutes by (centrifuge ALC PK 130). The cell pellet was once washed in an equal volume of sterile Ringer solution, then ringer was removed, the sample was finally suspended in an equal volume of sodium alginate solution (2%) (Sigma, Milan, Italy, product A2033 61% mannuronic acid, 39% guluronic acid), which previously degassed and sterilized. used in the feeding system, was loaded with 100 ml of alginate cell suspension and placed on the encapsulator. The settings microencapsulation conditions used were:

- 6.7 ml / min flow rate
- 1000 Hz vibration frequency
-1800 electrode voltage

Alginate droplets containing yeast cells were cured in 200 ml of 0.5mol / L CaCl2 solution (4:1 ratio with suspension of alginate cells) and left under stirring for 20 minutes to obtain mono disperse cross-linked microcapsules.

The suspension was left for 30 minutes at room temperature, the settling of the microcapsules was observed, therefore the volume of the upper phase was gently sucked and subsequently discarded. To restore the initial concentration of cells, the alginate (MC) microcapsules were routinely stored at 4°C for further experiments.

In this experiment the same previous procedure applied, the difference is that *S. boulardii* incubated at 40°C for overnight, next day stressed at 50°C for 5 min, then cells capsulated.

Another microencapsulation method was used by using the core shell method. Sample preparation was done in the same way as the previous method, while 200 ml of alginate solution (2%) was placed in sterile bottle and 50 ml of mixed alginate with *S. boulardii* sample filled in sterile syringe.

The settings microencapsulation conditions used were:

-7.01 ml/ min flow rate.

-1300 Hz vibration frequency.

-2200 electrode voltage.

Another settings microencapsulation conditions used were:

-3.51 ml/ min flow rate.

-700 Hz vibration frequency.

-2500 electrode voltage.

Then the sample collected, microcapsules were routinely stored at 4°C for further experiments.

Figure 2: Microincapsulator B-395 Pro

4.2 Enumeration of microcapsules

one ml of *S. boulardii* microcapsule diluted in 9 ml ringer solution. then in a series of gradually less potent dilutions was obtained up to 105. an aliquot of 1 ml of appropriate decimal dilutions were spread on tryptone soya extract agar. All plates incubated at 28°C for 5days.

To verify the efficacy of the microencapsulation, a sample of $CaCl_2$ solution was taken immediately after the microencapsulation process, by taking 1 ml of $CaCl_2$, diluting it in 9 ml solution of the ringer solution, then in a series of gradually lower dilutions the solution was obtained up to 105, 1 ml of appropriate decimal dilutions on tryptone soya extract agar, all dishes incubated at 28°C for 5 days

To identify the ratio between microcapsule cells and free cells, 1 ml of microcapsules dissolved in 9 ml of sodium citrate (0.2M), from 5-10 min. then diluted in 9 ml ringer solution a series of gradually less potent dilutions was obtained to 10^5. an aliquot of 1 ml of appropriate decimal dilutions were spread on tryptone soya extract agar. All plates incubated at 28°C for 5 days.

5.2 Microencapsulated cells heat resistance

This experiment is divided into two parts. The first part is to verify the lethal temperature and time of *S. boulardii*, by applying heat stress to *S. boulardii* free cells, using different sets of time temperatures. The second part is to check whether the heat resistance of *S. boulardii* cells can be increased using the microencapsulation by using several procedures, which will be explained later.

In the first part of the experiment: 1 ml of *S. boulardii* incubated in 50 ml TSB for overnight culture, then the sample centrifuged at 6500 for 15 min and washed once with 50 ml of ringer solution, then divided in 7 falcons each falcon contains 3 ml of the sample, each falcon labeled and treated, as explained in table 2:

Table 2: list of time temperature combination used in heat stress.

Temperature	**Time**	**Name**
Room temperature	Zero	Zero min
60°C	5	60°C5min
60°C	10	60°C10min
60°C	15	60°C15min
70°C	3	70°C3min
70°C	8	70°C8min
70°C	10	70°C10min

After heat stress each falcon diluted in 9 ml ringer solution until -6, then all dilutions spread on TSA agar and incubated at 28°C for 5 days

The second part of the experiment when heat stress applied on cells in microcapsule by using different procedures. In all procedure microcapsules samples divided into 4 falcons, 3 ml in each falcon, and labeled as table 3.

Table 3: Heat stress applied on cells in microcapsule form

Temperature	**time**	**Name**
Room temp	0min	0 min
Room temp	0 min	0min citrate
70°C	3 min	70°C3min
70°C	3 min	70°C3min citrate

0 min: Is the count of microcapsule without heat stress, 0min citrate: Is the count without heat stress and microcapsules diluted in sodium citrate, 70°C3min: after heat stress, 70°C 3min citrate: After heat stress and diluted in sodium citrate .

Samples "70°C 3min, 70°C 3min citrate", were incubated at 70°C for 3 min. While no heat stress applied on the other two samples, all 4 samples "0 min, 0min citrate, 70°C3min, 70°C3min citrate" were diluted to -5 and spread on TSA agar for 5 days at 28°C.

In this procedure, core shell microcapsules are treated with chitosan 1:10 (w / v) (sigma 445569) and are stirred at 4000 rpm for 15 minutes, then centrifuge at 6500 for 15 min, chitosan was removed and replaced with the original volume of the ringer solution, samples "70°C3min, 70°C3min citrate" incubated at 70°C for 3 minutes. While no heat stress was applied to the other samples. All samples were diluted to -5, all dilutions spread on TSA agar and incubated at 28°C for 5 days

In this procedure the previous procedure was followed the only difference is that UHT milk (full fat milk) used to wash chitosan instead of using ringer solution.

6.2. Conditions of gastrointestinal stress

The ability of the strains to survive under simulated gastrointestinal transit was tested according to (Vizoso et al. 2006) with some modifications, overnight cultures were recovered by centrifugation (6,500 rpm for 15 min) and washed once with ringer solution.

To create gastrointestinal conditions 3 solutions prepared:

1. 10 ml of simulated saliva juice (SSJ: 5 g/l NaCl, 2.2 g/l KCl, 0.22g/l $CaCl_2$ and 1.2 g/l $NaHCO_3$, 100 mg/l lysozyme, pH 6.9).
2. 9 ml of simulated gastric juice (SGJ: 5 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$ and 1.2 g/l $NaHCO_3$, 3g/l pepsin, pH 2.5) .
3. 8 ml of stimulated pancreatic juice (SPJ: 6.4 g/l $NaHCO_3$, 0.239 g/l KCl , 1.28g/l NaCl, 0.5% bile salts and 0.1% pancreatin , pH 7.0.

