DÉBORA CHRISTINA CNOSSEN

USE OF STRATEGIES TO ENABLE THE SPRAY DRYING OF *Lactococcus lactis*

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Magister Scientiae.

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Romans 11:36

SUMÁRIO

RESUMO

CNOSSEN, Débora Christina, M.Sc., Universidade Federal de Viçosa, novembro de 2018. **Use of strategies to enable the spray drying of Lactococcus lactis**. Orientador: Antônio Fernandes de Carvalho. Coorientador: Evandro Martins.

Dentre as bactérias do ácido lático (BAL) aplicadas à indústria de alimentos, estirpes de Lactococcus lactis destacam-se pelo alto potencial de acidificação, atividade proteolítica, produção de compostos aromáticos e inibição de patógenos pela síntese de bacteriocinas. Em algumas aplicações, a utilização de L. lactis como cultura iniciadora depende da sua preservação por desidratação, sendo a secagem por atomização uma alternativa promissora para tal finalidade. No entanto, essa técnica de secagem é pouco aplicada para L. lactis devido à sua baixa termorresistência e pronunciada morte celular durante o processo. Neste contexto, o objetivo deste trabalho foi aplicar estratégias para aumentar a tolerância de L. lactis ao processo de secagem por atomização. Por meio da triagem dos parâmetros operacionais de secagem, foram determinados os parâmetros do pó considerados ideais (atividade de água (Aw) = 0,198 e temperatura (T $°C_{powder}$) = 52 ° C) para garantir maior sobrevivência das células durante a secagem. Na sequência, avaliou-se o efeito dos teores de lactose e gordura na microencapsulação de L. lactis em matrizes lácteas. Os resultados revelaram que a adição de lactose no leite mostrou-se ideal para a secagem de bactérias sensíveis ao calor. Em contraste, o teor de gordura demonstrou pouca contribuição na sobrevivência do microrganismo. Por fim, utilizando os parâmetros ideais de secagem e uma eficiente matriz encapsulante, avaliou-se a influência da aplicação prévia de estresses progressivos sobre a sobrevivência de L. lactis ao processo de secagem. Os resultados indicaram um aumento na sobrevivência de L. lactis (~ 112,5%) após a secagem por atomização e durante o armazenamento, quando expostos à condição progressiva de estresse oxidativo. Coletivamente, os resultados denotam-se como estratégias potenciais para aumentar a tolerância de bactérias termossensíveis ao processo de secagem por atomização.

ABSTRACT

CNOSSEN, Débora Christina, M.Sc., Universidade Federal de Viçosa, November**,** 2018. **Use of strategies to enable the spray drying of Lactococcus lactis**. Advisor: Antônio Fernandes de Carvalho. Co-advisor: Evandro Martins.

Among the lactic acid bacteria (LAB) applied in food industry, Lactococcus lactis strains stand out due to its high acidification potential, proteolytic activity, production of flavor compounds and inhibition of pathogens by bacteriocins. In some applications, the use of L. lactis as the starter culture depends on its preservation by dehydration, being the spray drying a promising alternative for this purpose. However, this drying technique is poorly applied to L. lactis due to its low thermoresistance and pronounced cell death during the process. In this context, the aim of this work was to apply strategies to increase the tolerance of L. lactis to spray drying. By screening of drying parameters, the ideal powder parameters (water activity (Aw) = 0.198 and temperature (T $°C_{powder}$) = 52 ° C) were determined to ensure greater survival of cells during drying. In the sequence, the effect of lactose and fat contents on the microencapsulation of L. lactis in dairy matrices was investigated. The results revealed that the supplementation of milk with lactose seems to be ideal to drying of heat-sensitive bacteria. In contrast, the fat content has low contribution on the microorganism preservation. Lastly, by using of ideals drying parameters and encapsulating matrix, the influence of previous application of progressive stresses on the survival of L. lactis to the drying process was evaluated. The results showed an increase on the survival of L. lactis (~112.5%) after spray drying and during storage when exposed to the progressive condition of oxidative stress. Collectively, the results are shown as potential strategies to increase the tolerance of heat-sensitive bacteria to the spray drying process.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The spray drying technique is widely applied in food production due to its lower energy costs, flexibility and higher productivity. Although advantageous compared to other drying techniques, this technology is little used in the production of dehydrated lactic cultures.

Some lactic acid bacteria (LAB) used in the food industry are sensitive to spray-drying conditions and easily lose viability during the drying process and storage. However, the use of strategies as optimization of drying parameters, microencapsulation and cellular pre-adaptation to sublethal stress conditions are promising possibilities for the development of stable ferments at lower productive cost.

The Lactococcus lactis strains selected in this study show technological properties to industrial applications; however, they are very heat sensible. In this sense, the objective of this work was to adopt strategies to increase the tolerance of L. lactis to spray drying.

