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MASTER'S DEGREES IN

FOOD SCIENCE AND TECHNOLOGY

AND

NUTRITION AND FOOD TECHNOLOGY

MISSION IMPOSSIBLE: TO MAKE PROBIOTIC THE "KHUBZ"

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Dedication

This is thanks to my lord

(I dreamed but not expect, that I would arrive at this juncture of my life)

This dissertation is dedicated first and foremost to my God **"ALLAH"**, who is always with me

I also dedicate this book, to my mother, a smart and beautiful woman, who I miss her every day, and she was one of the first supporters **"Azeza"**

For my lovely husband, who is carrying my absence and after him **"Ismaael"**

For my lovely brother, who is the first supporter and the largest shareholder in all respects in my life **"Kamel"**

For my favorite teacher dear, who is for me one of my family, I am really happy and proud for being a student, my professor **"Gianluigi"**

For my love heart my little child, who has not forgotten me and always miss me, who still calls me Mama **"Mohammad"**

List of Content

List of Tables

List of Figures

MISSION IMPOSSIBLE: TO MAKE PROBIOTIC THE "KHUBZ"

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Abstract

In this study, we aimed to probiotication "khubz' and "Labneh" by preparation microcapsules of saccharomyces boulardii. We followed the procedure by Sheu and Marshall (1993) and modified by Truelstrup Hansen et al. (2002). It is water in oil emulsion technique for saccharomyces boulardii with average diameter of 15 µ that is a good diameter to reduce the gritty taste effect of bigger microcapsules produced by using other techniques like for example the extrusion, microcapsules contained about 50-60 yeast cells each one. and *S. boulardii* shows the same fermentation performance of saccharomyces *cerevisiae* and that microencapsulated saccharomyces *boulardii* cells show a slight reduction of fermentation performance compared to free ones, whereas in "khubz" probiotication the result show its more difficult to have this type of "khubz", instead of "labneh" probiotication with microcapsules the results show longest shelf life than "labneh" probiotocation with free cells of saccharomyces *boulardii*

1. INTRODUCTION

“Khubz” is the Arabic word to indicate “bread” but it also indicates shape and size of bread that is usually consumed in Arabic countries and, in particular, in Palestine. “Khubz” (i.e. bread) is produced as a circular flatbread, more or less 25 cm in diameter. Since it does not contain any added fat, it dries rapidly and is best eaten while still warm from the oven. It has a somewhat tough chewy texture. “Khubz” is consumed in Arabic countries during breakfast, lunch and dinner and it is consumed both to be coupled with a lot of different recipes and to be stuffed like a sandwich. It is usually prepared in Palestine by specialized small bakeries that produce and sell in the same place. In the Figure 1 is shown “khubz” and a typical producer of “khubz” in Bethlehem. According to this bakery shop in Bethlehem, daily is consumed 1,000 Kg of white flour to produce 700 single pieces of “khubz”. Ingredients are white flour, salt, water, sugar (to accelerate leavening and to browning) and yeast. Bread was previously used by some authors for its probiotication but just after oven (Altamirano-Fortoul, et al., 2012, Soukolis, et al., 2014). They used coating film containing microencapsulated probiotics. Probiotication of bakery is a very challenging because of high temperature (i.e. ranging between 180°C and 280°C) reached during baking. Other authors described the probiotication of bakery products, like for example biscuits, stuff cake or chocolate souffle (Mauriello, et al, 2016).

Figure 1. Bakery in Bethlehem (Palestine) that produce and sell “khubz”.

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).To consider strain as probiotic it must reach the intestine alive, and able to colonize 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).

1.1 Probiotics

Probiotic, pro-biotic that means “for” and “life”, respectively, are down from Latin and Greek words and according to the previous literature they start to define probiotic from 1965 by Lily and Stillwell, which defined as a substance resulting from microorganisms that enhance the availability of others microorganisms. In 2001 definition of probiotic was reported and established by the specialist working group, which are World Health Organization and Food and Agriculture Organization FAO\WHO, which defined probiotic as live special strain of rigidly selected microorganism when run in sufficient amount granted a health profit on the host (Hill, et al, 2014, Manigandan, et al, 2012). Probiotics could be yeast, bacteria, molds, but the majority is bacteria and under this majority the most common is (LAB) lactic acid bacteria (Suvarna, et al, 2005). probiotics includes *Bifidobacteria* e.g. *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* (Barry, et al., 2015).

Probiotics have been considered like a denomination of natural related to a lot of confirmed evidence of their implementation in human health products, and other commercial product “probiotic products” shown in the Figure 2 (Hill, et al, 2014). During the last decade the beneficial microbes have encompassed in different types of food with positive health functions where all manifested that make the research going up, to boost the notion that they are clinically offer health interesting (Guarnen F, et al, 1998; Manigandan, et al, 2012)

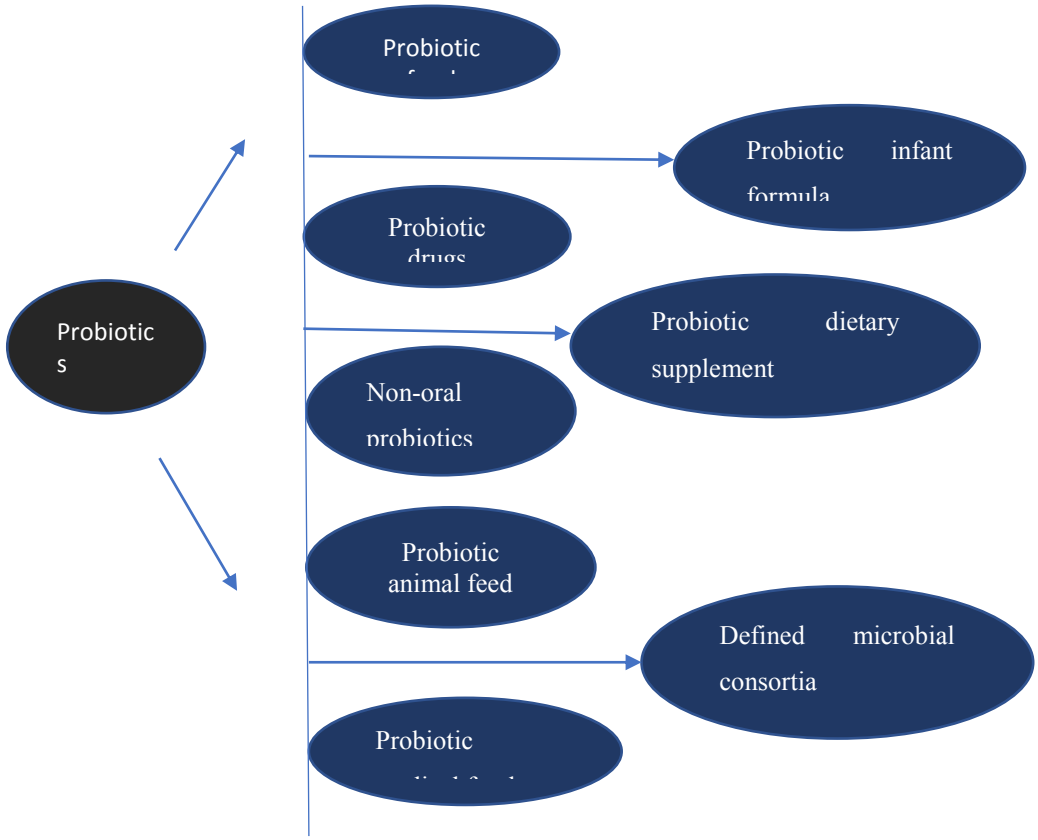


Figure 2. Gross framework of probiotics products, with different target groups, and all different type of probiotic must be cover under the safety, and the scientific evidence for their health benefits, which result in produce different product, target group and different used.

Nowadays a lot of probiotics are available for consumers without prescription as supplements or present in food products such as fermented milk, with uncertain benefits. Probiotics have subcategories according to the regulation in different countries related to their purpose used includes pharmacy as drugs, or food with probiotics for instance dietary supplements, foods, or in animal feeds fortified with probiotics and GMP (genetically modified probiotics), (Degnan FH, et al, 2008).

A complex and sophisticated microbiota is colonizing the GI tract of human in which they play a multiple remarkable function, so the proffer of probiotics as a part of human daily diet is creditable for the intestinal bacteria (Markowiak and Slizewsl,2017), and according to many clinical studies on the beneficial effect of probiotics on human gastrointestinal disorders for instance, diarrhea, IBS (irritable bowel syndrome), IBD(irritable bowel disease) and Gastritis, also there is other positive benefits result of the treatment of human disease such as diabetes mellitus type 2, insulin resistance syndrome, non-alcoholic fatty liver disease, obesity and atopic dermatitis included in allergic disease moreover, the affirmative implication of probiotic in the field of human health have been established by raise the work of immunomodulation. The efficiency and the effect of probiotic may relate to the strain species, the administrated quantity and the probiotics product components (Gibson, et al,1995)

The selection of probiotic strains covered under criteria and prerequisites. In the Figure 3 show the ideal properties of probiotics. Depending on the feedback of different institution, they are, Food and Health Organization

(FAO), Word Health Organization (WHO) and European Food Safety Authority (EFSA) table (11), strains must fulfill functionality, security standards, (Marck (FAO, 2002,EFSA J., 2005) ,and the ability of probiotic in technologically utility, which mean there are capable to stay alive also capable to retain their features during the conveyance (distribution and storage process) (Lee et al, 2009), the property of these probiotics are on the scale of particularly chosen strains of especially species (Pandei et al, 2015).