All solutions were sterilized by filtration (0.45 μm). To evaluate the survival of microcapsules at simulated gastrointestinal conditions, 15 ml of microcapsules were taken and divided into 5 falcons, 3 ml in each falcon, falcons labeled from A to E

To count the original microcapsules number falcon A counted without treatment, by diluted 1 ml of the sample in 9 ml ringer solution until dilution factor -5, all dilutions spread on TSA agar. The other falcons no B, C, D centrifuged at (6,500 rpm for 15 min), then ringer solution removed, 12 ml of SSJ solution added to all falcons, the ratio was 1:4, while falcon E SSJ solution added without removing ringer solution, all falcons incubated at 37°C in light stirring (80 rpm) for 5 minutes. After this treatment time, all falcons were centrifuged at 6500 rpm for 15 minutes except falcon E, and SSJ solution removed.

For falcon B, 3 ml of ringer solution added and 1 ml were taken and diluted in 9 ml ringer solution until -6 and all dilutions spread on TSA agar.

For the other remaining falcons 12 ml of SGJ solution added, the ratio was 1:4, then they incubated at 37°C in light stirring (80 rpm) for 2 hours. Then all falcons centrifuged, then the solution removed except E. For no C, 3 ml of ringer solution added, and 1 ml were taken and diluted in 9 ml ringer solution until dilution factor -6, all dilutions spread on TSA.

For the other falcons 15 ml of SPJ solution added, the ratio was 1:5, then falcons incubated at 37°C in light stirring (80 rpm) for 3 hours. All falcons centrifuged, and the solution removed and replaced with 3 ml ringer both falcons no D, E diluted until -6 and spread on TSA agar all plates incubated at 28°C for 5 days.

The same procedure was flowed for free cells of *S. boulardii*.

3.Results and discussion

1.3 Sacharomycess var *boulardii* in conditions of standard growth

After 48 hours of incubation at 28°C, the colonies were developed and observed under the microscope, showing the growth of *S. boulardii* colonies and rod-shape bacteria. *S. boulardii* was found in all plates, whereas rod shape bacteria found only in plates that had a high dilution factor from -1 to -5, while there was no contamination with rod shape bacteria in plates that had dilution factors -6 and -7, the same results have been observed, meaning that product contaminated with a rod-shaped bacteria and when exposed to a high dilution the contamination becomes less abundant. colony was picked from plate which has dilution factor -7 and streaked on TSA agar for 48 hr at 28°C, colonies looked white

and distinguishable (fig3), under microscope colonies appear spherical and clustered (fig4)

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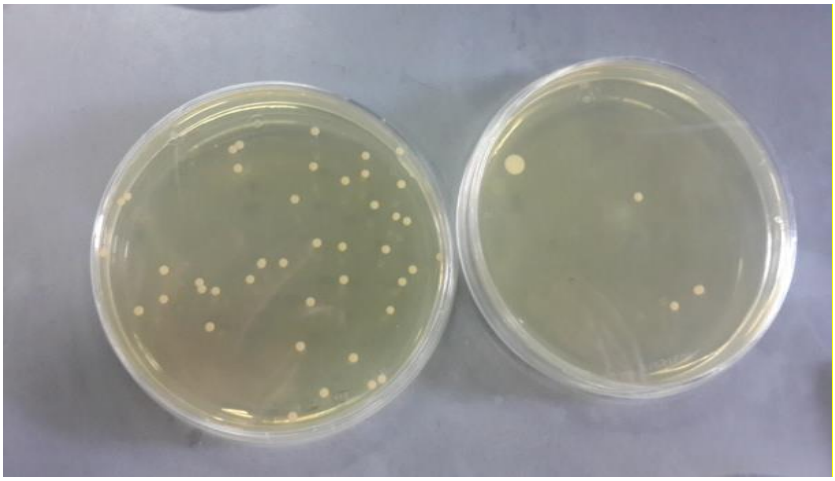


Figure 3: *S.boulardii* on TSA agar.

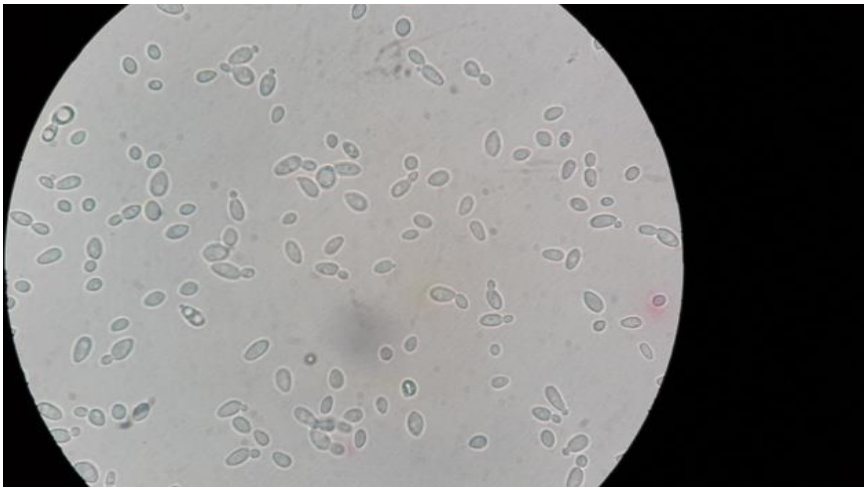


Fig 4. *S. boulardii* under microscope 40x

2.3 Optimal growth condition of *S. boulardii*

After 5 days of incubation at 28°C and 37°C the plates counted, results reported in table 4

Table 4: *S. boulardii* colonies count after 5 days of incubation at different media temperature combinations.

Name	**CUF/ml**	**CUF/ml**
TSB-TSA28	118*10^5	8*10^6
TSB-MEA28	70*10^5	8*10^6
MEB-TSA28	13*10^5	0*10^6

MEB-MEA28	10*10^5	1*10^6
TSB-TSA37	48*10^5	3*10^6
TSBMEA37	40*10^5	2*10^6
MEB-TSA37	35*10^5	2*10^6
MEB-MEA37	19*10^5	2*10^6

TSB: trypton soya broth, TSA: trypton soya agar, MEB: malt extract broth , MEA: malt extract agar .

