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Dried Probiotics for Use in Functional Food Applications

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1. Introduction

The increasing consumer demand for foods with health benefits has led the food industry to diversify its products. Most of the foods containing probiotic bacteria are dairy products, although there is a rapidly growing demand for incorporating probiotics in other segments of the food industry.

According to Food and Agriculture Organization (FAO, 2002), probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host. In order to produce such health benefits, it is essential that live probiotic bacteria survive after passing through the gastrointestinal tract and reach their site of action intact. Foods containing probiotic microorganisms are expected to have a promising future, but will require the development of new technologies, specifically developed to enable their use in innovative products in a wide range of industries. The challenge for the industry is to produce large enough amounts of viable and stable probiotic cultures for use as inoculums or to be introduced directly into a dried food.

Although specific numbers are not mentioned at the FAO definition, some studies demonstrated that high levels of viable microorganisms are recommended in probiotic foods for efficacy (Meng et al., 2007). However, maintaining such counts throughout preparation, processing and storage requires constant study by the scientific and industrial community.

Drying process are often used as a means to stabilize probiotics and facilitate storage, handling, transportation and subsequent use in functional foods (Santivarangka et al., 2008). Drying by spray-drying and freeze-drying are the most commonly used techniques for dehydrating probiotic cultures and lactic acid bacteria starter cultures (Betoret et al., 2003), although other methods such as vacuum oven drying, fluid bed drying or combinations of these may also be used.

Drying processes are a major cause of loss of viability of probiotics, and in the specific case of freeze-drying, the freezing step causes additional stress on the bacterial cells, making the

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lipid fraction of the cell membrane more susceptible to damage during the process. Although the exact mechanisms of cell inactivation during drying processes are not yet fully elucidated, it is known that bacterial cells consist of 70 to 95% water, and its removal poses serious physiological obstacles to the survival of cells (Santivarangka et al., 2008).

Survival of microorganisms during drying processes and subsequent storage, depends on factors, such as species and strain, drying conditions, inoculums and culture media, the use of cultures pre-adapted to the stress inherent to the processing conditions and the use of cryoprotective agents (Champagne et al., 2005). Examples of such agents include trehalose, sucrose, glycerol, skim milk powder, whey, betaine, gum acacia (Meng et al., 2007) and exopolysaccharides, the latter of which are produced by the cells themselves as a protective mechanism in response to stress situations (Ruas Madiedo et al., 2002). The use of fibers and prebiotics as multi-functional protective agents and vehicles for probiotics / symbiotics has gained wide interest over the past few years (Saarela et al., 2006).

This chapter describes some methods to increase the viability of probiotics, along with new uses and applications in the functional food industry. Dehydration methods, such as freeze-drying, spray-drying and the use of cell protective additives will be introduced. Finally, the use of probiotics in ice cream after drying by freeze-drying and the use of probiotics in dark chocolate after drying by spray-drying will be discussed.

1.1 Drying processes

There is currently a real interest on the part of the food processing, chemical and pharmaceutical industries and in agriculture in the use of products containing sensitive compounds or living cells, due to beneficial functional properties and effects on the environment and human health (Ivanova et al., 2005). These products are used in dehydrated form to increase stability, and may be either encapsulated or attached to a support for this purpose (Anal & Singh, 2007). They can be developed for therapeutic use by oral administration in capsules/tablets, or as direct starter cultures, as functional supplements or as biocontrol agents. Commercial dried probiotic cultures obtained by processes the cost-benefits of which are interesting may help to expand the market and enhance the use of these microorganisms in several other applications (Fu & Chen, 2011).

Drying is an ancient method of food preservation and the term is generally used to refer to the withdrawal of moisture from a substance. It is used in the drying of particulate solids, pastas, solutions and provides the greatest diversity among the unit operations used in foods (Ratti, 2001). Conventional hot air drying allows to extend the shelf life for long periods of time, however, the quality of the products obtained is drastically reduced when compared to traditional drying. Drying by convection may, however, offer an efficient alternative in terms of costs and transport, although studies on the intrinsic factors and optimization of extrinsic processes should be carried out to minimize losses that may occur during the hot drying process (Fu & Chen, 2011; King & Su, 1994).

During thermal processing, stress caused by heat and by dehydration may be significant to the cells. Both forms of stress at lethal levels cause permanent loss of viability. Excessive heat causes denaturation of the structure of macromolecules or breaks the bonds between monomeric units. Stress caused by dehydration affects principally the cytoplasmic membrane by changing fluidity or the physical state of the membrane, in addition to causing lipid peroxidation. Microorganisms subjected to dehydration by heat would undergo simultaneously the two types of stress in a synergistic manner (Fu & Chen, 2011).

Culturing conditions and the cellular growth phase have great influence on viability during drying, with the cells in the stationary phase being more resistant than in any of the other stages. For that reason, this is the stage of development that has been used in different drying processes, such as: spray drying; fluid bed drying; vacuum drying; as well as freeze drying (Fu & Chen, 2011).

When the water is removed at a fast rate, the microorganism has not the time it needs to adapt itself through genetic expression or by adjusting its metabolism. An osmotic pressure response can be induced by stress conditions and leads to accumulation of compatible solutes, as well as to a cross-response with the effects of the other stress conditions. Pre-adaptation to heat induces the heat-shock response of proteins and enzymes, such as chaperones and proteases. The first help to stabilize the RNA and repair denatured proteins, while the proteases degrade denatured proteins, with both reactions leading to heat stress resistance (Fu & Chen, 2011).

The industrial use of a certain and specific culture sometimes does not allow to replace this same culture by another and, for that reason, pre-adaptation offers the opportunity to increase survival against adverse conditions (Fu & Chen, 2011).

Freeze-drying, also known as lyophilization or cryo-desiccation, was initially used as a commercial technique in the 1930's for the preservation of blood plasma and instant coffee, however, from that time onward, it has been being used for the preservation of other foods and products (Food Today, 2009). It is the best method for removing water and generally the water content falls to levels lower than 4% (Muller et al., 2009).

For the lyophilization, the food or suspension medium was quickly frozen, resulting in the formation of small ice crystals, while at the same time the non-frozen phase becomes concentrated. Large crystals formed during slow freezing might damage the structure of the product or penetrate the cell walls (Stapley, 2009; Food Today, 2009). A number of factors affects the efficacy of cryopreservation of microorganisms, but one of the most important conditions is the composition of the suspension medium for freezing (Hubalek, 2003). The solid state of the water during lyophilization protects the primary structure and shape of the product with minimal volume reduction. In spite of the numerous microbiological advantages and the high quality of the products as compared to other methods, lyophilization is always considered a slow and the most expensive dehydration process (Ratti 2001; Peighambardoust et al., 2011), in addition to also being seen as less favorable for the production of larges masses of dry cells (Fu & Chen, 2011).

In lyophilization, the frozen material is exposed to a head space with partial vapor pressure a slightly below the partial equilibrium vapor pressure of the ice at the temperature of the material. This is achieved by placing close to the food a condensation unit that causes the sublimation of the ice crystals (primary sublimation) and also desorption of non-crystalline water present in the food matrix (secondary drying). The condensation surface temperature should be significantly lower than that of the drying material (Stapley, 2009). In conventional lyophilization, the vacuum in the lyophilization chamber is maintained by a vacuum pump, the function of which is to remove non-condensable gases, since these reduce the transfer rate of the water vapor from the food to the condenser. For being endothermic, in sublimation heat must be applied to the food to maintain and accelerate the process. (Stapley, 2009; Food Today, 2009), without thawing the food and maintaining its rigidity. However, the temperature of the food should always be kept below the

temperature of collapse, since the latter is related to the glass transition temperature (T_g). Several researchers have verified that the temperature of collapse lies 2-3K above T_g or between the values of T'_g and the phase transition temperature of the cytoplasmic membrane (T'_m), though it varies as a function of moisture content (Stapley, 2009). The collapsing phenomenon occurs when the solid food matrix can no longer maintain it own weight, leading to a drastic structural change which is marked by a decrease in volume, an increase in stickiness of dry powders, loss of porosity, etc (Chuy & Labuza, 1994, Ratti, 2001).

A comparison between the reduction in volume during lyophilization and conventional drying shows that in the first case this reduction is minimal (5 to 15%), whereas it is excessive in the case of air drying (close to 80%), and that this behavior is observed in several foods. In the case of hot air the reduction is accompanied by wrinkling, deformation or color changes indicating a collapse of the food dehydrated by this method. Also according to data shown by Ratti (2001), in conventional drying the temperature of the product remains above T_g throughout the entire process, reason why collapse and inferior quality of the final product are expected.