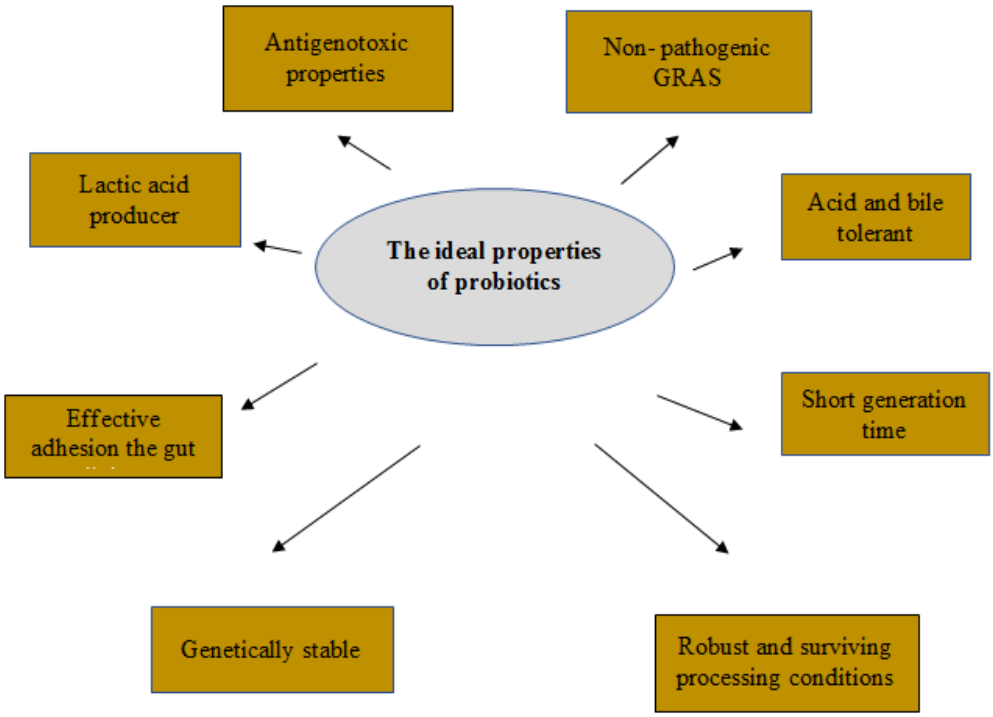


Figure 3 The ideal properties of probiotics microorganisms.

Probiotic plays several roles in human organisms, mainly its effects to make the equilibrium state between pathogenic and beneficial bacteria (EFSA J. 2017, Schachtsiek et al,2004). Another advantage of the used probiotic is related to their biological benefits, which include:

Microbiological functionality

The definitive aims of microbiological interference out of probiotics is to get better or recover the microbial homeostasis in human body. The flexibility of microbiome against infestation through external strains is based on the availability of functional unoccupied housing by endogenous microbial cells, and so as increase in unoccupied functional housing there is increase in the chance of growth of pathogens infestation, colonize and post infection. Therefore, probiotic microorganisms useful in ameliorate or get back microbial balance in two ways. Firstly, by occupied the functional housing that are remain non-occupied by endogenous community, and in this way decrease the opportunity to be occupied by pathogens. In this scenario probiotic participate in the influence of homeostasis. Other contribution of probiotic microorganism in the internal balance is via production different products such as, short chain fatty acid, i.e. (butyric acid, propionic acid, acetic acid), probiotic bacteria such as, *Lactobacillus* genus bacteria able to produce bacteriocin (small peptide, has antimicrobial activity), however probiotic bacteria (e.g., *Lactobacillus and Bifidobacterium)* may produce derivatives of bile acids, which term de-conjugated bile acids, show a strong antibacterial impact, (Markowiak et al, 2017), and also production of highly active stress compound such as, hydrogen peroxide. Which all participate and increase bacteriostatic agent toward the pathogen (vandenplas et al, 2015). The beneficial effect of probiotics microorganism through possible different mechanisms with different distribution of these mechanism among species and strains such

as, among probiotic microorganism they ability to regulation GUT motility and trnsite, and production of SCFA and acid, whereas at species level their ability to produce vitamin and enzymes activity and others, at strains level, their ability to effect on immunological and neurological systems (Hill, et al, 2014).

Nutritional participation

Some specific gut microorganisms participate in the availability of some different vitamins such as vitamin k, pyridoxine, vitamin B12, and some of B group vitamins ex: (biotin, thiamin, niacin) are produced by Bifidobacterium adolescentis, and *Bifidobacterium pseudocatenulatum*, (Sanders et al, 2007, Nova et al, 2007, Ouwehand et al, 1999). This type of participate enhance a good health benefits on their host, and thus consider as a probiotic effect.

Physiological participation

Probiotic microorganism enhances the gastrointestinal motility and transit, reduction the total amount of gases that make bloating and disturbance in the GI tract, improvement the iron absorption and reduce the amount of serum cholesterol via bile salt hydroxylase (vandanplas, et al, 2015)

Immunological participation

The ability of probiotic microorganism to increase the activation of macrophage concentration, and antibody grade, (Helena Parracho et al, 2007), and some probiotic strains their ability to produce IL-6.

Possible mechanisms of probiotic effect or action

There are several health benefits attributes to the probiotic microorganism, and their effects are varied, start from the lowest such as decrease or alleviation of constipation to the prohibition the main life threatening such as cancer, IBS

The most human probiotics are cover under the following genera: Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium, Enterococcus, and some special yeast strain affiliation to Saccharomyces additionally, strains of (G+) gram positive bacteria affiliation to the genus of bacillus bacteria and they generally used in the production of probiotics product (Simon et al, 2005)

There is several terms introduced in different country as a regulation for probiotics in their present inside the food such as GRAS term (Generally Regarded As Safe), should have in microorganism utilize for consumption aims according to USA which is organized by FDA (Food and Drug Administration) whereas EFSA inserted the definition of QPS (Qualified Presumption of Safety) which include extra standard related to the access the integrity the type of bacteria where used as a supplement (Anadón et al,2006, Gaggia et al, 2010), there is different type of probiotic microorganism apply in human nutrition, include species of Lactobacillus, Bifidobacterium, (LAB) Lactic Acid Bacteria, and other type of microorganism as are listed in table (1) (Anadón et al,2006, Gaggia et al, 2010, EFSA J. 2013).

1.2 *Saccharomyces cerevisiae* var. *boulardii*

The microorganisms 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 and non-colonizing yeast, vegetative cells of tested yeasts are cylindrical, of dimensions 2–3μm x 5–8μm. The yeast produces no filaments in slide cultures on potato glucose medium (McFarland 2010). According to the study of McFarland and Bernasconi, this strain of yeast is able to grow well at high up to 37°C compared to other strains belong to Saccharomyces sp who favor lower temperature. However, it was shown that S. boulardii strains are able to adopt a filamentous mode of growth upon exposure to a poor nitrogen source. It was first isolated from Lychee fruit in Indocina in 1923 by French scientist Henry Boulard and a freeze-dried form was marketed by Laboratoires Biocodex (Montrouge, France) in 1962 as probiotic. Several studies have shown that, in humans, *Saccharomyces boulardii* is an efficacious and safe probiotic, which has proven efficacy against some enteric diseases including, but not limited to, *Clostridium difficile*-associated disease and rotavirus diarrhoea (McFarland, 2006; Grandy et al., 2010). the potential mechanism of action of this type of yeast is based on its activity for inhibition of pathogen attachment in intestine, inhibition of action of microbial toxin (Mansour-Ghanaei et al 2003), stimulation of immunoglobulin A and secretion of increased levels of secretory immunoglobulin A (IgA) and IgA antitoxin A, and competition for attachment sites (Pothoulakis et al 1993) , and trophic effects on

intestinal mucosa (Castagliuolo et al 1999; Qamar et al 2001). A specific study (performed in children) evaluated the effect of Saccharomyces boulardii in the prevention of *Clostridium difficile* diarrhoea. The risk of documented Clostridium difficile diarrhoea was lower in the *Saccharomyces boulardii* group compared with the placebo group, but the difference was of borderline statistical significance (RR: 0.3; 95% CI: 0.1–1.04) (Kotowska et al 2005). The results of this study show that S. boulardii significantly reduces the risk of diarrhoea in patients treated with antibiotics for various purposes (but mainly respiratory tract infections). However, they do not allow conclusions about the efficacy of S. boulardii in preventing diarrhoea attributable to any single antibiotic class. Results from one recent RCT suggest that *S. boulardii* effectively prevents diarrhoea caused by amoxicillin in combination with clavulanate as well as intravenously administered cefuroxime. Although no adverse effects were observed, the administration of S. boulardii is not without risk. One caveat about S. boulardii is that it can cause fungemia (Zunic et al 1991) Most complications have occurred in immune compromised subjects or in patients with other life-threatening illnesses managed in intensive care units. The strain used for this research was “CNCM-I-1079”. S. cerevisiae boulardii (CNCMI-1079 strain) is a non- pathogenic yeast widely used inhuman medicine to prevent and treat intestinal disorders, such as infectious and antibiotic-associated diarrhea (Buts and DeKeyser, 2006; Czeruckaetal ,2007). Its role in gut function has also been highlighted in physiological studies on swine, and trials as a feed additive in husbandry condition shave shown its positive effects on weaned pigs (Le Bonetal,

2010). The aim of that preliminary study was to investigate the effect of increasing dietary supplementation of *S. cerevisiae boulardii* on the apparent digest-ibility, growth performance, caecal fermentation, carcass characteristics and meat quality of broiler rabbits. This study shown that protected live yeast(S. cerevisiae boulardii, CNCMI-1079 strain)was resistant to the pelleting process and to the passage through the rabbit digestive tract as far as the caecum where it showed an 86% survival rate in the 600mg/kg supplementation level group (L. Rotolo et al 2014).

1.3 General introduction on microencapsulation

Cell microencapsulation has its roots in the primordial microbial immobilization technique, known as the procedure of physical confinement of cells to a certain defined space with the main purpose of preserving their viability. Through the tailoring of innovative techniques and the research on new suitable encapsulating materials, microencapsulation has become a sophisticated technology for the entrapment of living cells for a variety of applications. It can be defined as the engineered inclusion of cells into functional matrices to form solid objects with variable size (1-1000 μm), shape (e.g. spherical, droplet, irregular) and morphology (e.g. coated/non-coated matrix or reservoir microcapsules, single/multi-core, single/multi-layers). Currently, cell microencapsulation still accounts many applications in the research field, as suggested by the constant growing in scientific publications, along with the scaling-up of this technology for the industrial development. First research on living cell immobilization dated back to 70-80’s, when, for exemplum, it was demonstrated that this procedure

enhanced aspartase activity in E. coli (Chibata et al., 1974) or when yoghurt bacteria (Lactobacillus delbrueckii and Streptococcus thermophilus) were entrapped in different alginate particles with the aim to improve a continuous pre-fermentation of milk for yoghurt production (Prévost and Diviès, 1987; 1988a and b). The use of immobilized microbial cells in many biotechnological processes was found to be profitable over the utilization of same cells in free form (Rathore et al., 2013). This practice was found advantageous in improving the production of some metabolites, facilitating the separation of cells from fermentation products, enhancing fermentation process due to higher cell density achieved, permitting the reuse of immobilized cells and finally, but not least, it proved to endure cells against environment stress-related factors prolonging cell viability. Because of the versatility of immobilization, and later of the microencapsulation, to be applied for the entrapment of many different microbial cells, this technology is still explored in as many different applications. Some exempla, including the production of biofuels, environmental decontamination, novel food development and enzymes, vitamins, food and pharmaceutical products are included in table 1.