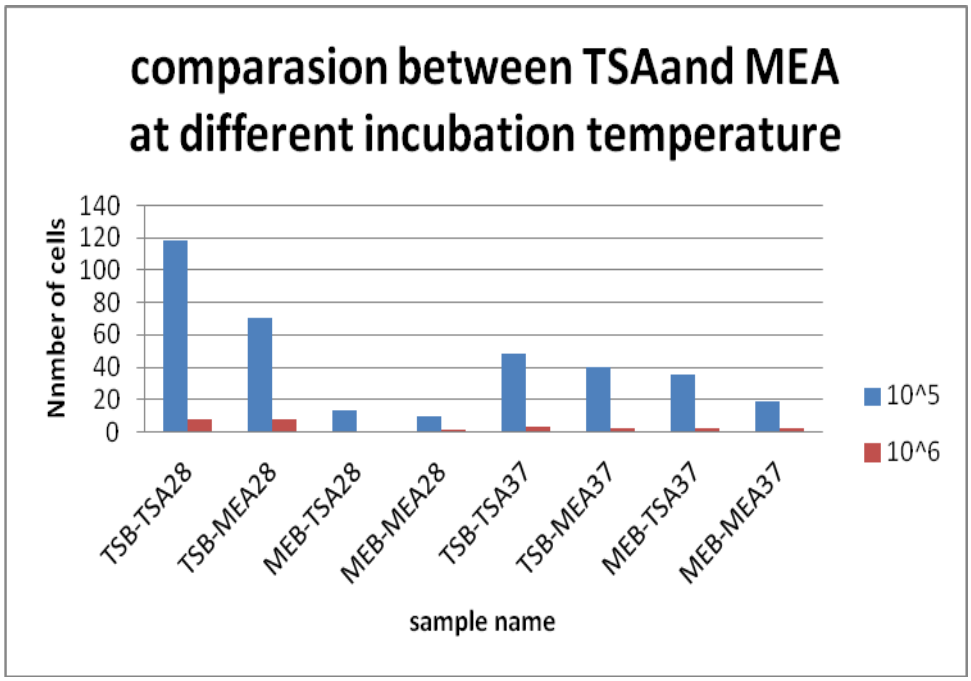
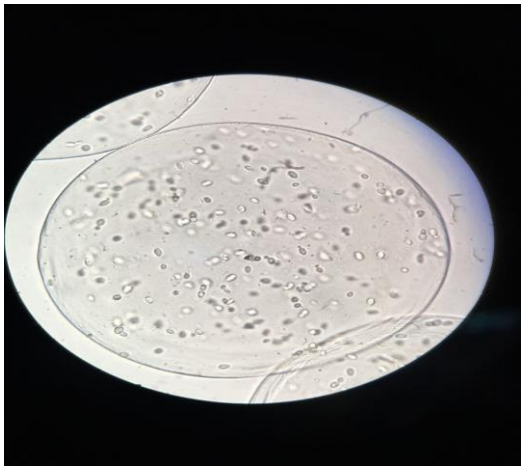


Fig5: Comparison of cells count between broth, media were used at different incubation temperatures.

The best growth condition for *S. boulardii* could be reported as best incubation broth is TSB and best incubation temperature is 28°C and best agar to spread on is TSA and best incubation temperature is 28°C.

3.3 Morphology of microcapsules

To evaluate the morphology formed of microcapsules, they were observed under an optical and an electron microscope, they show microcapsules with a medium diameter. Moreover, the size of microcapsules remained unchanged during their storage under refrigeration conditions at 4°C after removing the supernatant and replacing it with the same volume of Ringer (fig 6).



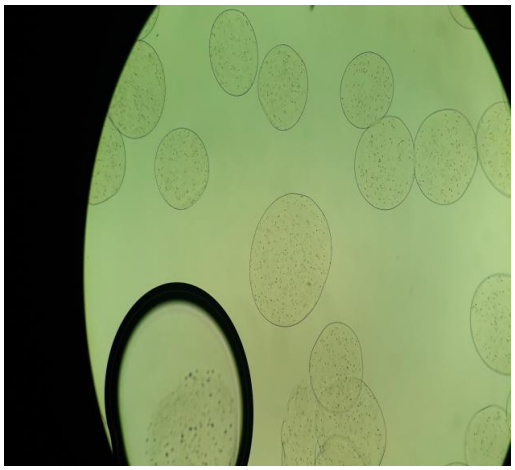
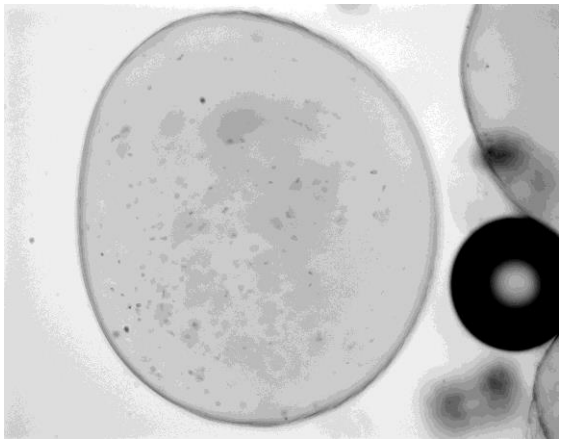


Fig 6.a.Microcapsule under microscope40x Fig6.b.Microcapsule under microscope 10x



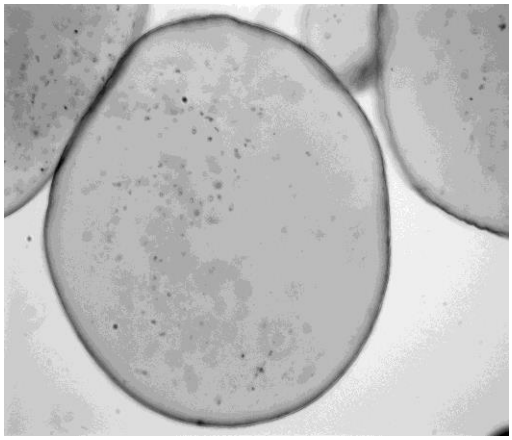


Fig 6c microcapsule in chitosan

4.3 Effectiveness of the microencapsulation and enumeration of microcapsules

For all microencapsulation were performed in this work were subjected for the evaluation of the efficiency of the microencapsulation process, the total *S. boulardii* load was calculated before and after the microencapsulation process. All of the lots showed excellent results in terms of encapsulation efficiency, this is confirmed by counting the cells in CaCl2.

For each microencapsulation process free cells were counted by spread plates method on TSA agar, in each step of microencapsulation process: When it is inculcated in TSB, in

alginate solution, after microencapsulation process and when microcapsules were disaggregated in sodium citrate.

To estimate the number of *S. boulardii* in each capsule and the effectiveness of microencapsulation process EE% was calculated for each process. The yeast counts were expressed in CFU/ml. the result of each microencapsulation were reported separately and labeled by "first microencapsulation", "second microencapsulation", "micro 30°C", "micro 40°C", "core shell microencapsulation."

-For the first experiment (first microencapsulation) the results were summarized in table 5.