With regard to costs, lyophilization is generally seen as an expensive method of preservation (Peighambardoust et al., 2011), which depends on the type of material, the products, the packaging, the capacity of the processing plant, duration of the cycle, etc. The total cost is about 4 to 8 times greater than that of hot air drying. However, studies indicate that, when an analysis is made of the total energy involved in the different processes, the inclusion of the expenses with home storage, lyophilization is advantageous. Furthermore, comparing the several steps involved, the added value and improvement in the quality of raw materials and the differentiated products, such as is the case of probiotics, lyophilization should not be considered prohibitive in terms of cost (Ratti, 2001). Lyophilization involves four main steps: freezing; vacuum; sublimation and condensation, with each contributing to the total energy consumption. Any development in the classical process to reduce energy costs should focus on improving heat transfer in the sublimation phase, reduce time and consequently expenses with vacuum and avoid the use of condensers. Technological developments such as microwave heating, desorption freeze-drying using desiccants, atmospheric fluidized bed freeze-drying, atmospheric spray-freeze drying have not yet been totally incorporated by the industries due to a series of technical problems (Ratti, 2001).

Probiotic lyophilized or atomized yogurts and fermented products have been described by various authors and, in these cases, the viability and activity of the bacteria are important to obtain the beneficial effects, in addition to maintaining the original authentic qualities of the products (Kumar & Mishra, 2004; Peighambardoust et al., 2011). Different approaches can be used for evaluating the survival or activity of the dehydrated cells. Among these, one can cite the determination of cell viability in itself, the determination of the metabolic capacity and the determination of the ability of the microorganisms to tolerate stressing environmental conditions, such as acidity and bile (Fu & Chen, 2011). Lyophilization and drying by atomization are among the most used processes to maintain these properties. Since lyophilization utilizes milder conditions, the survival rates are higher than those obtained with atomization (Wang et al., 2004), with the specific exception of the damage caused by freezing. The physical mechanism of dehydration and cryoinjuries caused by freeze-drying are different from what occurs in heat-drying, as well as the damage on the

cell structure (Fu & Chen, 2011). In the case of *L. paracasei*, for example, lower temperatures applied during freezing stage of the lyophilization process lead to lower survival rates probably due to stress during freezing (Bauer et al., 2011). Protection of the cells during the different steps is of fundamental importance (Semyonov et al., 2010) and the effect of protectants should be studied on a case-by-case basis as a function of the lineages used (Carvalho et al., 2004). However, atomization for the commercial production of cultures allows to reduce costs (Peighambardous et al., 2011; King & Su, 1994) and increase the yield. (Zamora et al., 2006). In spite of being economic and effective, spray-drying inflicts high a mortality rate the cells as a result of the simultaneous action of heat, oxygen and dehydration stress (Anal & Singh, 2007; Fu & Chen, 2011).

Atomization (spray-drying) is considered a long-term preservation method for lactic bacteria and dates from 1914 when it was described in a study by Rogers on dehydrated lactic cultures. The concept of spray-drying was patented by Samuel Percy in 1872 and industrial use with milk started in 1920. The drying rate and continuous production capacity are useful for large-scale production. In spite of this, it is commercially less employed due to the low survival rates during drying, low stability during storage and difficulty to rehydrate the product (Peighambardoust et al., 2011). Spray-drying, a convective drying process (Fu & Chen, 2011) is a unique process in that the particles are formed at the same time they are being dehydrated. The final product should be compatible with the quality standards for particle size and particle distribution, residual moisture content, density and shape of the particles. During the process, dry granulated particles are obtained from a paste. By atomization the moist product in the shape of small droplets is passed at high speed through a flow of hot air between 150 a 200^0C. The atomized droplets have a large surface area, which results in a very short drying time when exposed to hot air in the drying chamber. There are several types of atomizers and a classification would probably be based on the kind of energy involved: centrifugal, kinetic, pressure or sound energy (Vega-Mercado et al., 2001). Selecting the configuration of the atomizer depends however, on the nature and viscosity of the feeding flow, as well as on the characteristics of the dehydrated product. In the period of constant drying rate, the temperature of the product and the thermal inactivation are limited to the temperature of the wet bulb and if the evaporation rate is high, the cells are protected against the high temperatures of the air in the chamber (Fu & Chen, 2011 ; Peighambardoust et al., 2011). The falling rate period of the particle is important and the optimal residence time is the time necessary for complete removal of moisture with a minimum increase in the temperature of the dry product (Peighambardoust et al., 2011).

The preparation of dry starter cultures is a long process which involves various steps from culturing up to storage in powdered form (Peighambardoust et al., 2011). During drying by atomization, bacteria may suffer damage to their cytoplasmatic membrane due to the high temperatures and survivors with sub-lethal injuries become more sensitive to the storage conditions, losing viability during storage time (Corcoran et al., 2004). Several investigators have reported that increased outlet temperatures reduce the survival of the microorganisms upon completion of the spray-drying process, with this being one of the most important parameters affecting the viability of the starter cultures (Peighambardoust et al., 2011). Other pre-drying processes, such as emulsification, homogenization and pressure applied prior to atomization may cause injury or inviability by 2 to 3 log cycles. For that reason, it is

suggested that probiotic bacterial or yeast cultures be added only after these processes are completed. In addition to mechanical stress, the high atomization pressures lead to the formation of smaller particles which are subject to inactivation caused by dehydration and heat, thereby emphasizing the importance of selecting the best atomizer (Fu & Chen, 2011).

Another alternative drying method is vacuum drying, which is practiced at low temperatures. This method is applied using temperature ranges between 30 and 80°C and may cause great cell losses due to heat damage. However, by working slightly above the triple point of water, temperatures close to 0°C would be obtained. This process is called Controlled Low-Temperature Vacuum Dehydration (CLTV), and is interesting for sensitive food ingredients and can be used for drying microorganisms such as *L. acidophilus* (King & Su, 1994). Compared to spray-drying, other processes using lower drying temperatures allow for greater and better survival of microorganisms (Fu & Chen, 2011).

Survival rates are species-specific, depend on the drying method used and experiments are essential to attain maximum survival under a given drying condition (Fu & Chen, 2011; Otero et al., 2007). The different behavior of the cells during dehydration may be explained by differences in the cell membrane (Bauer et al., 2011). Indeed, Bauer et al (2011) have demonstrated differences in behavior between probiotics by comparing controlled low-temperature vacuum dehydration (LTVD) and lyophilization (Freeze-drying - FD). For the survival of *Bifidobacterium lactis Bb-12*, FD would be the method of choice, for *L. paracasei* there wasn't any difference between the two methods and for starter culture *L.delbruekii ssp bulgaricus* manufactured using the LTVD, the culture exhibited higher survival rates.

The loss of viability during storage increases with temperature between 4 and 37°C (Sunny-Roberts & Knorr, 2009). The mechanism behind the loss of cell viability during the storage period depends on a series of factors, including temperature, moisture content, protectors and oxidative stress. Since there is generally no heat stress during storage, the mechanism of death must be different from that which occurs in thermal processes (Fu & Chen, 2011).

1.2 Cell protectors

Initiation bacterial cultures (starters) are of great importance for the industry for the production of fermented foods, with drying being the most used method to prepare and store these microorganisms. However, this practice may cause injuries that cause a drop in the number of viable cells and consequent losses in industrial production. In this way, a well-succeeded drying process depends on the capacity to maintain a sufficient number of cells that contains and preserves the same characteristics of the original culture (Santivarangkna et al., 2008).

A method commonly employed to increase the survival of bacterial cultures is the addition of protective agents. These agents have been previously added to drying media to protect the cells against adverse processing and storage conditions. Among these protectors may be cited skim milk powder, whey, trehalose, glycerol, betaine, saccharose, glucose, and lactose, in addition to some polysaccharides (Meng et al., 2007).

Several hypotheses have been proposed to explain the effect of these protectors on the process of cell dehydration. Among these, the presence of compatible solutes, such as sugars, quaternary amines and some amino acids, may equip the cells against hyperosmotic stress, which occurs during the drying process, bearing a direct relationship between the accumulation of these solutes and survival under adverse conditions (Kets et al., 1996). According to Santivarankna et al. (2008), it is believed that the protective effect of these

solutes occurs due to their preferential exclusion, which is the main mechanism of protection of macromolecules against moderate loss of water. Still according to the same authors, the preferential exclusion occurs where a macromolecule has greater affinity with water than with the solute, with the consequence of the latter becoming in excess on the surface of the macromolecule.

Cell growth is the first step in the preparation of dehydrated starter cultures, but its viability during drying and the subsequent storage period may be affected by the growth conditions and growth medium (Santivarankna et al., 2008). It has been suggested that adverse conditions such as heating or the application of osmotic stress during the microbial growth phase may leave the lactic acid bacteria more tolerant to dehydration processes. In the study published by Tymczyszyn et al. (2007), *Lactobacillus bulgaricus* were pre-adapted in a low-water activity medium, containing lactose and polyethylene glycol during the cellular growth phase and it was observed that both conditions at different concentrations brought about an increase in the survival of the cells subjected later on to the vacuum drying process.