In the field of food science and technology, immobilization/microencapsulation technology gradually became a tool for the improvement of the performances of microorganisms of technological interest (e.g. starter cultures, biocatalysts) as well as a strategy for the protection of microorganisms considered beneficial (i.e. probiotics) for human and animal health.

Table 1. Applications and purposes of immobilized/encapsulated microbial cells in different technological processes

.Encapsulated cells	Immobilization/encapsulation carrier	Purposes of application	Reference
		Biofuel production	
Clostridium acetobutylicum SE25	AquaMats AO	Enhancement of butanol production	Li et al., 2016
Synechocystis sp. PCC6803	HFE-7500 oil	Increment of cell cyanobacteria photosynthetic growth and production of lactate	Hammar et al., 2015
Environmental decontamination			
Anthracophyllum discolor Sp4 CCCT 16.5	Alginate-CaCl2	Degradation of herbicide atrazine	Elgueta et al., 2016
Ogataea polymorpha VKMY-2559	Organic silica sol-gel	Purification of methanol containing industrial wastewater	Kamanina et al., 2016
Lysinibacillus fusiformis B26	Agar and calcium alginate	Decolourization of synthetic dye	Dogan et al., 2016
Production of ingredients for food industry			
Lactococcus lactis; Lactobacillus plantarum NCIM 2084; Lactobacillus delbrueckii NCIM 2365;	Hexamethylene diisocyanate and glutaraldehyde and chitosan; Polypropylene matrix and chitosan; Palmitoylated alginate	Enhancement of lactic acid production	Groboillot et al., 1993; Krishnan et al., 2001; Rao et al., 2008
Saccharomices cerevisiae; mixed starter culture from Loog-Pang (Thai rice cake)	Carboxymethylcellulose-alginate- CaCl2; Silk cocoons	Enhancement of ethanol production	Talebnia and Taharzadeh, 2007; Khamkeaw and Phisalaphong, 2016
Candida guilliermondii	Alginate- CaCl2	Xylitol production	Carvalho et al., 2003
Enterococcus faecium A2000; Lactobacillus curvatus MBSa2	Alginate-CaCl2	Enhancement of bacteriocin production	Ivanova et al., 2000; Barbosa et al., 2015
Streptomyces sp. RCK-SC	Polyurethane foam (PUF)	Enhancement of pectinase production	Kuhad et al., 2004

Fermentation of food matrices			
Lactoferm ABY 6;	Chitosan coated alginate-CaCl2	Fermentation of whey-based substrate and improvement of bacterial survival	Krunić et al., 2015
Lactobacillus plantarum PCS 26	Alginate-CaCl2	Fermentation of apple juice and prolongation of probiotic viability	Dimitrovski et al., 2015
Lactobacillus plantarum and Staphylococcus xylosus	Alginate-starch	Improvement of cells viability during meat thermal treatment for fermented sukuc preparation	Bilenler et al., 2017
Probiotic Lactobacillus delbrueckii and Streptococcus thermophilus	Chitosan coated alginate-CaCl2	Elucidation of fermentative ability of encapsulated starter cultures and protection of probiotic strain	De Prisco et al., in submission
Protection of probiotics and enhancement of their functions			
Lactobacillus reuteri CGMCC 1.3264	Fe3O4 particles	Enhancement of reuterin production	Lui and Yu, 2015
Lactobacillus reuteri DSM17938	Alginate-CaCl2	Improvement of cell viability during storage	De Prisco et al., 2015
Lactobacillus gasseri and Bifidobacterium bifidum; Lactobacillus plantarum TN8	Chitosan-alginate-CaCl2	Improvement of cell viability during GI simulated transit	Chávarri et al., 2010; Trabelsi et al., 2013
Development of new foods			
Lactobacillus rhamnosus GG; Bifidobacterium animalis BB-12	Whey proteins; cellulose acetate phthalate	Probiotic fruit juices	Doherty et al. 2012; Antunes et al., 2013 al.,
Lactobacillus reuteri DSM 17938	Chitosan coated alginate-CaCl2	Probiotic chocolate soufflè	Malmo et al., 2013
Lactobacillus casei ATCC 39392	Calcium alginate-resistant starch	Probiotic cream filled cake	Zanjani et al., 2012

1.4 Generalities on microencapsulation technology

As previously mentioned, microencapsulation develops from immobilization that can be defined as the technology of holding sensitive compounds in/onto functional matrices (Figure 4). This research mainly addressed the use of entrapment and containment procedures.

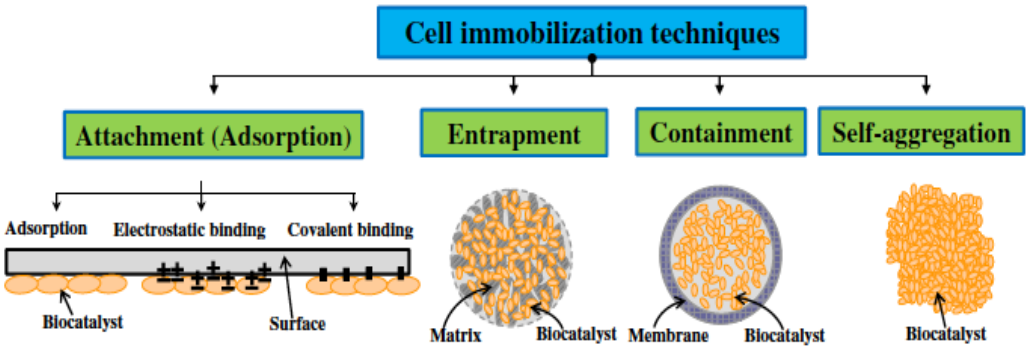


Figure 4. Types of cell immobilization techniques. Image by Abbel-Rahman et al., 2013

In the practice, microencapsulation step includes the complete envelopment of pre-selected core materials in its liquid, solid or gaseous phase within a defined natural or synthetic porous or impermeable membrane by using different techniques (De Vos et al., 2010; Whelehan and Marison 2011). What results from this process are the microcapsules, solid particles that can differ for their size (Figure 5) and morphology (Fig. 2.3) depending on materials and techniques applied to produce them. Capsule sizing can vary among macro (>1000 μm), micro (1-1000 μm) or nano (<1 μm) ranges. Encapsulation technology applied to microbial cells, which are typically 1-4 μm in size, is usually referred to as microencapsulation (Gawkowski and Chikindas 2013).

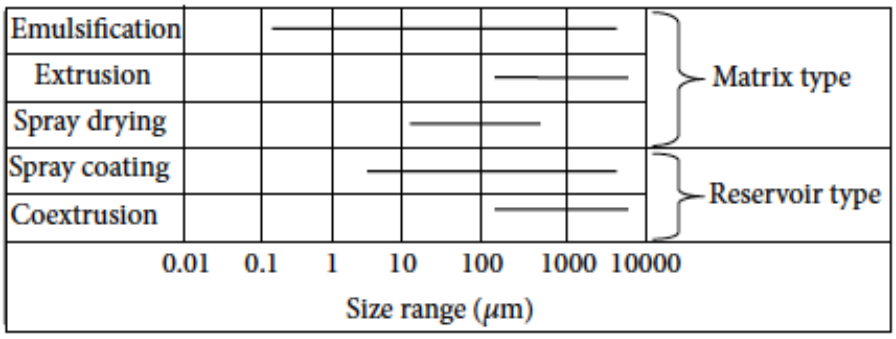


Figure 5. Size range provided by the main techniques for cell encapsulation for food and pharmaceutical application. Image by Solanki et al., 2013.

According to their morphology, microcapsules can be classified in five main different types (Gharsallaoui et al. 2007; Solanki et al., 2013) as illustrated in figure 6.

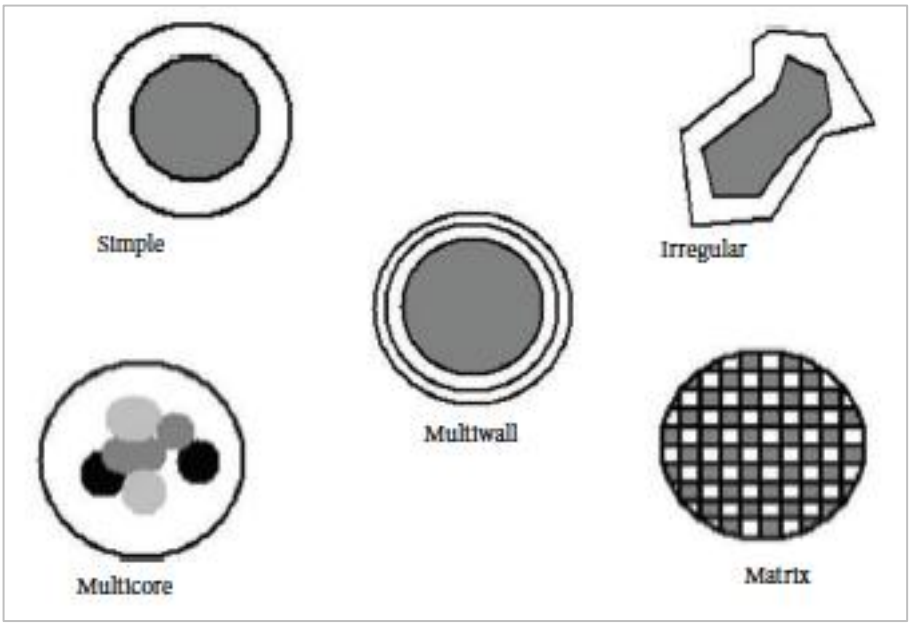


Figure 6. Different types of microcapsules used in food industry. Image by Solanki et al., 2013.

In the "simple" type, also referred as core-shell or reservoir microcapsule type, cells or other sensitive compounds are retained in the core of the capsules completely surrounded by a continuous entrapping membrane. Both the diameter of the core and the thickness of the membrane can vary.

In the “multicore” system the encapsulated ingredients form two or more aggregations, as usually occurred in emulsification techniques (Whelehan and Marison, 2011). When capsules are not characterized by a well-defined and spherical morphology they are defined “irregular” shaped. In “matrix” type microcapsules a physical separation of the encapsulating agent and the encapsulated ingredient does not exist since the latter is homogeneously dispersed in the encapsulating agent and then in the entire capsule surface. All microcapsule types reported above can be coated by applying a further layer of a coating agent that can create bonds with capsule material due to physical or chemical reactions; resulting particles can be referred as “multiwall” microcapsules.