Table 5: Count of cells before, during and after microencapsulation process for the first microencapsulation.

Name	**10^4 CFU/ml**	**10^5 CFU/ml**	**10^6 CFU/ml**
S. boulardii	un	84	10
S. boulardii in alginate	un	80	8
Microcapsules	67	6	0
Microcapsules in citrate	un	77	6

Un: uncountable, 0 no growth

Fig 7

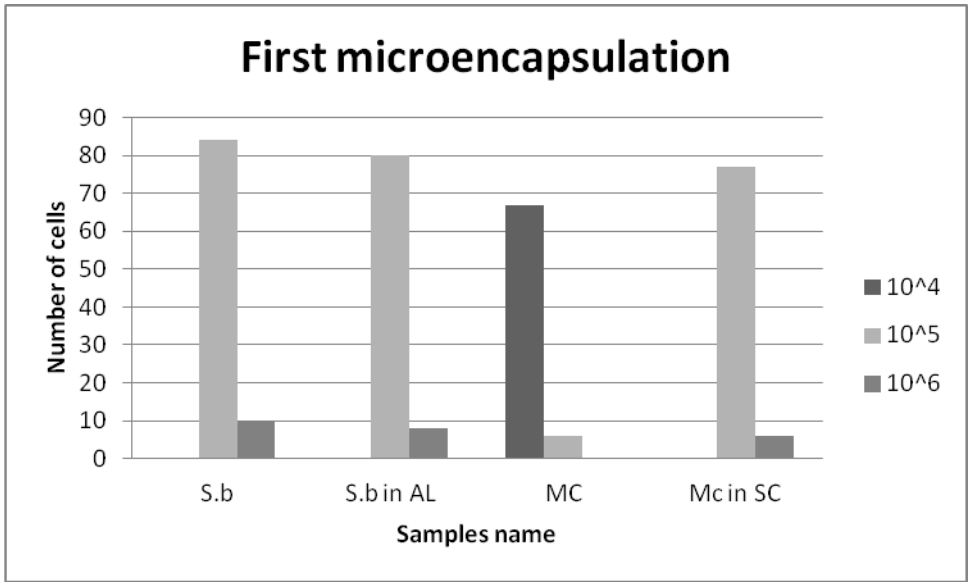


Fig 7: Count of cells in each step of microencapsulation, were *S. boulardii* count in TSB (S.b), *S. boulardii* in alginate(S.b in AL), after microencapsulation (MC), and disaggregation of microcapsule (MC in SC).

The results showed that approximately the same number of *S. boulardii* cells prior to the microencapsulation proces were almost equal to the number of cells after dissagregation of capsules in sodium citrate. The small decrease in cell counts after microencapsulation could be associated with loss of cells during sample washing, number of cells in CaCl2 were 160 *10^1, most of *S. boulardii* cells were coated sucsessfully, this method of microencapsulation was effective in converting free cells into microcapsules % EE was 96.28%.

-The same counting was applied on the second microencapsulation, the process labeled as the second microencapsulation.

The second microencapsulation results were reported in table 6.

Table 6: Count of cells before, during and after microencapsulation process for the second microencapsulation.

Name	**10^4 CFU/ml**	**10^5 CFU/ml**	**10^6 CFU/ml**
S. boulardii	Un	138	14
S. boulardii in alginate	un	135	12
Microcapsules	70	6	0
Microcapsules in citrate	un	127	10

Un: uncountable, 0 no growth

Fig 8

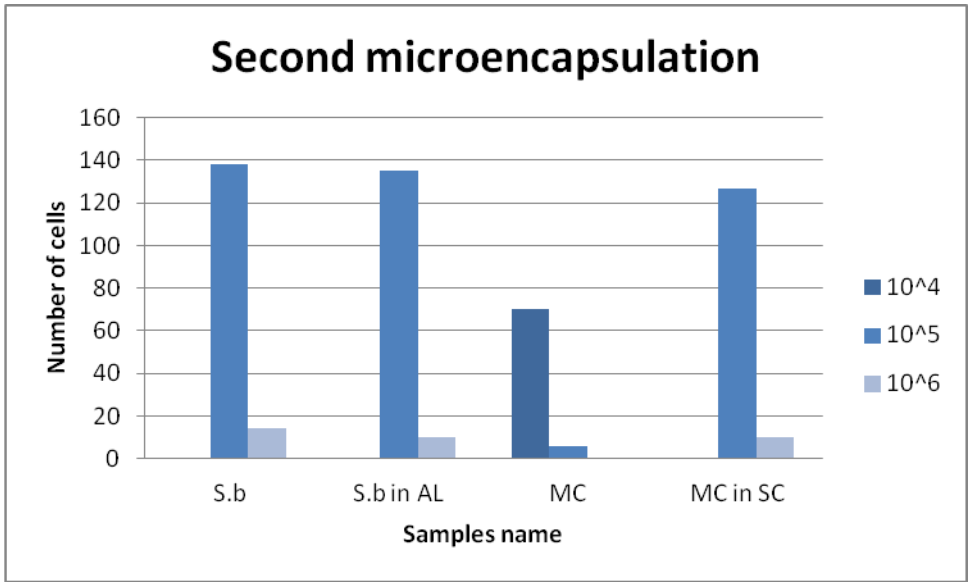


Fig 8 : Count of cells in each step of microencapsulation, were *S. boulardii* count in TSB (S.b), *S. boulardii* in alginate(S.b in AL), after microencapsulation (MC), and disaggregation of microcapsule (MC in SC).

The results showed that approximately the same number of *S. boulardii* cells prior to the microencapsulation were almost equal to the number of cells after dissagregation of capsules in sodium citrate. The small decrease in cell counts after microencapsulation could be associated with loss of cells during sample washing, number of cells in CaCl2 were 170 * 10^1, most of *S. boulardii* cells were coated successfully. This method of microencapsulation capsule was effective in converting free cells into microcapsules % EE was 94.55%.

-In this experiment *S. boulardii* incubated in TSB at 30°C for overnight culture, then the same procedure of microencapsulation was followed, the result of micro 30°C reported in table 7.

Name	**10^4 CFU/ml**	**10^5 CFU/ml**	**10^6 CFU/ml**
S. boulardii	un	80	7
S. boulardii in alginate	un	76	7
Microcapsules	47	7	0
Microcapsules in citrate	un	72	5

Un: uncountable, 0 no growth.