In this aspect, the presence of carbohydrates in the growth medium has been shown to be of great importance. Panoff et al. (2000) observed that the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the presence of sugars such as lactose, saccharose, trehalose and cryoprotectants, such as glycerol, causes the cells to adapt themselves to freezing and thawing in the lyophilization process with a consequent increase in cell survival. In the study by Carvalho et al. (2004), supplementation of the growth medium of *L. bulgaricus* with glucose, fructose, lactose and mannose, resulted in increase in the level of protection conferred by the agents used in the drying medium as compared to non-supplementation. Furthermore, the data suggest that cell growth in the presence of several sugars produces cells with different morphological and physiological characters, which represent distinct levels of resistance to the various stress treatments investigated. The osmotic stress caused by the solutes in this growth stage may favor the assimilation of the protectants inside the cells, which in its turn leads to a reduction of the osmotic effects that dehydration causes (Kets et al., 1997), since the protectant accumulated within the cell reduces the osmotic difference between the internal and external environment (Capela et al., 2006).

On the other hand, several lactic acid bacteria (LAB) produce exopolysaccharides (EPS), which may remain either attached to the cell wall (capsular) or be excreted in free form (mucus). The majority of the studies involving the production of EPS by the LAB bacteria focus on their use in the food industry as viscosity agents, stabilizers, emulsifiers or gelling agents (De Vuyest &Degeest, 1999), although some authors have suggested the importance of their role in the mechanism to protect the cells against dehydration processes (Ruas Madiedo et al., 2002). It has been observed that the production of this metabolite requires the utilization of substrates and energy, and since this polysaccharide is not considered essential to the cell, it is believed that its production is connected to an important biological function, such as for example, obtaining a selective advantage in the environment (Looijesteijn et al., 2001). In this manner, the presence of certain carbohydrates such as gum acacia, may favor their production and consequently increase bacterial survival in subsequently adverse conditions (Schiavão-Souza et al., 2007).

Protective agents in the drying medium may be available both in the form of sugars that are not utilized in the growth medium when there is no washing stage, or from the build-up of compatible solutes. However, in most cases they are additionally mixed or used as drying medium to increase the viability of bacterial cultures (Santivarangkna et al., 2008).

The damage caused in biological systems by drying processes should be attributed to: denaturation of critical main components, such as DNA and RNA (Linders et al., 1997); change in the physical state of the lipid membrane; and changes in the structure of more sensitive proteins. The removal of water from region of the polar chains of the phospholipids, causes the closure of these chains, forcing the encounter of acyl groups, favoring the formation of Van der Waals interactions. As a result, the lipid may pass from the crystalline liquid to the gel phase and with rehydration, this change occurs again. For that reason, in the process of the membrane going through these phase changes, there are regions that become defective, turning the membrane leaky (Leslie et al., 1995).

With the addition of protectants, such as the disaccharide trehalose before drying, a reduction in the membrane transition temperature is observed. This occurs due to the replacement of water between the front end of the lipid groups, which prevents the change of phase and consequent leakage during rehydration. In addition, these sugars have the capacity to form a vitreous state, in which they present high viscosity and low mobility, leading to an increase in the stability of the material to be preserved (Crowe et al., 1996). In lyophilization processes, the presence of cryoprotectors may raise the vitreous transition temperature and this way, the viable cells become able to reach this state of stability without nucleation of intracellular ice, which might cause damage inside the cell by the crystals formed (Meng et al., 2007).

The damage inflicted on dehydrated probiotic cultures during drying may be aggravated during their storage, which would make them undesirable or unfit for further industrial use. Teixeira et al. (1996) observed that after storage of dried cells of *L. bulgaricus*, the unsaturated fatty acids/saturated acids ratio of the lipid membrane diminishes considerably, thereby evidencing the damage caused by lipid oxidation. This oxidation may result in some physical changes in the functions and structure of the membrane. The increase in the proportion between saturated fatty acids brings about an increase in the transition temperature and consequently leads to a reduction in the fluidity of the membrane up to a certain temperature and an increase in leakage through the membrane during rehydration. In addition to this, it is known that the consequence of a great number of biological oxidations results in the formation of free radicals and that these may reduce the hydrophobicity of fatty acids due to the introduction of hydrophilic groups, and thus, weaken the hydrophobic interaction with the proteins of the membrane, which may be essential to their activity (Santivarangkna et al., 2008).

Nonetheless, the use of protectants in drying medium may reverse this pattern. Some sugars like sorbitol, maltose and mannitol are also effective protective agents capable of providing cultures with protection against oxidation damage inflicted over long periods of storage. Sunny-Roberts & Knorr (2009) observed an increase in the viability of *Lactobacillus rhamnosus L*GG stored at room temperature in dried form in the presence of trehalose supplemented with monosodium glutamate (MSG), indicating a possible antioxidant action attributed to a combination of compounds. The possible mechanisms suggested behind this antioxidant protection provided by sugars are reported for being free of free radicals and metal quelants, and having capacity to form complexes of hydrogen peroxide and restrict the viscous flow of oxygen diffusion (Santivarangkna et al., 2008).

Additionally, it is suggested that temperature is one of the more critical parameters for microbial survival during storage (Gardiner et al., 2000), and that this effect may be directly related with the speed or rate of the biochemical and physiological reactions that occur

inside the microorganisms. Dehydrated probiotics are generally stable when stored at temperatures of 4ºC. However, the stability of dried probiotics kept at non-refrigeration temperatures is strongly recommended when these are used to supplement products such as milk powder, cereals, confectionery and chocolate, in addition to significantly reducing the costs with transportation and storage (Foerst et al., 2011).

Studies have shown that the use of sugars during drying and in the course of subsequent storage may result later in higher survival at higher storage temperatures. Foerst et al. (2011) evaluated the storage at different temperatures of *L. paracasei* F19 vacuum-dried in the presence and absence of protectants and observed that when drying was done in the presence of sorbitol, the microorganism may be stored at room temperature without any significant loss of its viability. Ananta et al. (2005) obtained similar data and information on the survival for *Lactobacillus rhamnosus* LGG atomized with reconstituted skim milk (RSM).

Trehalose (α-D- glucopyranosyl, α-D- glucopyranoside) is a non-reducing disaccharide synthesized by procaryotic and eucaryotic organisms, such as yeasts, bacteria, molds, algae, plants and invertebrate insects (Conrad et al., 2000). Several studies have demonstrated that trehalose is one of the best protein stabilizers as compared to other compatible solutes (Crowe et al., 1996) under the identical conditions of drying and storage, because of its uncommon capacity to stabilize proteins in their native conformation by changing the water environment surrounding the peptide (Magazú et al., 2005). Trehalose may interact directly with the polar chains of the phospholipids of the membrane via a hydrogen bridge between the hydroxyl groups of the sugar and the phosphate groups of the lipids, stabilizing them under adverse conditions (Welsh, 2000). Zayed & Ross (2004) reported a 10% increase in the viability of *L. salivarius* when lyophilization of these microorganisms was performed in the presence of 15% trehalose.

It was observed that the production of trehalose by organisms that synthesize it is directly linked to the cell protection mechanism under adverse conditions. Li et al. (2009), observed that under stressing conditions such as heating, the genes responsible for the codification of its biosynthesis are activated, promoting their formation and release into the environment, consequently providing protection to the cells. The study by Mamose et al. (2010) supports the hypothesis that the presence of trehalose before freezing, in addition to reducing the damage caused by this procedure also favors the formation of several processes for the recovery from post-freezing damage.

In addition to the mono-and disaccharides, polysaccharides have been shown as promising for use as cell protectants. In spite of the size of their molecules, they can be inserted between the phospholipid chains, changing the transition temperatures of the membranes. This polysaccharide–phosphoplipid interaction depends on the flexibility of its structure. In polysaccharides with rigid structure, the interaction is dependent upon size, whereas for those with a flexible structure, the negative steric effects of increasing degrees of polymerization is counterbalanced by the elasticity of the structure (Santivarangkna et al., 2008). The use of gum acacia in the drying medium of probiotic cultures, has been proven promising. Desmond et al. (2002) obtained a survival rate 10 times greater when the microorganism *L. paracasei* NFBC 338 was dried by spray-drying in the presence of 10% (g/v) gum acacia as compared to drying without the polysaccharide. In the study by Lian et al. (2002), four lineages of bifidobacteria were successfully subjected to spray-drying with gum acacia.