Besides capsule sizing, microcapsules morphology is an important parameter to be tailored during cell encapsulation, since many studies underlined the correlation between microcapsule morphology and their performances. (Rule, et al,2007; Rocha-Selmi, et al,2013)

The different types of microcapsules are produced from a wide range of wall materials and by a large number of different microencapsulation processes such as spray-drying, spray-cooling, spray-chilling, air suspension coating, extrusion, centrifugal extrusion, freeze-drying, coacervation, co-crystallization, liposome entrapment, interfacial polymerization, emulsion or micro-channel and membrane emulsification. (Anal & Singh, 2007; Gharsallaoui et al., 2007; De Vos et al, 2010). Among these, extrusion, emulsion and spray drying techniques in particular

are the most suitable to be scaled-up for industrial production of cell microcapsules (Burgain et al., 2011).

Since many comprehensive reviews (Ross et al., 2005; Anal & Singh, 2007; De Vos et al., 2010; Rokka and Rantamäki, 2010; Martín et al., 2015) address the main aspects of microencapsulation techniques, including methodologies and materials used for cell encapsulation, this section will only give the most relevant information about materials (alginate, chitosan and xanthan gum) and technique (vibrational nozzle technology) selected for the present research.

1.5 Microencapsulation technique to increase probiotic viability

As mentioned before to consider strain as probiotic it must reach the intestine alive, and able to colonize 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).

As these probiotics are proved for its health benefits includes antiinfection, anticarcinogen attribute, improve lactose intolerance and enhance nutritional properties, they need to reach colon in range of 10^6 -10^7 and at the time of food consumption is 10^7 to 10^12 for their therapeutic agent (évalo-Villena, Marí, 2017).

Microencapsulation of probiotics process efficiency can be evaluated by several factors such as right choice of capsules attribute (material),final beds diameter as it increase the ability of pancreatic enzyme to digest become less and cause unsuitable flavor and mouth feel coating of capsules that provide physiochemical attribute and increase the efficiency of encapsulation by sealed it without cracks or gaps(Ragavan et al 2019) and the microbial cells count that firstly added to encapsulation cells as microbial number increase, and according to the previous study the most viability of probiotics yeast showed by microcapsules coated with whey protein is eighty nine percent on 60th days and the most famous biomaterial used for microencapsulation is alginate that have adhesive properties.

The main components used in the microencapsulation probiotics are numerous such as alginate and its arrangement used mainly in LAB, starch rich in amylose used for strengthening the capsules work, blend of xanthan-gelan which is contra versa with alginate this mixture is withstand to low PH conditions, Chitosan similar to alginate and dissolve at pH < 6 and utilize for gelatins capsules as coating material (Morreale et al, 2019)

There are multitude technologies to prepare microencapsulation are vary in conformation and the size depend on the type of technology and material used, one of this techno is spray draying its inexpensive, a lot of production, small capsules and according to many research they proposed the moisture content and water activity are 4-5g\100g and 0.15- 0.30 respectively *(*évalo-Villena et al,2017), however it disrupts viable cells due to use high temperature. Freeze drying is a perfect choice for delicate material as beneficial bacteria its expensive and cell damage if not do rightly and other techniques such as spray cooling, freeze drying, spray coating, emulsification, liposomes, coacervation, extrusion, and among all this techno the most utilization in academic research are spray drying, extrusion, and emulsification techniques (Ragavan et al 2019).

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 probiotic should release 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 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 gL-1) and chitosan (4 gL-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 2 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.

Whereas emulsification technique was used in our experimental. Emulsification is a process of dispersing one liquid (dispersed phase) cell\polymer suspension, in a second immiscible liquid (continues phase) oil\organic phase by including core material in the first liquid (dispersed phase) and used hardening such as calcium chloride (Figure 7). Emulsification microsphere result in vary size and are affected by the speed of stirring and the rate of addition cross-linking solution. The most stumbling block is the toxicity of organic solvent to microsphere cells, so used oil is less toxic than organic solvent, but also removal the oil is more difficult than organic solvent (Rathore, et al 2013, Chopde, et al, 2014)

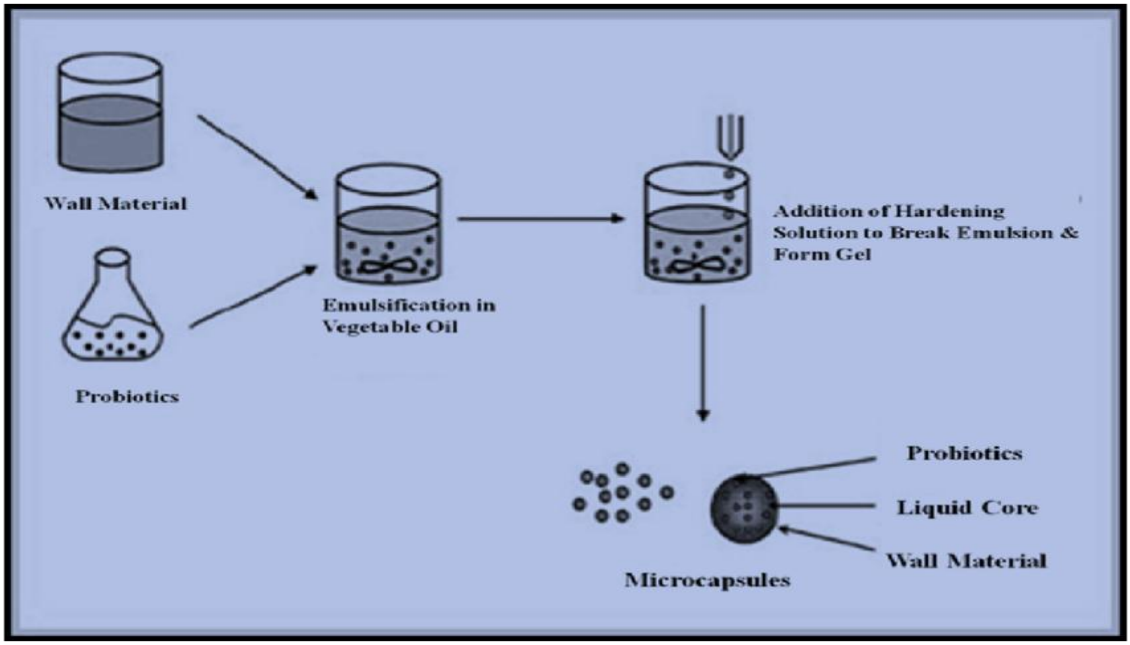


Figure 7. frame draw for emulsification technique as a type of encapsulation process. Image by Chopde, et al, 2014.

1.6 Labneh

Concentrated yogurt known as “Labneh”, very famous in the Middle- East, widely used as spreading in sandwich. Produce from mixing set fermented milk “yogurt” from cow milk, “labneh” result from draining proportion of whey protein, at the end of draining the percentage of fat and total solid are 9% - 11%, 23% - 25% respectively, characterize as semi- solid and it is color is white to creamy paste, smooth texture and the taste between sour cream and cottage cheese (crossing). Another description, it has sharp flavor due to diacetyl result from fermentation (Al-Kadamany et al,2002, Mihyar et al, 1999)

2. MATERIALS AND METHODS

2.1 Isolation of yeasts (*Saccharomyces cerevisiae* and *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*, figure 8) in the form of capsule for sale in pharmacies, which was cultivated, subsequently in Yeast Peptone Dextrose (YPD) broth (1% Yeast extract, 2% Peptone, 2% Dextrose) at 25°C. After checking the purity of the strain, the sample was stored in Yeast Peptone dextrose (YPD Agar) (1% Yeast extract, 2% Peptone, 2% Dextrose, 1.5% Agar), for all the experiments described below. In figure 9 is represented slide used for cell counting by using light microscope.

Figure 8. *S. boulardii* used for experiments

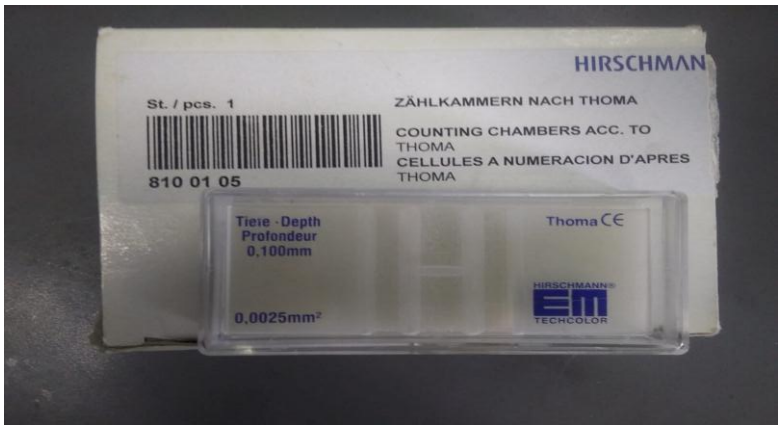


Figure 9. Micrometer slide used to calculate CFU|ML of free cell

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 107. an aliquot of 1 ml of appropriate decimal dilutions were pouring on tryptone yeast peptone dextrose agar. All plates incubated at 25°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.

A culture of a non-probiotic Saccharomyces cerevisiae was used for some experiments. Microorganism was isolated on YPDA at 25°C from a commercial bakery yeast.

2.2 Microencapsulation of *Saccharomyces boulardii* by water in oil emulsion

Firstly, yeast cells were microencapsulated following the protocol of Poncelet et al. (1992), who described the formation of alginate beads, in which gelation is due to the presence of calcium carbonate in the alginate. Calcium carbonate is water insoluble but during the stirring in presence of oil, glacial acetic acid is added, and it becomes soluble and promote

alginate gelation. This protocol is known with the name of “internal gelation”. Following the procedure is detailed:

- Prepare 25 ml of *S. boulardii* culture in YPDB;

- Centrifuge the culture to obtain a cell pellet;

- Suspend the pellet in 25 ml of 1.7% sodium alginate containing 0.0625 g of CaCO3 powder;

- Mix the suspension with 100 ml of vegetable oil (soybean or sunflower) containing 1 g of Tween 80;

- Homogenize by using mechanical stirring at 300 rpm for 20 min;

- After stirring add 25 ml of vegetable oil containing 150 µl of glacial acetic acid during mechanical stirring (600 rpm) for 10 min;

- Pour the suspension in a glass cylinder (200 ml) and wait for phases separation;

- Discard upper oil and water phases.