Fig 9

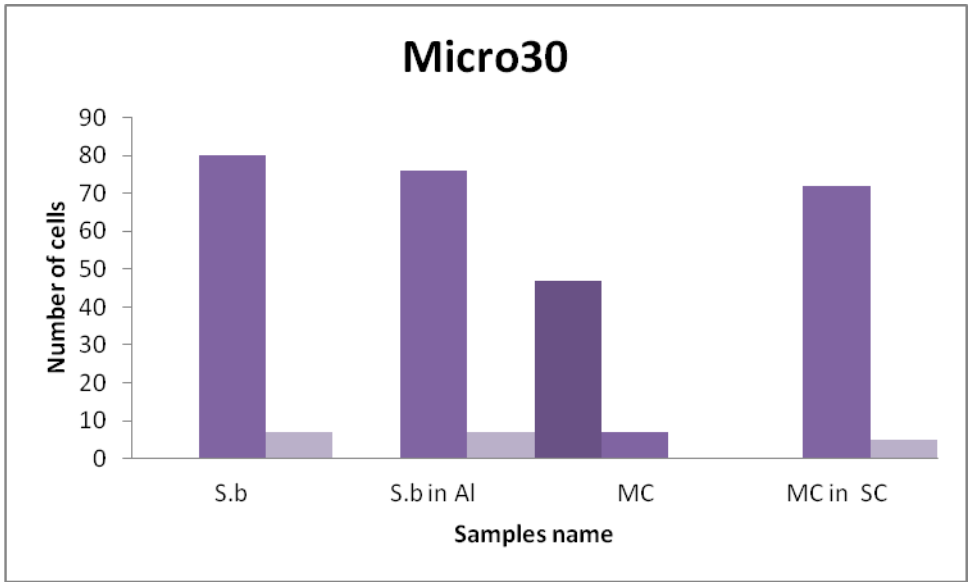


Fig 9: Count of cells in each step of microencapsulation, were *S. boulardii* count in TSB (S.b), *S. boulardii* in alginate (S.b in AL), after microencapsulation (MC), and disaggregation of microcapsule (MC in SC).

The results showed that approximately the same number of *S. boulardii* cells prior to the microencapsulation were almost equal to the number of cells after dissagregation of capsules in sodium citrate. The small decrease in cell counts after microencapsulation could be associated with loss of cells during sample washing, number of cells in CaCl2 were 166 * 10^1, most of *S. boulardii* cells were coated successfully. This method of microencapsulation capsule was effective in converting free cells into microcapsules % EE was 94.78%.

-In this procedure incubation conditions and treatment were different, *S. boulardii* counted in TSB (incubation temp 40°C), *S. boulardii* counted in alginate, and after heat stress for 5 min at 50°C, also microcapsules and after microcapsule disaggregation in sodium citrate this procedure labeled as . micro 40°C.

The results of micro 40°C reported in table 8.

Table 8**:** Count of cells before, during and after microencapsulation process for. micro 40°C.

Name	**10^4 CFU/ml**	**10^5 CFU/ml**	**10^6 CFU/ml**
S. boulardii	un	55	3
S. boulardii in alginate	un	46	2
S. boulardii heat stress	un	49	3
Microcapsules	22	2	0
Microcapsules in citrate	un	44	2

Un: uncountable, 0 no growth

Fig9:

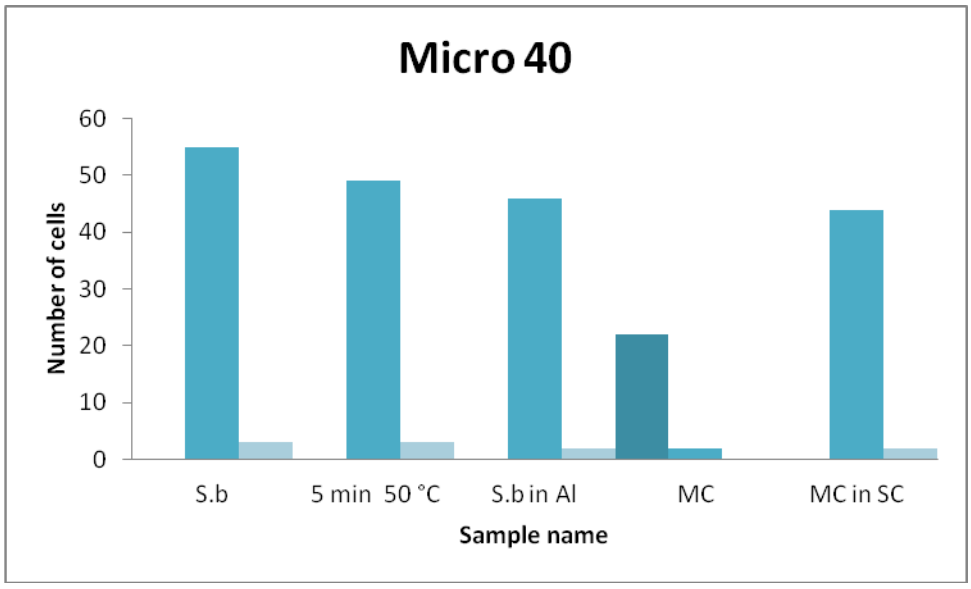


Fig 9: Count of cells in each step of microencapsulation, were *S. boulardii* count in TSB (S.b), *S. boulardii* in alginate(S.b in AL), after heat stress for 5 min at 50°C after microencapsulation (MC), and disaggregation of microcapsule (MC in SC).

The results showed that approximately the same number of *S. boulardii* cells prior to the microencapsulation were almost equal to the number of cells after dissagregation of capsules in sodium citrate. The small decrease in cell counts after microencapsulation could be associated with loss of cells during sample washing, number of cells in CaCl2 were 162 * 10^1, most of *S. boulardii* cells were coated successfully. This method of microencapsulation capsule was effective in converting free cells into microcapsules % EE was 94.28%.

-The last microencapsulation was performed by using core shell technology, its labeled as core shell, after core shell microencapsulation microcapsule coated with chitosan as a double layered microcapsules, the results of core shell microencapsulation were reported in table 9

Table 9: Count of Core shell microencapsulation : Count of cells before, during and after microencapsulation process.