Reconstituted skim milk (RSM) is another carrier matrix utilized in the production of dehydrated probiotics. In studies investigating the protective effect of different food systems in the survival of cells subjected to drying, the results obtained with RSM stood out in comparison to those yielded by other compound such as gelatin and starches (Lian et al., 2002). Fu & Chen (2011) suggest that their effective protection may be related to the presence of lactose in the composition of RSM, since this is the disaccharide that interacts with the cell membrane and helps to maintain its integrity in a similar way as non-reducing disaccharides do with trehalose and saccharose. However, this mechanism has not yet been totally elucidated as yet. In another way RSM might create a porous structure in the lyophilized product, which would make its rehydration easilier (Carvalho et al., 2004).
Nonetheless, researchers are satisfactorily using formulations containing RSM and other compounds to increase the survival of probiotics subjected to drying and subsequent storage for later use. Corcoran et al. (2004) evaluated the influence of RSM combined with polydextrose on the lactobacillus viability during spray-drying and observed that these combinations produced an increase in the survival of these cells during storage. Likewise, Ananta et al. (2005) used reconstituted skim milk at a concentration of 20% (w/w) for spray-drying *Lactobacillus rhamnosus* GG, and found that the incorporation of raftilose and polydextrose to this medium, resulted in an increase in the survival of the probiotic after drying.
For that reason, the use of compounds that may protect bacterial cells against drying processes constitutes an important strategy for the industry, which desires to obtain probiotics with high counts to be added to several types of foods, in a way so as to provide and guarantee the benefits this type of product.

2. Perspective of trehalose and gum acacia on the survival of *Lactobacillus casei* subsp. *paracasei* LC-1 culture upon lyophilization and application in ice-cream

2.1 Use the trehalose and gum acacia on the survival of *Lactobacillus casei* subsp. *paracasei* LC-1

Lyophilization has been widely used to produce probiotics in powder form. Lyophilization is a process based on the sublimation of water, in which bacterial cells are first frozen and then dried under high vacuum (Leslie et al., 1995). Since water plays an important role in stabilizing the structure and maintaining the functional integrity of cellular macromolecules, its removal causes damage to the surface of cell walls and membranes, thereby compromising the viability of the bacterial cells (Ruas Madiedo et al., 2002).
For most probiotic bacterial cultures of commercial interest to the dairy industry, skim milk powder is frequently selected as the drying medium because it prevents cellular injury by stabilizing the cell membrane constituents, in addition to creating a porous structure in the freeze-dried product that makes rehydration easier (Carvalho et al., 2004).
The addition of protective compounds such as trehalose, sucrose, betaine and gum acacia is another option for maintaining the viability of microorganisms during freezing, drying and storage (Carvalho et al., 2004). Leslie et al (1995) showed that the survival of freeze-dried *Escherichia coli* DH5a and *Bacillus thuringiensis* HD-1 was respectively 60% and 45% higher in the presence of trehalose compared with its absence. Guergoletto et al (2010) observed a positive and significant effect of trehalose on the survival of *Lactobacillus casei* subsp. *paracasei* LC-1 subjected to vacuum drying. However, in these studies the effect of these compounds was evaluated separately.

Our group evaluated the effect of trehalose and acacia gum on *Lactobacillus casei* subsp. *paracasei* LC-1 culture upon completion of lyophilization. Table 1 shows the different concentration of trehalose and gum acacia, their coded values and the results of cellular viability calculated from the number of colonies on MRS Agar plates, before and after lyophilization using skim milk and whey media. The highest survival rates were obtained with the media containing 5% trehalose and 4.5% gum acacia, which statistically corresponds to the center point. The response surface plot (Figure. 1) shows the experimental data distributed in the intervals studied and clearly shows that the highest survival rates of LC-1 are located in the region around 5% trehalose and 4.5% acacia gum.
Conrad et al. (2000), reported a survival rate of 90% of *L. acidophillus* cells after lyophilization in a drying medium containing 5% (w/v) trehalose. Desmond et al (2002) observed that the use of 10% (w/v) gum acacia in the medium used for drying *L. paracasei* in a spray-dryer increased survival of the probiotic by 10 times compared to the medium without gum acacia.
Schiavão-Souza et al. (2007) evaluated the use of 4.5% and 5.5% gum acacia in the media of skimmed milk powder to optimize EPS production by *Lactobacillus casei* and observed that the addition of 4.5% gum acacia, favored the production of exopolysaccharides (EPS). According to Ruas-Madiedo et al. (2002) the production of these compounds by lactic bacteria may be considered as a protection mechanism against stress situations, such as dehydration processes, which may explain the higher survival rates obtained in our study.

2.2 The use of *L. casei* (LC-1) protected with trehalose and acacia gum in ice cream processes

Ice cream is an appropriate food matrix for the addition of probiotic and prebiotic ingredients. Some studies have demonstrated that it is possible to produce ice cream of high sensory quality with added probiotic microorganisms (Shah, 2000). Among other innovative products as a functional food, ice cream has a promising market and a huge growth potential. However, the addition of the probiotic microorganism should not change the sensory attributes of the product.

Trehalose (x1)	Trehalose (% w/v)	Gum acacia (x2)	Gum acacia (%w/v)	Cellular viability before lyophilization (log cfu)	Cellular viability after lyophilization (log cfu)
-1.0	2.5	-1.0	3.5	10.3	10.2
-1.0	2.5	1.0	5.5	10.5	8.4
1.0	7.5	-1.0	3.5	10.2	8.0
1.0	7.5	1.0	5.5	10.6	9.7
0.0	5.0	0.0	4.5	10.8	11.0
0.0	5.0	0.0	4.5	10.6	10.9
-1.4	1.5	0.0	4.5	10.3	10.0
1.4	8.5	0.0	4.5	10.5	9.6
0.0	5.0	-1.4	3.1	10.7	9.9
0.0	5.0	1.4	5.9	10.4	9.5

Table 1. Variables trehalose and gum acacia: actual (% w/v) and coded values (x1 and x2) and the survival of *Lactobacillus casei* subsp. *paracasei* (LC-1- values in log cfu.g^{-1}) in culture media containing milk solids, before and after lyophilization

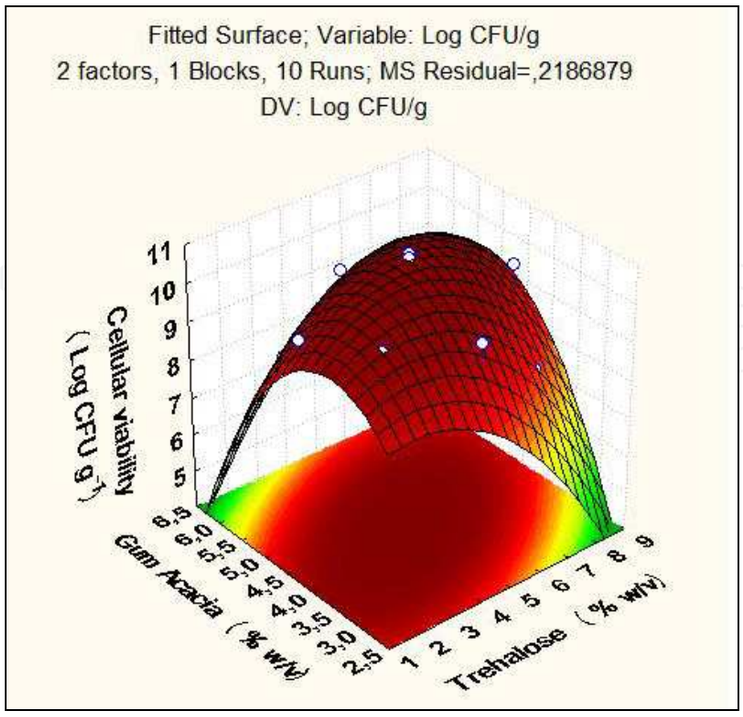

Fig. 1. Response surface plot obtained after lyophilization of *Lactobacillus casei* subsp. *paracasei* LC-1 culture suspended in a skim milk and whey medium, for the percentage of variables trehalose and gum acacia, with the viability of LC-1 expressed in log cfu.g^{-1} as response.

Our group evaluated the viability of Lactobacillus casei (LC-1) in the free form and/or protected with trehalose and gum acacia in ice cream throughout 98 days storage at -20°C, as well as the acceptability from a sensory viewpoint. Figure 2 shows the results of the free and protected L. casei populations over a total 98-day storage period -20ºC. At the beginning of storage, populations of 9.20 and 9.80 log CFU.g-1 were observed, which after 98 days of storage had been reduced to 6.90 and 8.17 log CFU.g-1 for free L. casei and protected L. casei, respectively. There was a significant difference ($p<0.01$) in the L.casei populations of the two treatments tested at the end of the storage period investigated. Hekmat & McMahon (1992) verified a decline by 2 logarithmic log cycles in the L. acidophilus counts in ice creams after 17 weeks storage at -29°C. Similar results were observed in our experiments with free L. casei.