In the further procedure yeast cells were microencapsulated in alginate using the method described by Sheu and Marshall (1993) and modified by Truelstrup Hansen et al. (2002). Briefly, 100 ml of yeast culture in the early stationary phase were harvested by centrifugation at 6500 rpm for 15 min. The cell pellet was washed once in an equal volume of a sterile quarter-strength Ringer solution (Oxoid) (Ringer). The obtained wet cell pellet was suspended in sterile 2% alginate (Sigma, product n. A2033) solution. The

cell suspension was mixed with 100 g sunflower oil containing 0.5% Tween 80 (Sigma, product n. P8074). Different variations on the basic procedure were carried out, in particular the ratio of the components of mix; cells, alginate solution, oil. Moreover, mix was homogenized by using stirring or Ultraturrax homogenization. The scheme of the general procedure is reported below:

- Prepare 25 ml of *S. boulardii* culture in YPDB;

- Centrifuge the culture to obtain a cell pellet;

- Suspend the pellet in 30 ml of 2% sodium alginate containing;

- Mix the suspension with 180 ml of vegetable oil (soybean or sunflower) containing 1 g of Tween 80;

- Homogenise by using mechanical stirring* (Figure 10), or Ultraturrax** (figure 11).

- After adding about 30 ml of $CaCl_2$ during mechanical stirring (600 rpm) for 20 min***;

- Pour the suspension in a glass cylinder (200 ml) and wait for phases separation;

- Discard upper oil and water phases.

* 600 rpm

** 25000 rpm for 45 sec.

*** Calcium ions, necessary for the alginate gelation, were added in two consecutive steps: first, 60 g olive oil containing 0.5% Tween 80 and 62.5 mM CaCl2, stirring at 300 rpm for 20 min, and then 40 ml buffered peptone solution added with 0.05 M CaCl2. Microcapsules were harvested by one-night sedimentation at room temperature and washed once in a double volume of a sterile quarter-strength Ringer.

All experiments described above were performed using freshly prepared microcapsules. All microcapsules samples were routinely kept at 4°C.

For some further experiments yeast microcapsules were treated with 5% chitosan. Briefly, a variable aliquot of alginate yeast microcapsules were added to a 5% solution of chitosan in 0.1 M acetic acid. All was mixed for 40 min at 100 rpm at room temperature. After that capsules coated with chitosan were separated by centrifugation (4000 rpm for 10 min at room temperature) and used for further experiments.

Figure 10. plate heater was used as a way for mechanical stirring by using magnetite

Figure 11: Ultraturrax homogenizer

2.3 Preparation of "khubz"

Aliquots of 150 g of white flour (Figure 12) were used for the preparation of "khubz". In particular, 90 ml of quarter strength Ringer solution was added to flour in which yeast culture (free cells of Saccharomyces cerevisiae, free or encapsulated cells of Saccharomyces boulardii) was previously suspended. A dough stands spiral mixer (Figure 13) was used for dough preparation and leavened for about 16 hours at 25°C (Figure 14). After leavening each dough was cut in three portions and each one was rolled out to form a circle of about 25 cm in diameter and about 0.5 cm thickness. Samples were baked in a preheated oven (SMEG electric ventilated oven) at 250°C for 5 min (until leavening and surface browning of "khubz"). The temperature of "khubz" was immediately decreased after baking by storage for 1 h in 10°C incubator. Samples were collected after

leavening and after baking (product at 10°C) and they were subjected to live counting by agar plating.

Figure 12. type of flour used during all the research experiment

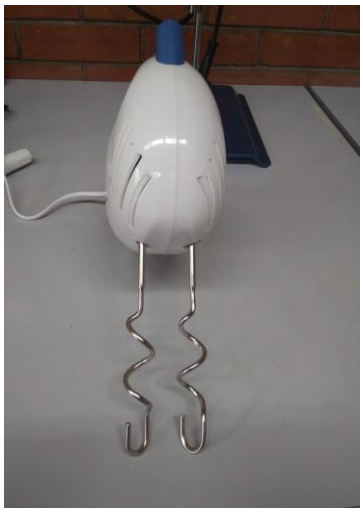


Figure 13. A dough stand spiral mixer, was used for dough preparation

Figure 14. shows the leavening of dough inside the incubator at 25°C for 24 h. the first line from the bottom represent the height of dough after preparation, whereas the second line from the bottom represent the leavening of dough after incubated at 25°C for 24 h.

2.4 Preparation of "labneh"

A commercial yoghurt (5% in fat, figure 15), was introduced in nylon socks to promote whey draining for 24 h at room temperature. After this period yoghurt was extracted by socks, loaded in plastic container and stored at 4°C.

Figure 15. the commercial yogurt used to prepare "labneh"

2.5 Viable yeast counting

We used a pouring technique as a method to determine the number of living cells in a given volume, for a given samples for all experiments, it was used to test the viability of each : free yeast cell , microcapsules before (by using ringer) and after disgregation (by using 2% sodium citrate), dough after leavening and after backing "Khubz", and for labneh mixed with free and other mixed with microcapsules yeast at different days of storage (first day of preparation , after three days, after ten days. where 9 ml of sterile ringer was putted in each tube by sterile pipette (10 ml), then start the preparation of a serous dilution 1 ml of the diluted material of the previous step is used to make the subsequent dilution. Then a fixed amount of serous dilution (generally 1 ml) from a sample is placed in the center of sterile Petri dish using a sterile pipette. YPD cooled agar (approx. 15mL) is then poured into the Petri dish containing the inoculum and mixed well. After the solidification of the agar, the plate is inverted and incubated

at 25°C for 24-48 hours. For accurate counts, the optimum count taken within the range of 30-300 colonies/plate.

2.6 Morphology and counting of microcapsules

Morphology

Yeast cells and microcapsules were checked under microscope after the viable count was done for free cells (biomass) and microcapsules before and after disgregation. Optical microscope was used by special slides (counting cells per square) to counting the number of cells per ml

Counting 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 106. an aliquot of 1 ml of appropriate decimal dilutions were spread on yeast peptone dextrose agar. All plates incubated at 25°C for 24-48 h. 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. the solution a series of gradually less potent dilutions was obtained to 106. an aliquot of 1 ml of appropriate decimal dilutions were spread on yeast peptone dextrose agar. All plates incubated at 25°C for 24-48 h. For accurate counts, the optimum count taken within the range of 30-300 colonies/plate.

3. Results and Discussion

3.1 Isolation of microorganisms

The isolation of *Saccharomyces boulardii* from the commercial supplement and *Saccharomyces cerevisiae* from commercial bakery yeast was not difficult and after first plating procedure we obtained pure culture of both microorganisms. *Saccharomyces boulardii* was used for all further experiments while *Saccharomyces cerevisiae* culture was used for some comparative experiments as reported below.

Under optical microscopic observation both cultures showed the typical cell shape, that is spherical for *Saccharomyces cerevisiae* and spheroidal for *Saccharomyces boulardii* (Figure 16).

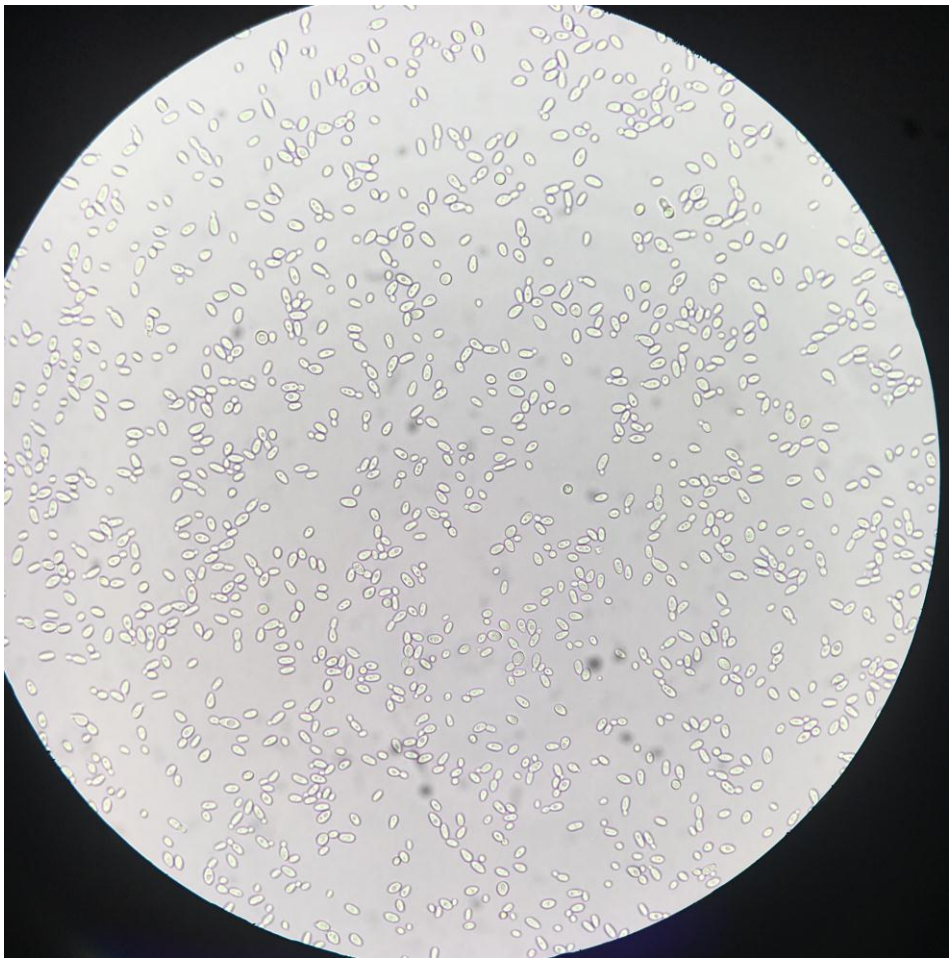


Figure 16. Cells of Saccharomyces boulardii observed under light microscope (400X magnification)

Strains were also subjected to growth monitoring in YPDB at 25°C for comparing purpose and results are reported in Figure 17. Both strains grew rapidly reaching the exponential phase in about 4 h and both showed a similar trend of growth.