Name	**10^3**	**10^4**	**10^5**	**10^6**
S. boulardii	Un	un	100	14
S. boulardii in alginate	Un	un	77	2
Microcapsules	Un	30	2	0
Microcapsule in chitosan	30	3	0	0
Microcapsules in citrate	un	un	76	7

Un: uncountable, 0 no growth

Fig:10

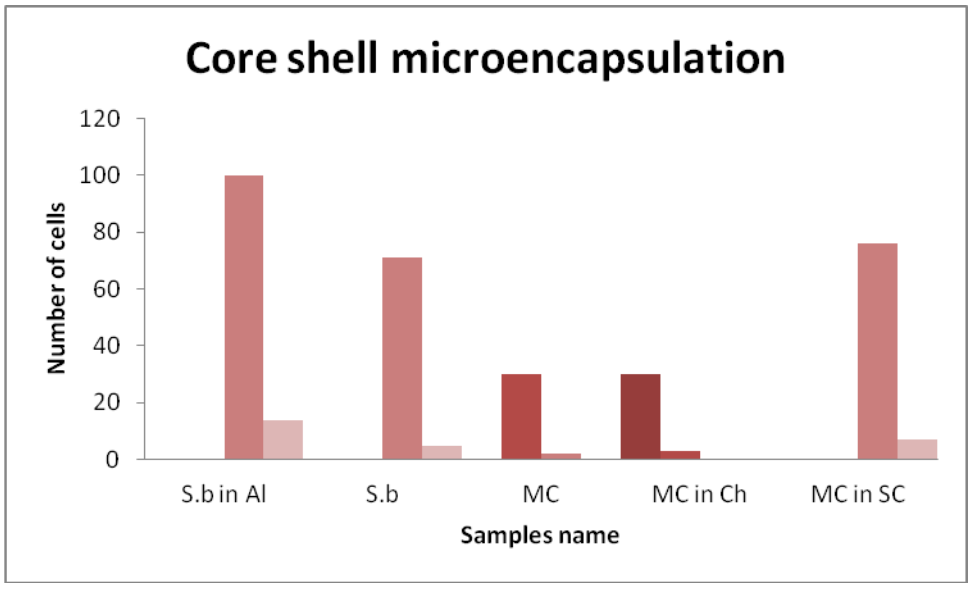


Fig 10 : Count of cells in each step of microencapsulation, were *S. boulardii* count in TSB (S.b), *S. boulardii* in alginate (S.b in AL), after microencapsulation (MC), and disaggregation of microcapsule (MC in SC)., and in chitosan (MC in SC).

The results showed that approximately the same number of *S. boulardii* cells prior to the microencapsulation were almost equal to the number of cells after dissagregation of

capsules in sodium citrate. The small decrease in cell counts after microencapsulation could be associated with loss of cells during sample washing, number of cells in CaCl2 were 120 * 10^1, most of *S. boulardii* cells were coated successfully. This method of microencapsulation capsule was effective in converting free cells into microcapsules % EE was 98.78%.

The resultes of all microcencapsulation were compared and sumarized in table 10.

Table 10: Count of all microencapsulation and EE% for each microencapsulation process .

Name	**10^3 CFU/ml**	**10^4 CFU/ml**	**10^5 CFU/ml**	**EE%**
First	un	67	6	96.28%
Second	Un	76	6	94.55%
Micro3°0C	Un	47	7	94.78%
Micro 40°C	Un	22	2	94.28%
Core shell	Un	30	2	98.78%

Fig 11

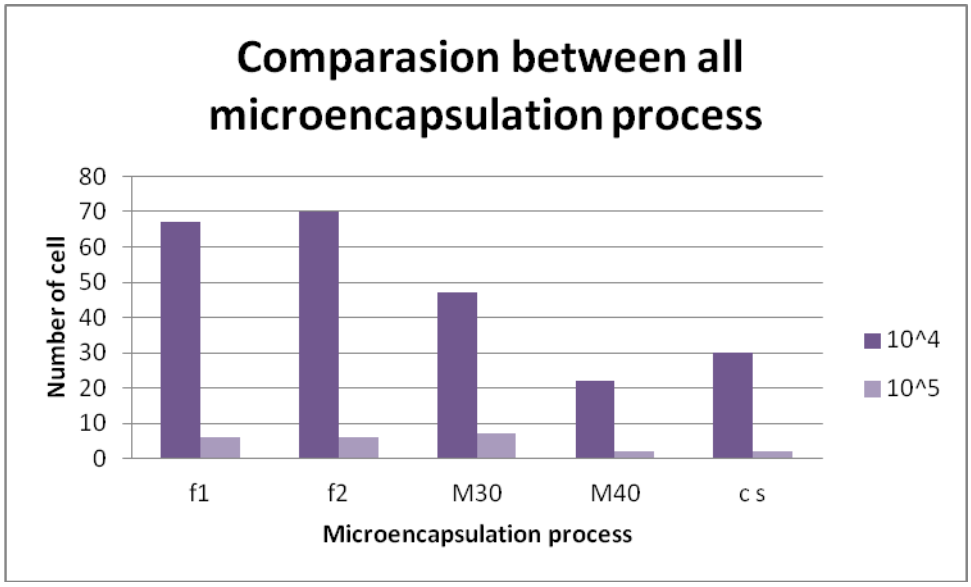


Fig 11: Comparison between all microencapsulation process in number of microcapsule production

Fig 12

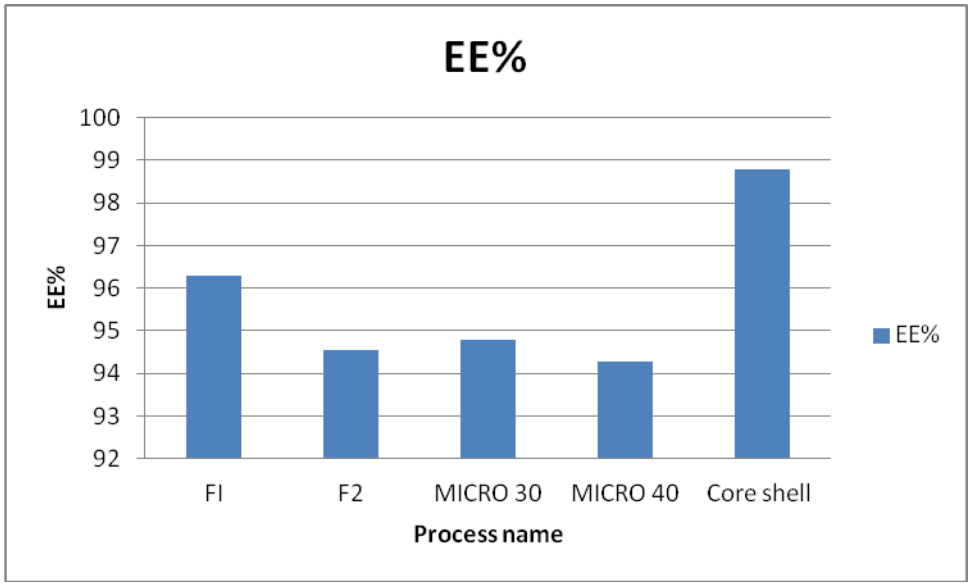


Fig 12: Compasion in EE%for all microencapsulation process

The variation in results were related to the difference in the initial microorganism content before microencapsulation process, while core shell technology has the highest EE% .