The reduction in the population of probiotic microorganisms in ice creams during shelf life was observed by Homayouni et al., (2008); Vasiljevic & Shah (2008). The drop in the bacterial population of may be the result of freezing, causing cell injuries and eventually cell death (Cruz et al., 2009). One strategy to diminish cell injuries is the use of cell protectants, such as trehalose or gum acacia. Studies such as those by Linders et al., (1997), Conrad et al., (2000) reported the action of these substances on the protection of probiotic microorganisms during dehydration processes such as lyophilization. Our results show that the use of trehalose and that of gum acacia as cell protective agents was effective in maintaining a high population of L. casei, with a reduction by only one logarithmic cycle after 98 days storage at -20 °C in ice cream.

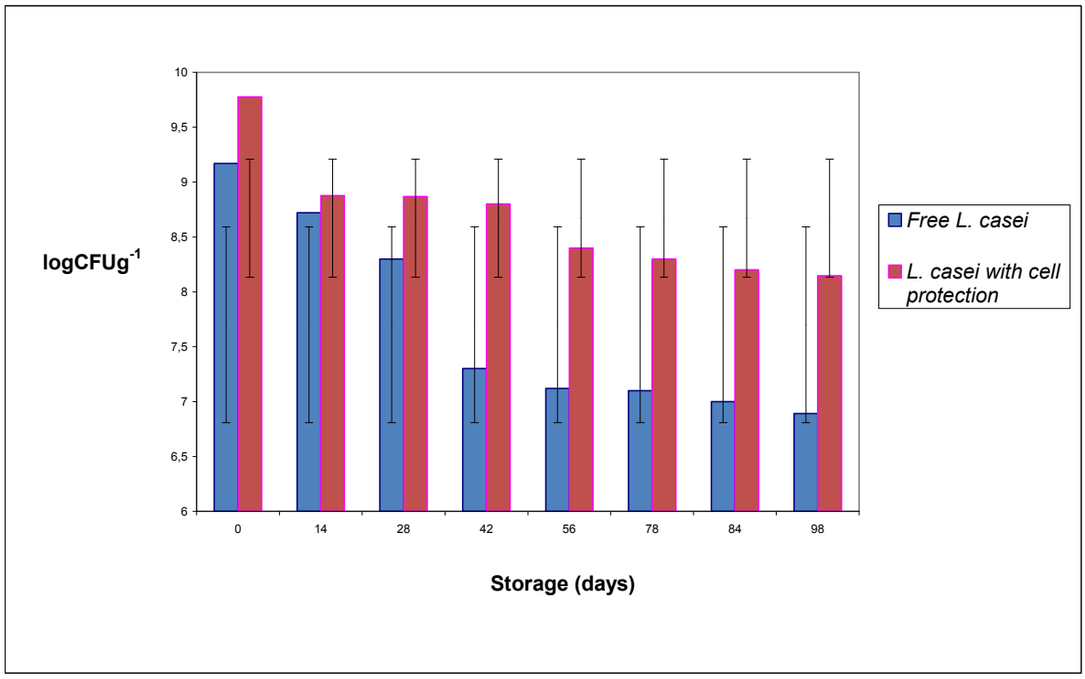

Fig. 2. Means and standard deviations of the viability of *L. casei* in ice cream during 98 days storage at -20°C.

Producing an ice cream containing probiotic of high sensory acceptability is a difficult task, since it requires a great deal of technical knowledge and most often functional ice creams present low sensory performance, when compared to conventional ice cream (Cruz et al., 2009).

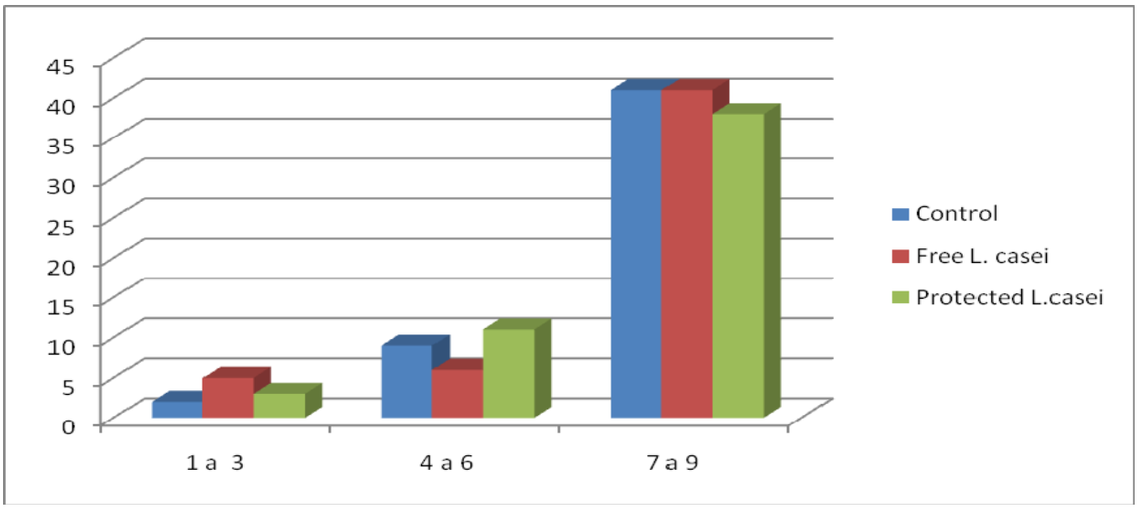

Fig. 3. Frequency hystogram for the attribute taste of the control ice creams, with addition of free and protected *L. casei*.

The results of sensory analysis of the taste of probiotic ice creams protected with trehalose and gum acacia are depicted in Figure 3. The three types of ice creams evaluated (control,

added with free *L. casei* and added with protected *L. casei*) exhibited a sensory profile similar in terms of the attribute taste and did not statistically differ from each other ($p<0.05$). Figure 3 shows that the product received liking ratings between 7 and 9 ("liked" to "liked a lot"); thus, the addition of the probiotic microorganism (in the free and in the protected form) did not affect the taste of the finished product. A similar result was observed by Homayouni et al., (2008), who found that the addition of microencapsulated *L.casei* in ice cream did not affect the sensory quality of the product.

In view of all this, one may conclude that trehalose and gum acacia have excellent perspectives as an industrial application as bacterial cell protectants.

3. Perspective of spray-drying and vacuum-drying processes for the preservation of *Lactobacillus casei* (Lc-1) and use in chocolate

3.1 Combination of atomization and vacuum chamber on *Lactobacillus casei* (Lc-1) drying

The group of *Lactobacillus casei* formed by strains of *Lactobacillus casei / paracasei* and *Lactobacillus rhamnosus,* in addition to possessing probiotic properties proven through clinical trials (Buriti & Saad, 2007) are also species that show good survival characteristics to drying processes by atomization. Corcoran et al. (2004) and Ananta et al. (2005) obtained dried cultures from *Lactobacillus rhamnosus* GG, while Gardiner et al. (2000) demonstrated enhanced survival to drying of *Lactobacillus paracasei* NFBC 338 compared to *Lactobacillus salivarius* UCC 118 showing adequacy of this group to this dehydration process.

As discussed on section "Cells Protectors" the growing and drying media have an effect on microbial survival during dehydration and the use of reconstituted skim milk (RSM) as drying vehicle can be inexpensive and effective alternative. Authors such as Corcoran et al. (2004), Gardiner et al. (2002) and Simpson et al. (2005) obtained high survival rates (greater than 70%) of lactic acid bacteria produced by drying by atomization using RSM.

Among the factors that influence the preservation of dried lactic acid bacteria - apart from temperature -, moisture control is indispensable, since according to the studies of Kurtmann et al. (2009), a gain in moisture content results in loss of Lactobacillus during storage. The authors demonstrated that one of the causes of the drop in the live lactobacillus population is lipid oxidation of the plasma membrane constituted by unsaturated phospholipids (Maness et al., 1999). Having this in mind, the aim of the present study was a combined use of spray and vacuum-drying to reduce the final moisture content of fermented powder.

Prior to drying, the lactobacillus were multiplied in three different concentration of skim milk powder (10, 20 and 30%). There were no differences in the amounts of *L. casei* between the fermentations with 20 and 30% reconstituted skim milk powder (RSMP), with mean values of, respectively, 9.36 and 9.41 (log cfu.ml^{-1}). The concentration of the probiotic was lower in fermentation with 10%(w/v) RSMP with 9.07 (log cfu.ml^{-1}). This behavior can be explained by the reduced availability of carbon sources (lactose) and nitrogen sources (milk proteins) necessary to the growth of the microorganisms (Pham & Shah, 2008).