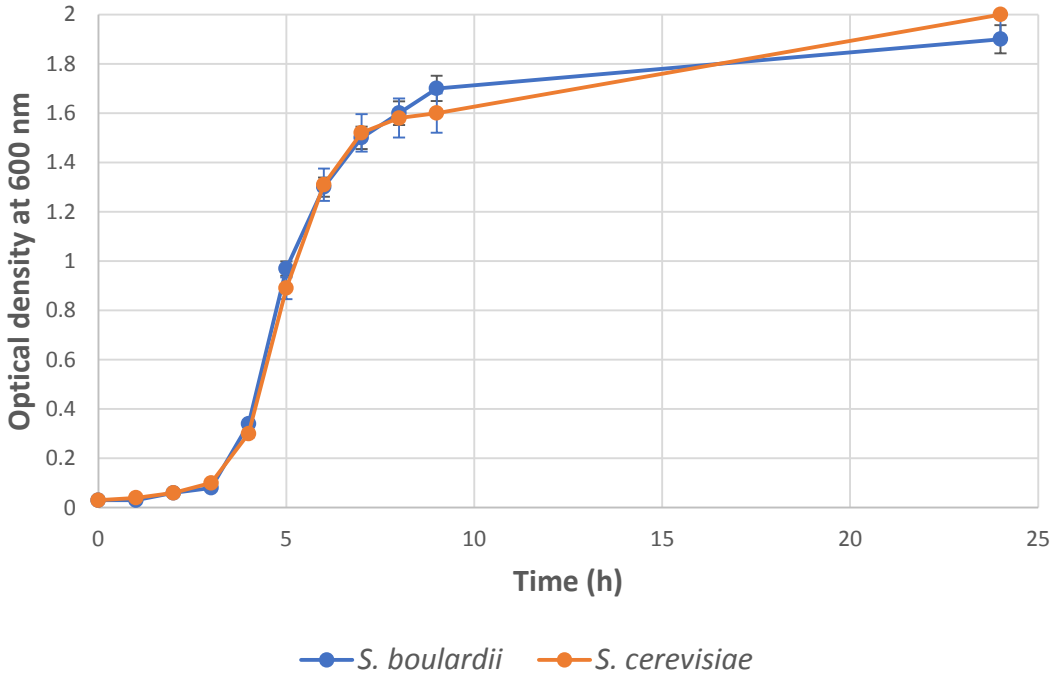


Figure 17. Growth curve of *Saccharomyces boulardii* and Saccharomyces cerevisiae in YPDB at 25°C. Average of three replicates and standard deviation.

3.2 Microencapsulation of Saccharomyces boulardii *cells*

Cultures of *Saccharomyces boulardii* were microencapsulated by using emulsion technique (water in oil). In figure 18 is depicted the Ultraturrax homogenizer and the mixture for microencapsulation.

Figure 18. Ultraturrax and the mix of oil, alginate and yeast cells.

After each microencapsulation experiment, cultures were subjected to analysis to determine morphology of microcapsules, yield of encapsulation, cell survival during the time. Firstly, results of microencapsulation procedure by using internal gelation protocol showed that after the addition of acetic acid to dissolve calcium carbonate, no phases separation occurred (Figure 19), even after 24 h of incubation. Moreover, optical microscope observation of emulsion didn't show clear microcapsules formation (data not shown). *Saccharomyces boulardii* was previously microencapsulated by other authors by using internal gelation procedure (Wentao Qi et al., 2019) and they obtained microcapsules, but paraffin oil was used. We used in our protocol sunflower oil and it could be the reason for what we did not reached the phases separation. Moreover, the same authors obtained emulsion by using a mixer while we used the homogenizer. It is possible that our microcapsules reached a smaller dimension compared to that obtained by these authors.

By applying the protocol in which $CaCl_2$ as gelling agent is used, we had phases separation. In particular, water is the lower phase, oil is the upper phase and microcapsules are in the intermediate phase (Fig. 20). The results of encapsulation of *Saccharomyces boulardii* led to microcapsules containing both yeast cells and small oil droplets. In Figure 21 are represented pictures of microcapsules observed under light microscope.

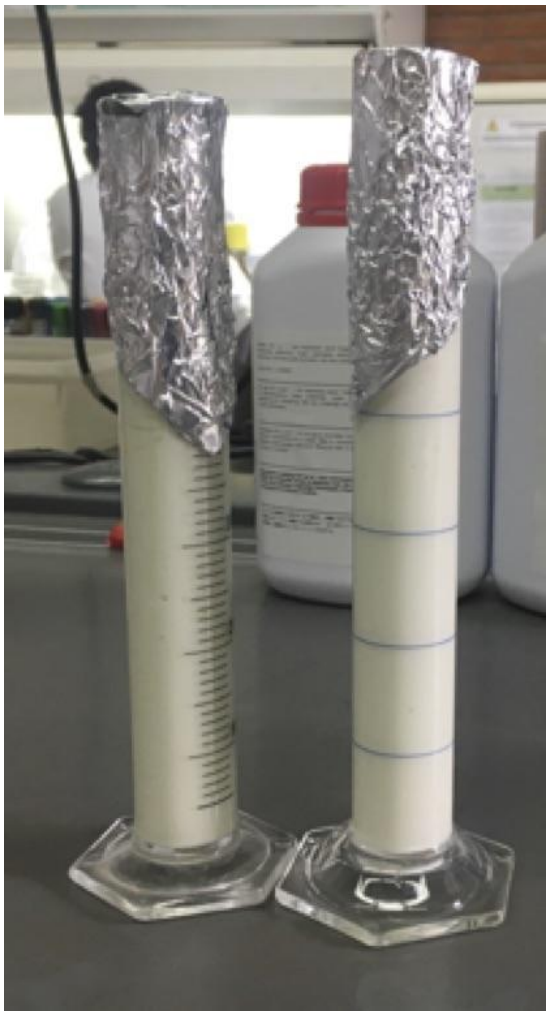


Figure 19. Microcapsules obtained by internal gelation procedure. No separation of phases occurs after 24 h.

Microcapsules showed an average size of 15 μ (± 5 μ) calculated on the size of about 50 microcapsules, obtained by different processes, and by using a microscope micrometer (Figure 22). Images show that about 50-60 yeast cells are included per each microcapsule and that small droplets of oil, presumably vegetable oil from emulsion process, are entrapped in the microcapsules. Size and presence of oil droplets agree with results obtained by other authors (Kakran and Antipina, 2014).

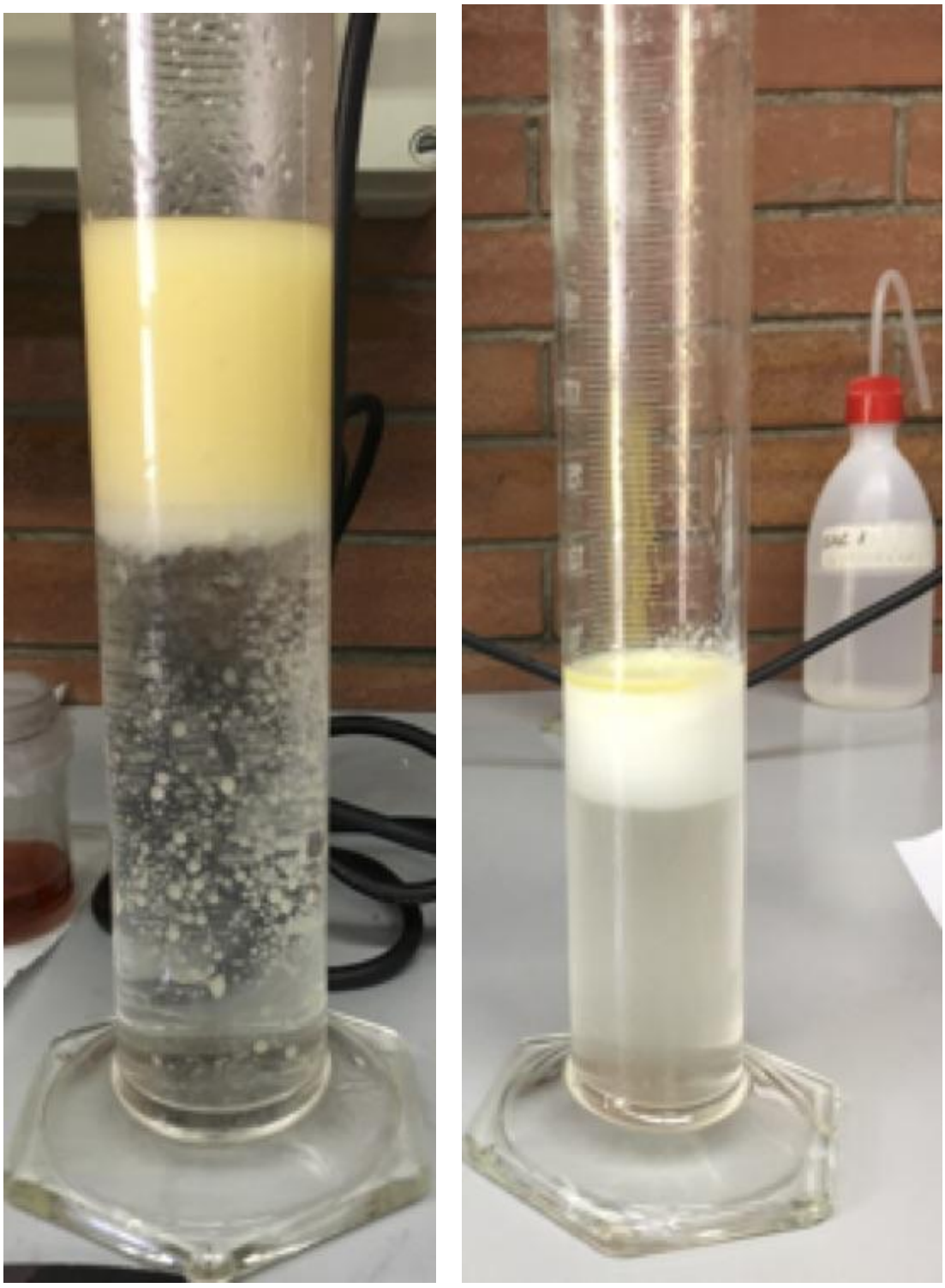
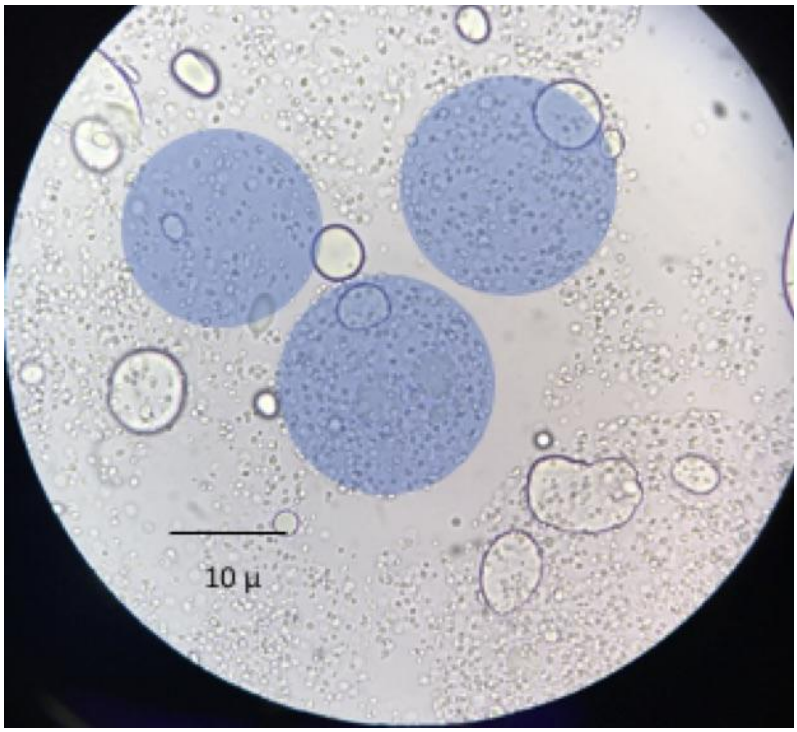


Figure 20. Separation of phases after microencapsulation procedure by using $CaCl_2$ like gelling agent.