5.3 Heat stress on *S. boulardii* in form of free cells and microencapsulated cells form

The first part of result was *S. boulardii* heat stress before microencapsulation.

S. boulardii was stressed at different time temperature combinations and then spread on TSA at 28°C for 5 days, the result summarized in the table in table 11.

Table 11: Count of *S. boulardii*. cells after different time temperature heat stress.

Name	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
Zero min	UN	UN	UN	UN	69	4
60°C5min	27	0	0	0	0	0
60°C10min	0	0	0	0	0	0
60°C15min	0	0	0	0	0	0
70°C3min	0	0	0	0	0	0
70°C8min	0	0	0	0	0	0
70°C10min	0	0	0	0	0	0

UN: uncountable; 0: no growth.

Results showed *S. boulardii* is heat sensitive, at different time temperature combinations, the only possible growth when cells stressed at 60°C for 5 min, after this time temperature combination, every stress consider as lethal for *S. boulardii* free cells.

The second part summarizing the results of heat stress (3 min at 70°C on cells in microencapsulated form. Heat stress applied on microcapsules with one layer (first second, micro 30, micro 40), and double layer(core shell + chitosan, core shell+ chitosan in milk).

The results of microcapsules by using one layer produced by first microencapsulation, after heat stress where reported in table 12.

Table 12: Count of cells in form of one layer microcapsules produced by first microencapsulation after 3 min heat stress at 70°C.

Name	**10^4CFU/ml**	**10^5CFU/ml**	**10^6CFU/ml**
0 min	67	6	0
0min citrate	Un	77	6
3min 70°C	0	0	0
3min70°C citrate	0	0	0

Table 13: Count of cells in form of one layer microcapsules produced by the second microencapsulation after 3 min heat stress at 70°C.

Name	**10^4CFU/ml**	**10^5CFU/ml**	**10^5CFU/ml**
0 min	70	6	0
0min citrate	Un	127	10
3min 70°C	0	0	0
3min70°C citrate	0	0	0

Table 14: Count of cells in form of one layer microcapsules produced by micro 30°C after 3 min heat stress at 70°C.

Name	**10^4CFU/ml**	**10^5CFU/ml**	**10^5CFU/ml**
0 min	47	7	0
0min citrate	0	72	5
3min 70°C	0	0	0
3min70°C citrate	0	0	0

Table 15: Count of cells in form of one layer microcapsules produced by micro 40°C after 3 min heat stress at 70°C.

Name	**10^4CFU/ml**	**10^5CFU/ml**	**10^5CFU/ml**
0 min	22	2	0
0min citrate	Un	44	2
3min 70°C	0	0	0
3min70°C citrate	0	0	0

Count of double layers of micro capsules by shell core microencapsulation technology, by using chitosan as second layer.

Table 16: Count of cells in form of double layers microcapsules produced by core shell microcapsules after 3 min heat stress at 70°C.

Name	**10^3CFU/ml**	**10^4CFU/ml**	**10^5CFU/ml**	**10^5CFU/ml**
0 min	un	30	3	0
0min citrate	un	un	76	7
0 min in chitosan	30	3	0	0
3min 70°C	0	0	0	0
3min70°C citrate	0	0	0	0

Table 17: Count of cells in form of double layers microcapsules produced by core shell microcapsules preserved in UHT milk after 3 min heat stress at 70°C.

Name	**10^3CFU/ml**	**10^4CFU/ml**	**10^5CFU/ml**	**10^5CFU/ml**
0 min in chitosan	46	4	0	0
3min 70°C	0	0	0	0
3min70°C citrate	0	0	0	0

In all experiments, where single layer was used (matrix microencapsulation), the results showed no cells growth after heat stress, even in sample "micro 40°C", where the incubation time was 40°C for overnight culture. The sample stressed by heat for 5 min at 50°C to increase heat adaptation and improve heat resistance before microencapsulation process .

Also in experiments where double layers were used to increase protection of cells against heat stress, by using chitosan. Plates show no growth at all after heat stress, as there has been no change in cell resistance to heat stress.

S. boulardii heat resistance in double layer also tested in UHT whole milk, milk normally contains fats and proteins which able to increase cells protection against heat stress for probiotic cells. while the result in milk still negative and no growth at all in all plates after 5 days of incubation at 28°C. probiotic yeast had a lower heat resistance than probiotic bacteria. (Arslan ,.et al 2017).

6.3 Survivability rate in simulated gastrointestinal (GI) conditions

The results of viable counts and survival rates of *S. bouardii* cells after exposure to simulated GI conditions are show in fig12. *S. boulardii* is resistance for gastrointestinal passage. The only significant difference that was observed concerned the number of cells present in sample D when pancreatin and bile salt added and incubated for 3 hr at 37°C, that's means *S. bouladii* cells is generally resistance for gastrointestinal passage before microencapsulation.

Fig 12:

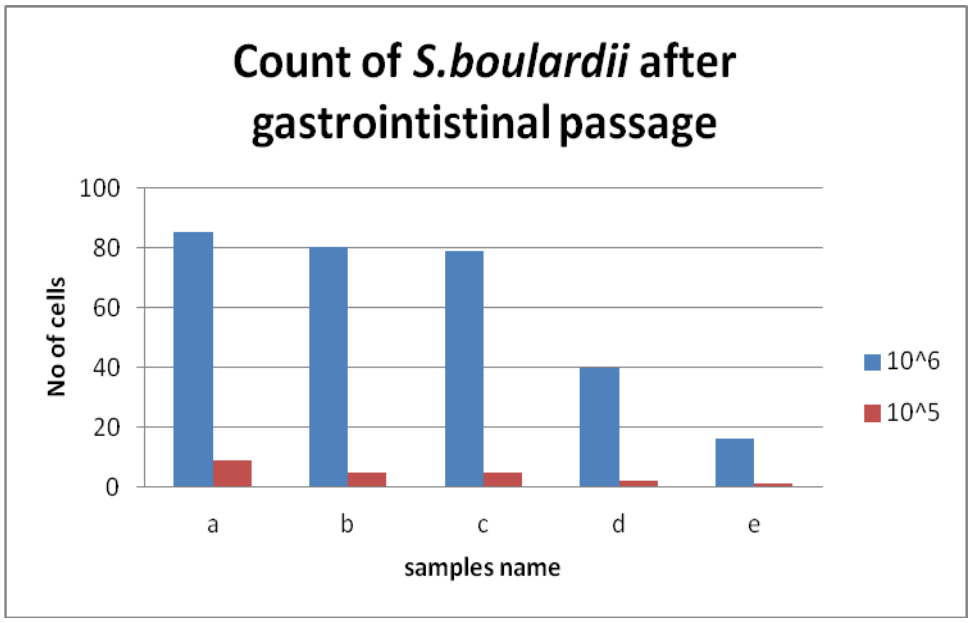


Fig 12: Count of *S. boulardii* free cells after gastrointestinal stress, a: Count of cells before stress, b: Count of cells after ssj solution for 5 min, c: Count of cells after adding ssj +sgj for 2 hr, d : of cells Count after ssj+sgj +spj, e: Count after ssj+sgj +spj. SSJ: Simulated saliva juice. SGJ: Simulated gastric juice. SPJ: Stimulated pancreatic juice.