Based on this, an experimental design was conducted evaluating all the variables that can influence the viability of microorganisms at spray drying method. The variables selected and the results obtained after drying are showed in Table 2. With the program Statisca 5.1, it was observed that the main factors to exert influence on the survival of *L. casei* (LC-1) were the inlet temperature (IT), the flow rate (FR) and the percentage of reconstituted skim milk powder (RSMP) (w/v).

Experimental test	X_1	X_2	X_3	X_4	IT (°C)	FR (ml.h^{-1})	% RSMP (m.v^{-1})	FAr	Log (cfu.g^{-1})
1	-1	-1	-1	-1	160	260	20	54	8.5
2	1	-1	-1	1	190	260	20	66	7.7
3	-1	1	-1	1	160	520	20	66	9.6
4	1	1	-1	-1	190	520	20	54	8.7
5	-1	-1	1	1	160	260	30	66	6.6
6	1	-1	1	-1	190	260	30	54	5.9
7	-1	1	1	-1	160	520	30	54	8.3
8	1	1	1	1	190	520	30	66	6.6
9	0	0	0	0	175	390	25	60	8.2
10	0	0	0	0	175	390	25	60	7.4

Table 2. Experimental Design $2^{(4-1)}$ and their viability of *Lactobacillus casei* (Lc-1) after spray drying method for spray drying method. Coded Independents variables: X_1, X_2, X_3, X_4, Original independents variables: Inlet temperature (IT), Flow rate (FR), % of reconstitute skim milk powder (RSMP) and Air Flow (FAr).

With the intention to obtains higher survival of the microorganism and choose the best conditions to dry these materials, a new experiment was conducted applying an experimental displacement with the best conditions. After this new adjustments it was observed that the conditions TE = 144°C; TA = 650 ml/h and %RSMP = 13.25 produced higher mean viabilities (9.95 log cfu.g^{-1}) with a variation coefficient (VC) smaller than 2%.

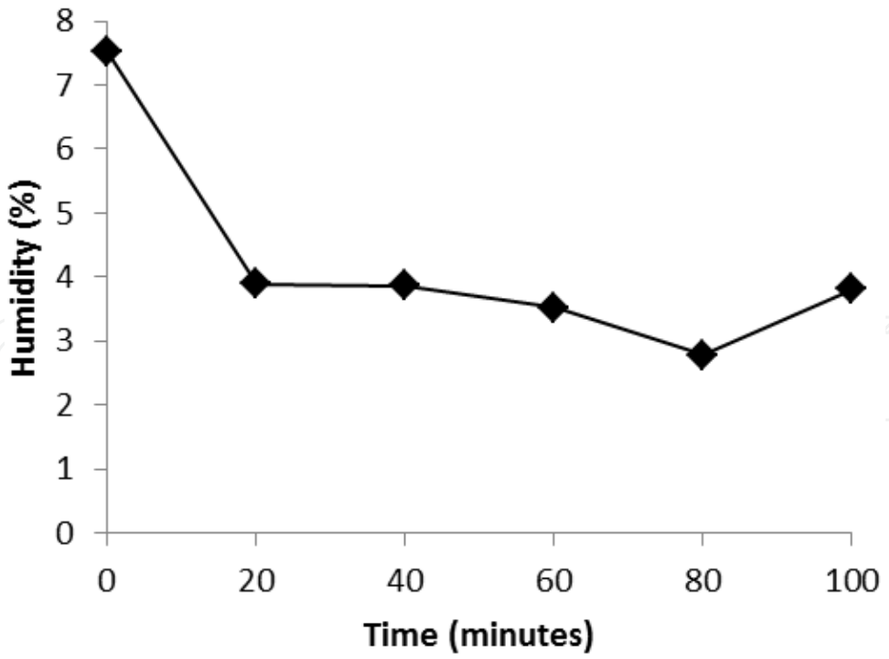

Fig. 4. Humidity values after vacuum chamber drying of dry fermentate (DF) at 45°C.

In spite of the satisfactory results as to the high concentration of viable lactobacillus after drying by atomization, the moisture content in % 7.2 ± 0.4 was greater than the maximum

recommended by Corcoran et al. (2004), which is 4% for the preservation of viable lactobacillus during storage in RSMP. An alternative was vacuum drying, which reduced the moisture level of the powders dried by atomization to values lower than 4%. Authors such as Santivarangkna et al. (2007) and Tymczyszyn et al. (2008) demonstrated that it is possible to dry lactobacillus in vacuum chambers and they employed temperatures lower than 45°C which are less aggressive to the microorganisms.

To determine the influence of vacuum dehydration time on viabilities and final humidity, the analyses were conducted every 20 minutes. There was no difference between the counts of *L. casei* (Lc-1) as related to drying times ($p < 0.05$). The mean value after drying was 9.96 ± 0.4 % (Log cfu.g^{-1}). The moisture level (in %) are demonstrated at Figure 4, and varied from 7.2 ± 0.4 at the beginning to 3.9 ± 0.2 (CV) after 20 minutes drying (quick stage). At 80 minutes the percentage of humidity reached 2.8 ± 0.2 (slow stage) and then recapture of humidity from the air occurred after 100 minutes drying, with the moisture content increasing to 3.8 ± 0.1.

Complementary drying of the vacuum-chamber dried fermentate (DF) did not cause loss of viability of the lactobacillus ($p < 0.05$) with a survival rate of 99.7 %. According to studies of Santivarangkna et al. (2007), vacuum drying causes damage to the cell envelop (cell wall and plasma membrane) of the lactobacillus, resulting in losses in viability during drying. These losses were not noted in this study, since the maximum drying time used was 100 minutes, while Santivarangkna et al. (2007) utilized 24 hours, which increases the contact of the bacteria with degradation processes such as: high temperature, enzyme action and lipid oxidation (Kurtman et al., 2009; Maness et al., 1999; Fennema, 1996).

These results makes vacuum drying a technologically feasible post-processing option for the control and standardization of the moisture level of lactobacillus dried by atomization without causing losses in terms of their viability. To produce the DF, a standardization process of the moisture content of the DF was performed in a vacuum chamber for 80 minutes at 45°C, which resulted in the standardized dry fermentate (SDF).

After fermentation and drying processes, 20g of powders were packaged in bi-oriented polypropylene (BOPP) packs measuring 11 x 11.5 cm and stored in a desiccator with silica at 25°C (Simpson et al., 2005).

In addition to being packaged in BOPP, the SDF was also wrapped in polyethylene compound film therephthalate/aluminum/low density (PET/Al/PEBD) with 58 μm thickness and 11 x 11.5 cm in size. The flexible films used and their respective oxygen transmission rates (O_2TR), expressed in cm^3 (CNTP)/m^{2}day and water vapor transmission rates (WVTR) expressed in g water/m^2 day at 38° C / 90% RH, were: PET/Al/PEBD (O_2TR = 0.32; WVTR = 0.036) according to Lima, Silva and Gonçalves (1999). The bi-oriented polypropylene (BOPP) has a WVTR of 4.6 to 6.2 and a O_2TR in the 1800 – 3120 range according to Teixeira Neto & Vitali (1996). The SDF packaged in two packages was named as compound standardized fermentate (CSDF).

The pronounced drop noted up to the sixth week in the viability of the DF (control, dried only by atomization) can be explained by to the high initial level moisture (7.2%) of the samples and according to Corcoran et al. (2004) moisture levels should be kept below 4% to preserve the lactobacillus during storage. Kurtman et al. (2009) revealed in their studies that the best relative humidity level for storing lactobacillus is 11%, which fits the low moisture levels required by storage. After 6 weeks, the difference in the DF counts (control) and the

SDF was 2.9 logarithmic cycles. The DF with initial moisture content of 7.2% maintained only 2.2 x 10^6 cfu/g, whereas the SDF (3% initial moisture) preserved 1.9 x 10^9 cfu.g^{-1} greater than DF ($p < 0.05$) and according to Ishibashi & Shimura (1993), a probiotic level should be 10^7 cfu.g^{-1}.

Since both DF as SDF were kept under the same storage conditions and departed from the same ($p< 0.05$) amount of viable *L. casei* cells (1.2 x 10^{10} and 1.0 x 10^{10} cfu.g^{-1}, respectively), it may be stated that the post-processing in a vacuum chamber used to obtain the SDF exhibited the largest number of survivors as compared to the control for 42 days.