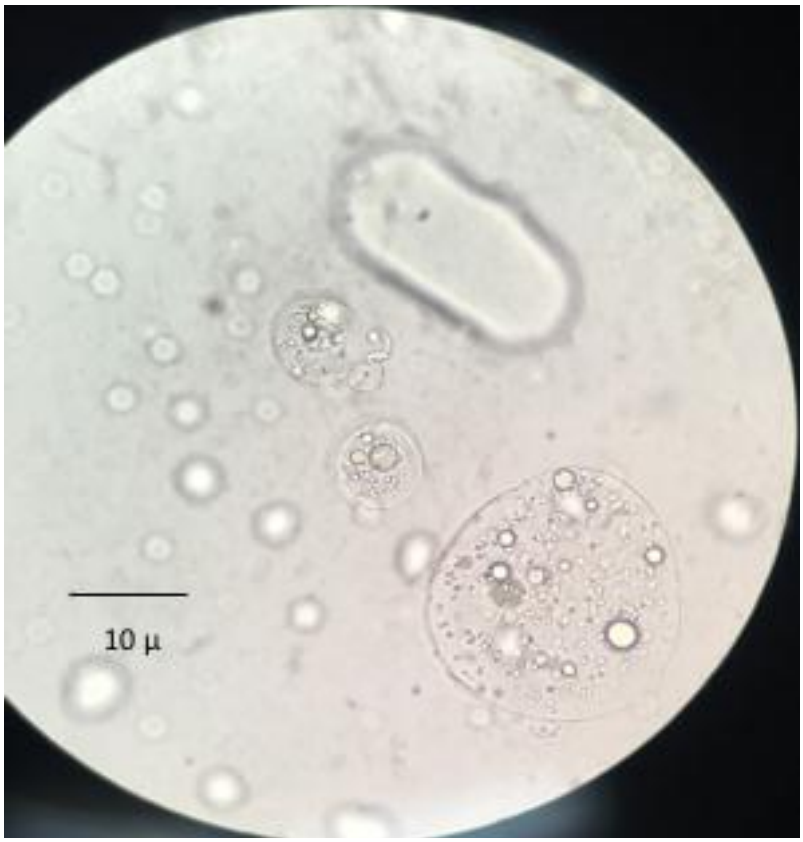


Figure 21. Images captured by observation under light microscope (400X magnification) of microcapsules obtained by emulsion technique. Blue circles in the first photo highlight the presence of microcapsules.

Figure 22. Micrometer slide used for numbering and size calculation of microcapsules.

In table 2 are reported results of microencapsulation under different conditions. In particular, the values of: average diameter of microcapsules, encapsulation efficiency (EE) and survival during the time (15 days) are reported.

Table 2. Values of different characteristics of microcapsules investigated in function of the values of parameters of microencapsulation process.

Condition of microencapsulation	**Average diameter μ ±SD**	**EE %**	**Survival during the time (15 days) %**
Mechanical stirring for 20 min	45 ±5	80	92
Mechanical stirring for 30 min	45 ±6	82	92
Mechanical stirring for 60 min	45 ±5	85	92
Ultraturrax 10000 rpm for 60 sec	37 ±3	79	90
Ultraturrax 12000 rpm for 60 sec	35 ±3	80	80
Ultraturrax 18000 rpm for 60 sec	28 ±2	80	83
Ultraturrax 25000 rpm for 60 sec	25 ±2	65	82
Ultraturrax 10000 rpm for 45 sec	37 ±3	79	90
Ultraturrax 12000 rpm for 45 sec	35 ±3	80	80
Ultraturrax 18000 rpm for 45 sec	28 ±3	80	83

Ultraturrax 25000 rpm for 45 sec	25 ±1	75	85
Ultraturrax 10000 rpm for 30 sec	38 ±3	68	95
Ultraturrax 12000 rpm for 30 sec	38 ±3	68	92
Ultraturrax 18000 rpm for 30 sec	36 ±3	67	92
Ultraturrax 25000 rpm for 30 sec	35 ±3	67	93

According to the results obtained we decided to use this condition of microencapsulation: Ultraturrax at 25000 rpm for 45 seconds.

3.3 Probiotication of "khubz".

First experiments of dough mixing for Arabic bread ("kohbez") was carried out by using free cells of *Saccharomyces cerevisiae* and *Saccharomyces boulardii* and microencapsulated cells of *Saccharomyces boulardii*. Volume of leavening was calculated per each experiment and results showed that both microorganisms in free form gave the same volume of leavening, while microencapsulated cells of *Saccharomyces boulardii* gave 90% lower volume (Figure 23).

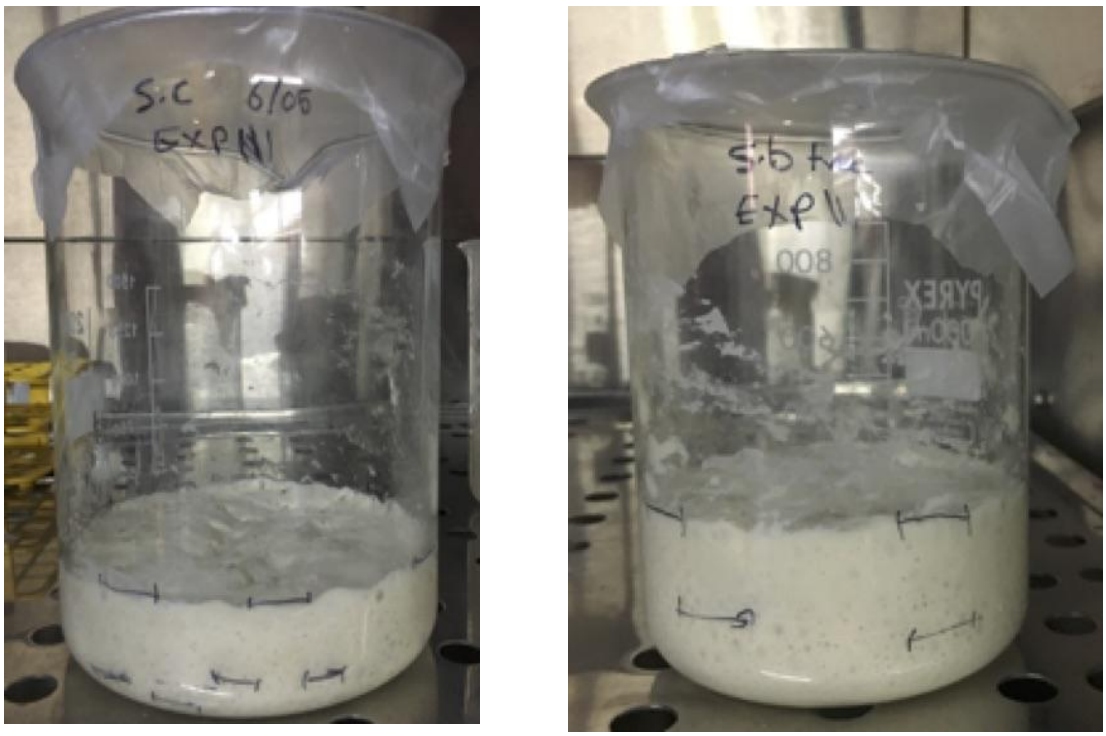


Figure 23. Leavening of dough obtained by using free cells of *Saccharomyces cerevisiae* (panel on the left) and *Saccharomyces boulardii* (panel on the right).

In the table 3 are reported results of leavening experiments comparing the performance of *S. cerevisiae* and *S. boulardii* (in the free and encapsulated form). As reported before, it is clear that the fermentation performance of the starter culture is slightly affected by the microencapsulation and that *S. boulardii* can be used in the free form for the normal leavening process, indeed no significant difference ($P<0.05$) was registered for the increasing of volume by comparing results of two starters in free form. There are no previous studies on wheat dough leavening to compare our results. However, results of previous studies on the fermentation by using encapsulated starter cultures showed that encapsulated cells have a slower metabolic activity compared to free cells. It could be due to both slow

penetration of substrate into the microcapsules and to a slow release of metabolites out of the microcapsules.

Table 3. Volume increasing (average value obtained by 5 independent replicates) after 16 hours of fermentation of dough prepared with different starter cultures.

Starter culture and form	**Volume increasing (cm^3)***
Saccharomyces cerevisiae free cells	730^A
Saccharomyces boulardii free cells	760^A
Saccharomyces boulardii microencapsulated cells	650^B

*Different letter means significant difference as determined by t-Test ($P<0.05$).

However, some authors showed that after a prolonged fermentation process the encapsulated microorganism can reach the same level (in terms of growth and metabolism) of the free culture.

Because preliminary results showed that the leavening performance of *S. cerevisiae* did not show significant difference ($P<0.05$) compared to *S. boulardii*, further experiments were carried out just by comparing free and encapsulated cells of *S. boulardii*. On the basis of three different experiments, the curves of leavening of two doughs prepared with free and encapsulated *S. boulardii* cells, respectively, were designed (Figure 24).