The results of viable counts and survival rates of *S. bouardii* cells in microcapsule form after exposure to simulated GI conditions are show in fig13. According to the results, the highest rate of cell release from capsule was 120 minutes after its incubated in Simulated gastric juice .

Fig 13

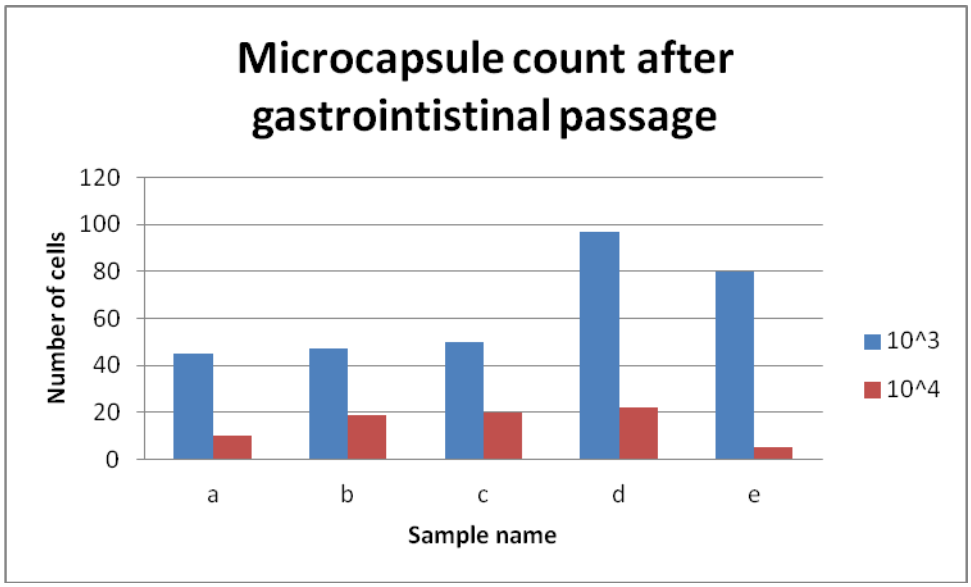


Fig 13: Count of cells *S. boulardii* in microencapsulated form after gastrointestinal stress, a: Count of cells before stress, b: Count of cells after ssj solution for 5 min, c: Count of cells after adding ssj +sgj for 2 hr, d : of cells Count after ssj+sgj +spj, e: Count after ssj+sgj +spj. SSJ: Simulated saliva juice. SGJ: Simulated gastric juice. SPJ: Stimulated pancreatic juice.

The microencapsulation technique had effect on the counts of *S. boulardii*. The highest count and survivability were determined in microcapsules and microencapsulation provided survivability at the end of 120 min. Microencapsulation provides the protection against bile salts and pancreatin.

Free yeast cells also had good survivability in simulated gastric conditions. It is known that *S. boulardii* is resistant to gastric acidity (Hebrard et al., 2010). It was reported that *S. boulardii* encapsulated with alginate bead slightly decreased after 180 min simulated gastric digestion test (Gallo, Bevilacqua, Speranza, Sinigaglia, & Corbo, 2014).

4.Conclusion

This research was focused on the microencapsulation of *S. boulardii,* by matrix as single layer and core shell techniques as double layers. It can be concluded from the results that the *S. boulardii* had high protection in simulated intestinal system. *S. bouladii* cells is generally resistance for gastrointestinal passage before microencapsulation.

S. boulardii cells are generally sensitive to heat, even after the microencapsulation technique with one layer and double layers. The use of sodium alginate as a first layer of shell and chitosan as a second layer does not provide sufficient protection for *S. boulardii* cells against thermal stress. In order to produce thermal resistance cells, other substances other than sodium alginate can be used.

S. boulardii cells was resistant in gastric and intestinal system while it was heat sensitive. This study suggests that there is a need to increase the viability of probiotics during production, digestion and storage time by combined microencapsulation techniques.

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ملخص : استخدام الكبسلة المصغرة لميكروب S. boulardii

تُعرَّف الكبسلة المصغرة بأنها تقنية تتضمن مكونات حساسة (صلبة أو سائلة أو غازية) ضمن عدة مصفوفات حيث أن المكونات موصلة أو محاط بالكامل بالمصفوفات الواقية ، في هذا البحث تم استخدام كل من تقنيتي المصفوفة الأساسية والأساسات الدقيقة لتغليف S. boulardii باستخدام ألجينات الصوديوم كطبقة أولى والشيتوزان كطبقة ثانية.
تم الكشف عن معدل البقاء على قيد الحياة لخلايا S. boulardii الدقيقة في الحالات محاكاة الجهاز الهضمي (GI) باستخدام محاليل الجهاز الهضمي المختلفة: عصير اللعاب ، محاكاة عصير المعدة وعصير البنكرياس .
كما تم اختبار الخلايا المكبسلة تحت ظروف الإجهاد الحراري ، حيث تم استخدام درجات حرارة مختلفة لأوقات متعددة : 60 درجة مئوية لمدة 5 دقائق 60 درجة مئوية لمدة 10 دقيقة ، 60 درجة مئوية 15 دقيقة ، 70 درجة مئوية لمدة 3 دقائق 70 درجة مئوية ولمدة 10 دقائق.
أظهرت النتائج أن خلايا S. boulardii المختبرة تحت الضغط الحراري هي حساسة بشكل عام للحرارة ، حتى بعد تقنية الكبسولة الدقيقة مع طبقة واحدة وطبقات مزدوجة. إن استخدام ألجينات الصوديوم كطبقة أولى من الصدف والشيتوزان كطبقة ثانية لا يوفر حماية كافية لخلايا S. boulardii ضد الإجهاد الحراري. ومن أجل إنتاج خلايا المقاومة الحرارية ، يمكن استخدام مواد أخرى غير ألجينات الصوديوم. بينما توفر عملية الكبسلة الصغيرة حماية كافية لخلايا S. boulardii ضد محاليل الجهاز الهضمي .