Up to the sixth week, the evolution of loss in viability of SFS and CSDF remained the same with losses of 0.7 log cycles for both. However, there was a moisture gain in SDF from 3.1 to 4.1% from the third to the sixth week. Since the 4% moisture limit was exceeded (Corcoran et al., 2004), the loss in viability of SDF was no longer linear and increased between the sixth and the ninth month (Figure 5). Between day 42 and day 63, the loss of viable lactobacillus was 2.3 logarithmic cycles. From the ninth month onwards, there was a tendency toward equality in the counts of *L. casei* between SDF and DF. After 18 weeks, there were no differences in the counts ($p < 0.05$) of DF (control) and SDF, with 6.1 and 6.2 log cfu.g^{-1}, respectively.

The loss in viability of lactic acid bacteria with increasing moisture levels can be better explained by the increase in oxidation and enzyme hydrolysis during storage. According to Fennema (1996), the oxidation process depends on the moisture level and is reduced whenever the BET monolayer value is established. The gain in moisture in excess of the monolayer results in increased oxidation of the microorganism and consequent loss of viability of the lactobacillus (Fennema, 1996; Kurtman et al., 2009).

Acceleration of oxidation driven by the gain in moisture occurs through the increase in the solubility of oxygen and the swelling of the macromolecules exposing more catalytic sites. According to Maness et al. (1999), the bacterial cell membrane is constituted by polyunsaturated phospholipids and is susceptible to oxidation by attack of oxigen reactive species. In addition to oxidation, the gain in moisture favors enzyme hydrolytic action (Fennema, 1996), which explains the greater loss in viability of the lactobacillus in comparison to storage conducted at increasing water activity values, even under low oxygen concentrations (Kurtman, 2009).

The enhances preservation of CSDF compared to SDF can be explained by the lower water vapor transmission rate (WVTR) of the laminated PET/Aluminum/PE film used to package CSDF, whereas SDF was packaged in BOPP which has a higher WVTR. Since the dried lactobacillus are sensitive to the increase in moisture contents as described in studies by Kurtman et al. (2009). After 18 weeks, CSDF kept itself 1.5 logarithmic cycles higher than SDF. At the beginning of storage, SDF was 9.6 log (cfu.g^{-1}), and this value was reduced to 6.2 log (cfu.g^{-1}) after 18 weeks.

In CSDF there was an average loss of 2.2 logarithmic cycles in 18 weeks. Departing from 9.9 at the beginning and falling to 7.7 log cfu.g^{-1} at the end of 126 days. This concentration of viable lactobacillus is greater than the probiotic level established by Ishibashi & Shimura (1993).

These values were higher than those described by Nebesny et al. (2007) who maintained 5 log cfu.g^1 for 4 to 5 months storage at 30°C in chocolate added with lyophilized lactobacillus.

In the samples kept below 4% as recommended by Corcoran et al (2004), the behavior of loss of viability is linear (SDF up to the sixth week and CSDF). The D_{FSPC} value was 8.2 weeks, with this result being a little below those found by Ananta et al. (2005) who reported a D = 11.5 in storage for 6 weeks at 25°C with controlled relative humidity at 11%. Simpson et al (2005) selected a *Bifidobacterium thermophilum* NCIMB 702554 from among 16 other bifidobacterial strains. The selected strain maintained 7.3 log cfu.g^{-1} at 25°C for 90 days, an amount smaller than that obtained for CSDF in our study, which maintained 8.3 log cfu.g^{-1} viable *L. casei* cells for 84 days at 25°C.

To observe the aspects of the microparticles containing *L. casei* after drying by atomization, a scanning electron microscopy was performed at Microscopy Laboratory of Analysis. Figure 6 shows micrographs obtained at a magnification of 3000X (A) and 6000X (B). These microparticles are spherical in shape and of varying sizes. At various ends or extensions they had the appearance of "flat balls" with well-evident wrinkles on their surface as if they had been severely dehydrated. Lian et al. (2002) observed more spherical microparticles in the drying of *B. Longum* B6 at an inlet temperature of 100°C, indicating that high drying temperatures may be responsible for the deformations observed on the surface of the microparticles. Like with the images taken by our group, Crittenden et al. (2006) also verified in their work absence of free or non-microencapsulated cells in the micrographs at a magnification of 1000X and proposed that the microorganisms may have been effectively encapsulated within the microparticles.

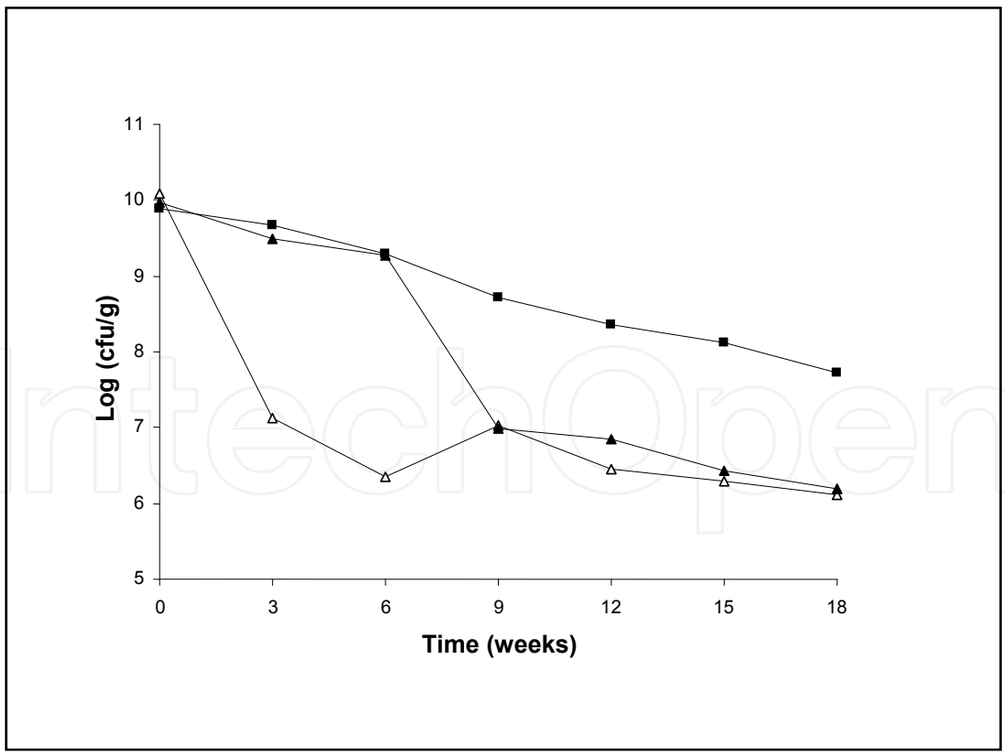

Fig. 5. Loss of viability of L. casei (Lc-1) at 25ºC during 18 weeks:(■) Compound standardized dry fermentate (CSDF), (▲) standardize dry fermentate (SDF); (△) Dry fermentate (DF)