The inoculum of free and encapsulated yeast culture was estimated to reach about $3.0x10^5$ CFU/g into the dough. After the fermentation period (about 16 h) we found, as expected, an increased number of yeast population in

the case of both free cells and the microencapsulated one, indeed we registered about 1.6 log cycle of cell growth.

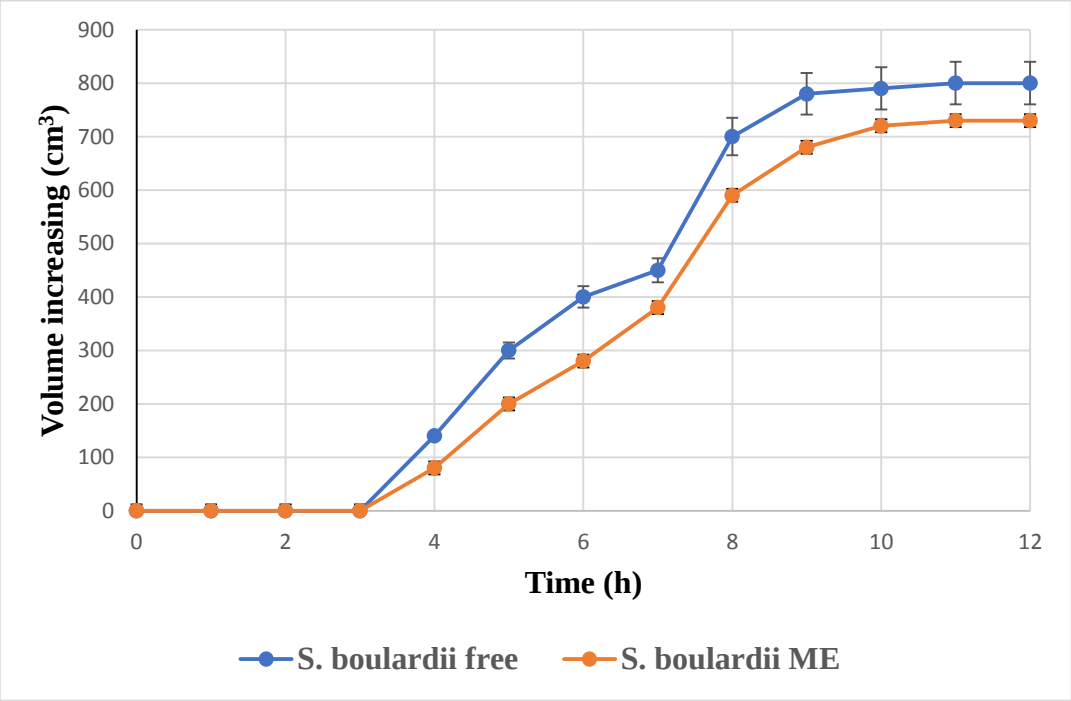


Figure 24. Trends of volume increasing of dough obtained by using free and microencapsulated *S. boulardii* cells.

Results of viable counting after the leavening period are reported in table 4.

Table 4. Viable yeast count of doughs, after fermentation period (about 16 h), obtained by leavening with different starter cultures.

Starter culture and form	**Viable count (CFU/g)***
Saccharomyces cerevisiae free cells	$2.5x10^{7\ A}$
Saccharomyces boulardii free cells	$1.2x10^{7\ A}$
Saccharomyces boulardii microencapsulated cells	$8.3x10^{6\ B}$ (whole capsules) $8.0x10^{6\ B}$ (disrupted capsules)

*Different letter means significant difference as determined by t-Test ($P<0.05$).

Interestingly, results showed no significant difference ($P<0.05$) between viable microencapsulated yeast count before and after disgregation of microcapsules, respectively. This result is not in agreement with previous results in which the viable count after disgregation gave a number 20-30 times higher of that obtained with whole microcapsules. Our result could be due to a spontaneous disruption of microcapsules during the viable counting process in which citrate is not used. This is because the presence of yeast cells instead of bacterial cells. On the other hand, accordingly, preliminary results showed no difference in viable yeast counting between whole and disrupted microcapsules just after their preparation.

Dough was used for “khubz” baking and some pictures of preparation are reported in figure 25. Results showed that yeast in free form did not survive to the baking process and that just 1-2 log cycles were found in bread prepared with the microencapsulated yeast. Experiments were performed 6 times and each time no live cells were found in bread produced with free yeast cells, while in the bread obtained with the starter culture in microencapsulated form 3 times on 6 we found live cells with the following counting results: 30 CFU/g, 70 CFU/g and 110 CFU/g. High variability of this result means that is difficult to control and standardize all the parameters considered and that probably some variables are missing. Because the baking was performed at 250°C per 5 minutes, reasonably the temperature in the core of the product did not overcome 120-130°C and the time of temperature decrease at 45-50°C is about 1 h at room condition (temperature of 25°C and no ventilation). To reduce the effect of heat stress

we reduced at 10 min the temperature decreasing time by the rapid refrigeration of the product just after the baking. Despite this shrewdness no change of previous results was registered. Results obtained unfortunately confirm the “mission impossible”, because the residual *S. boulardii* population is not enough to make probiotic the final product. Indeed, it is expected a live population of at least 1 million per gram of bread to consider it like a probiotic food.

3.4 Probiotication of labneh

Results of labaneh probiotication are given in terms of survival of yeast cells in the product during its shelf life. As reported above, labaneh is a dairy product appreciated in the Arabic countries and rarely it is produced and consumed in the western countries. In the figure 26 is depicted the draining step of commercial yoghurt to obtain labaneh. After 24 h of draining the product was collected and immediately used for probiotication experiment. In figure 27 is represented the graph of yeast cell survival during the shelf life of the product (20 days at 4°C). It is very clear that encapsulated cells have more chances to survive at the harsh condition of low pH and low water activity.

Figure 25. Steps of "khobz" preparation at laboratory scale. From left to right, line by line from higher to lower: 1. dough after leavening; 2. moulding; 3. start of baking; 4. during the baking; 5 - 6. final product.

Figure 26. Draining of commercial yoghurt for labaneh production.

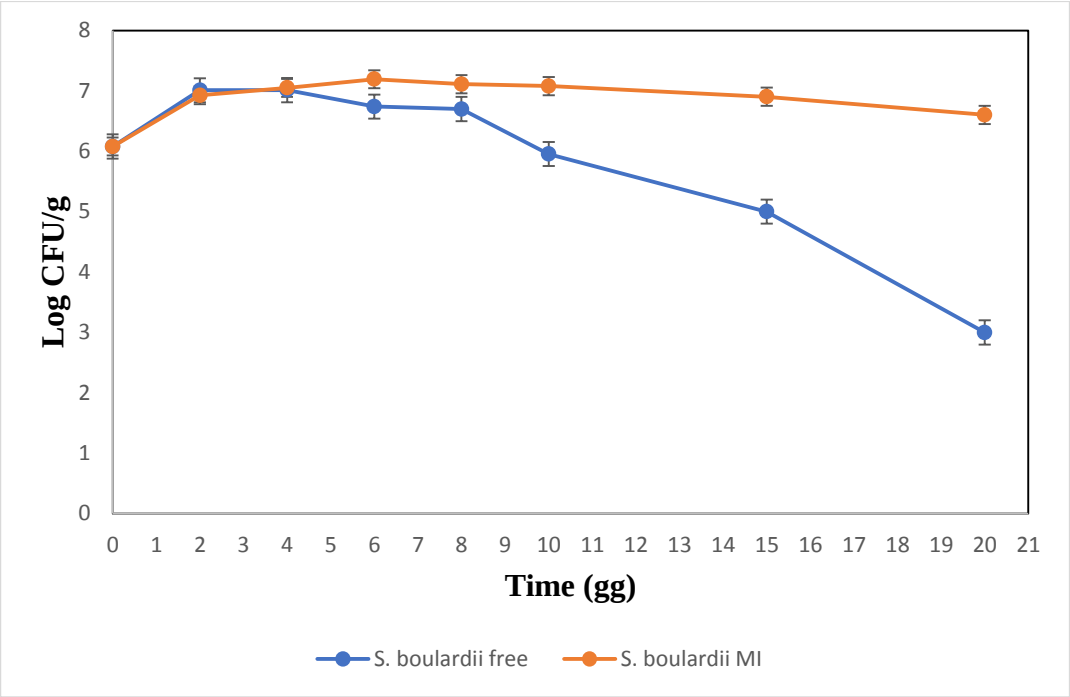


Figure 27. Curve of yeast survival during the shelf life of labneh.

4. Conclusions

In conclusion, we can say that water in oil emulsion is a suitable method for yeast cells microencapsulation and it can be used like it has been extensively used in the past for microencapsulation of probiotic bacteria. Microcapsules showed an average diameter of 15 μ that is a good diameter to reduce the gritty taste effect of bigger microcapsules produced by using other techniques like for example the extrusion. Our results showed that microcapsules contained about 50-60 yeast cells each one. Moreover, our results showed that *S. boulardii* shows the same fermentation performance of *S. cerevisiae* and that microencapsulated *S. boulardii* cells show a slight reduction of fermentation performance compared to free ones. Finally, we can say that to make probiotic the «khubz» is a big challenge and even though some bread showed live yeast cells it is not enough to make them probiotics. On the other hand, microencapsulated *S. boulardii* cells better survive compared to free ones during “labaneh” shelf life.

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المهمه المستحيله: عمل وتحضير بروبيوتيك خبز عربي

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الملخص

تهدف هذه الدراسة الى اعداد وتحضير خبز مدعم بنوع خاص من الخميره تم حوصلتها ب الميكروانكابسولاشن باستخدام صوديوم ألجينات وهذه الخميره هي سكارومايسس بولاردي حيت تم تجربه اكثر من بروتوكول لاعداد الميكروكابسول وجميعها مذكوره في الكتاب لكن اعتمدنا على بروتوكول Sheu and Marshall (1993) and modified by Truelstrup Hansen et al. 2002) وقمنا بتحضير ميكروكابسول بحجم 15 الى 20 ميكروميتر وتم حوصله داخل كل ميكروكابسول 50الى 60 خلية خميره وبناءآ على النتائج فإن اعداد وتحضير الخبز مدعم بالميكروكابسول الحصور بداخلها خلايا الخميره، وبعد اجراء زراعه الخميرة للخبز لم تظهر نتائج يمكن حسبانها ك خبز بروبيوتك ولكن بالمقابل قمنا بعمل بروبيوتيك لبنه بنفس الخميرة المحوصلة، حيث اظهرت النتائج مده صلاحية اعلى للبنه المدعمة بالخميرة المحوصلة.