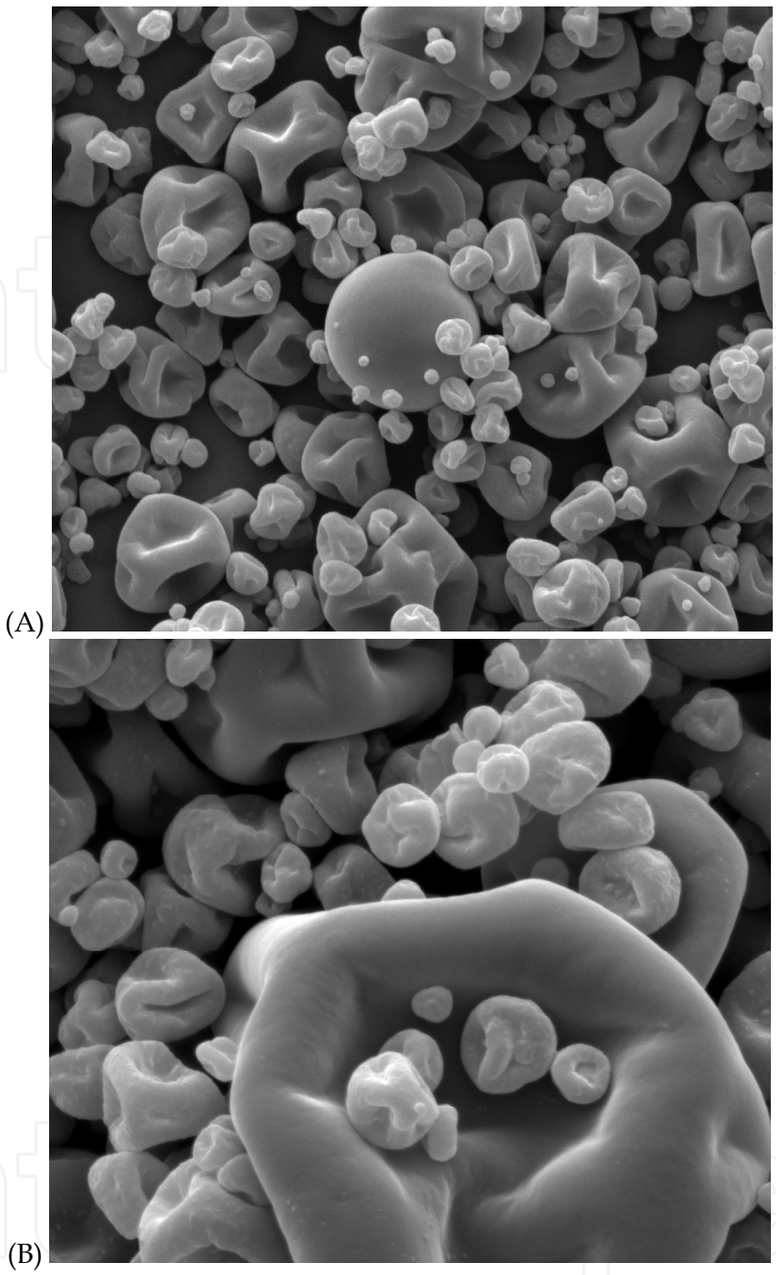

Fig. 6. Scanning electron micrographs at magnifications of (A) 3000x and (B) 6000x, respectively, of the microparticles of fermentate dried by atomization containing *L. casei*. Laboratory of Electron Microscopy and Microanalyses – State University of Londrina.

3.2 The use of *L. casei* (LC-1) microencapsulated by spray-drying in the processing of chocolate bars

The fermented powder obtained could be applied in several food products being chocolate an option that has been exhibited good results in terms of incorporating dried probiotics, since it melts at temperatures that microorganisms support, thereby avoiding loss of viability of the probiotic during the chocolate manufacturing process. Chocolate can further

preserve lactic acid bacteria, at high counts (greater than 10^7 cfu.g^{-1}), for 6 months storage at 18°C (Nebesny et al., 2005). Additionally, chocolate has an exclusive taste, aroma and texture which make it one of the most popular foods in the world, in addition to being a source of biologically active substances, such as polyphenols, which have been shown to possess significant antioxidant properties and to provide a beneficial impact on the human health, particularly on the cardiovascular system (Nesbeny, 2004).

The dried fermentate was produced by applying drying conditions that had been optimized with the aid of the experimental design $2^{(4-1)}$ and used to supplement bars of dark chocolate to produce a potentially probiotic product. Bars of dark chocolate 9 cm x 3 cm in size were produced with a standardized weight of 10.5g ±0.2g and the survival of *L. casei* to the production process was 85%, resulting in a concentration of 4.2 x 10^8 Cfu.g^{-1} chocolate. The consumption of 10g of the product would provide a dose of 4.2 x 10^9 Cfu, a value above the minimum limit of 10^7 Cfu.g or ml^{-1} for probiotic products (Ishibashi & Shimura, 1993). A desirable characteristic in a functional probiotic food is that the selected microorganism remains viable from the processing of the product until consumption at counts that are adequate to promote the desired benefit. Within this context, our group evaluated the viability of *Lactobacillus casei* (LC-1) microencapsulated by spray-drying in dark chocolate during 90 days storage at 4°C and 25°C. Lactobacillus counts were performed after 0, 30, 60 and 90 days storage. The results are presented in Figure 7.

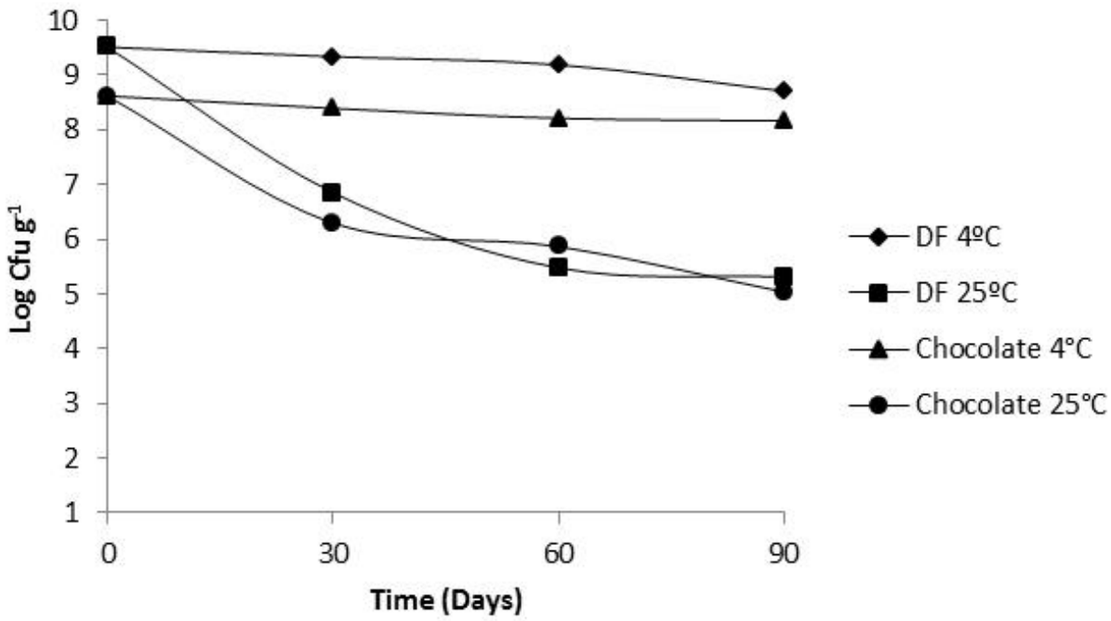

Fig. 7. Viability of *L. casei* in the Dry Fermentate (DF) and in little dark chocolate bars. (♦) DF at 4°C, (▲) Chocolate at 4°C, (■) DF at 25°C and (●) chocolate at 25°C.

After 12 weeks storage at 4°C (refrigerator temperature), there was a slight loss in viability of *L. casei,* but this did not surpass 0.5 log Cfu.g^{-1}. The survival rate was reduced from 8.6 log Cfu.g^{-1} to 8.2 log Cfu.g^{-1}, thus kept at a level above the minimum level of 7 log Cfu.g^{-1} (Ishibashi & Shimura, 1993). In storage at 25°C, the loss in viability was a little more abrupt and greater than 3 log Cfu.g^{-1}, falling back from 8.6 log Cfu.g^{-1} to 5.0 log Cfu.g^{-1}. These data reinforce that the damage to the cell caused by the drying process become evident during

storage of the product. The extent of the loss of viability depends on the storage conditions such as temperature, water activity, moisture content and osmotic tension (Nesbeny, 2007). The choice of the food matrix to which the probiotic will be added must be well analyzed, since the constituents of the food may negatively affect the viability of the microorganisms (Possemiers et al., 2010). Chocolate showed to be a good matrix for using probiotic microorganisms since it exhibits a survival profile quite similar to that of the DF.

4. Conclusions

At present, there are numerous products containing probiotic microorganisms, with the main commercial products being fermented milks and yogurts. The great obstacle to the use of these microorganisms in other food systems is the loss of viability during processing and storage. Within this context, the use of cell protectants enables the use of these microorganisms in several processes and formulations. In this chapter, previously unreleased studies conducted by our research group, using cell protectants in *Lactobacillus casei* (Lc1) and their use in different food systems, such as ice cream and chocolate were presented. The results show that the use of cell protective agents enables the addition of probiotics to different food matrices or food systems, thereby opening new possibilities for the development of foods that can positively affect consumer health.

5. Acknowledgment

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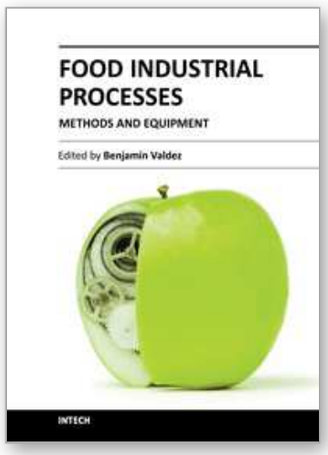

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The global food industry has the largest number of demanding and knowledgeable consumers: the world population of seven billion inhabitants, since every person eats! This population requires food products that fulfill the high quality standards established by the food industry organizations. Food shortages threaten human health and are aggravated by the disastrous, extreme climatic events such as floods, droughts, fires, storms connected to climate change, global warming and greenhouse gas emissions that modify the environment and, consequently, the production of foods in the agriculture and husbandry sectors. This collection of articles is a timely contribution to issues relating to the food industry. They were selected for use as a primer, an investigation guide and documentation based on modern, scientific and technical references. This volume is therefore appropriate for use by university researchers and practicing food developers and producers. The control of food processing and production is not only discussed in scientific terms; engineering, economic and financial aspects are also considered for the advantage of food industry managers.

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