This document is structured in four chapters: The Chapter 1 consists in a review contextualizing the challenges in the spray drying of thermosensible bacteria; the Chapter 2 describes the experimental procedure to determine the ideals powders characteritics and operational drying parameters to obtain higher cell viability; the Chapter 3 explores the effect of lactose and fat as encapsulating matrix and the Chapter 4 investigate how the adaptation of cells to progressive stresses can improve the L. lactis viability during drying and storage. This document is finalized with a general conclusion bringing the main findings, considerations and perspectives.

CHAPTER 1

LITERATURE REVIEW

Challenges in the Spray Drying of Latic Acid Bacteria: Understanding the Loss of Cellular Viability During the Process

ABSTRACT

Latic acid bacteria (LAB) belong to an important group of microorganisms with industrial interest acting both in the food and pharmaceutical industries. Generally, these cultures used in food processing are commercialized in dried form which guarantees reductions in transportation costs and space storage inside the industry. Spray drying is a promising technique to obtation of dried bacteria due to its versatility and lower energy cost. However, adverse conditions during the process are responsible for the low maintenance of cell viability. Therefore, the purpose of this review is to expose the principles governing the spray drying technique and understand how the physical changes involved in drying procedure can affect the cellular viability of LAB.

Keywords: Spray drying, Latic acid bacteria, Cellular viability, Drying parameters.

1. Introduction

The latic acid bacteria (LAB) is a group of microorganism that shows as general characteristic the production of lactic acid from the fermentation of carbohydrates and, depending on the metabolic pathway of fermentation, they are classified as strict homofermentative (produces only lactic acid), facultative heterofermentative (produces lactic and acetic acids) or strict heterofermentative (produces lactic, acetic and formic acids, ethanol and carbon dioxide) (Kajfasz & Quivey Jr., 2011). Besides these compounds, the LAB are also able to produce bacteriocins, hydrogen peroxide, exopolysaccharides, enzymes with lipolytic, proteolytic or stearic activities and molecules associated to flavor of some foods (Vlieg et al., 2006; Frantzen, Kleppen & Holo, 2017).

Some LAB species are also recognized as probiotics that, according to definition by FAO/WHO (2001), are live microorganisms that when administered in adequate quantities confer benefit to consumer health (Hotel & Cordobora, 2001; Pundir et al., 2013). The probiotics are associated to regulation of the digestive system, improvement of immunological parameters, control of cholesterol levels, prevention of diabetes, and sense of well-being through the reduction of anxiety (Cani, 2017).

The products of LAB metabolism can found application in the synthesis of polylactic acid (PLA) to biodegradable packaging production, cosmetics, repellent, medications and nutritional supplements (Smit, Smit, & Engels 2005; Papagianni, 2012; Rajendran et al., 2017; Song et al., 2017). Nevertheless, the food industry stands out by broad use of LAB in the production of fermented milks, vegetables and meats (Giraffa, Chanishvili, & Widyastuti, 2010; Peighambardoust, Golshan Tafti, & Hesari, 2011a; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015; Bintsis, 2018)**.**

From technological point of view, these bacteria are applied in food industry in order to define the sensorial characteristics of products, improve the texture and increase the water retention in fermented milks, develop glance in cheeses, act as biopreserver and inhibit the growth of spoilage and pathogen microorganism (Table 1) (Moraes et al., 2012; Frece, Cvrtila, Topić, Delaš & Markov, 2014; Frantzen, Kleppen & Holo, 2017; Song et al., 2017).

Depending on the metabolic characteristics of the LAB culture, it can be used to start the fermentation (starter culture) or act as a coadjuvant to accelerate the maturation or to modify the organoleptic properties of food (NSLAB culture). In both cases, the culture should be pure with high population density (~ 10^9-10^{11} CFU.g^{-1}) and the cells should be metabolically active or, in other words, be viable (Ishibashi & Shimamura,1993; Parente and Cogan, 2004; Huang et al., 2016a).

Table 1 - Lactic acid bacteria used in the production of several products

LAB strain	Application or technological function	Type of product	Reference
Lactococcus lactis	Fermentation of food, flavor	Cheese, yoghurt, sauerkraut	Song et al., 2017
	Bacteriocins	Bio-preservative, clinical applications	Bolocan et al., 2017
	Latic acid production	Pharmaceutical industry, polylactic acid (PLA)	Papagianni, 2012
Lactobacillus. delbrueckii spp. bulgaricus	Probiotic properties, texture and viscosity	Yoghurt, frozen yoghurt, dessert	Senok, 2009
Lactobacillus casei Shirota	Fermentation and probiotic properties	Fermented milk	Granato et al., 2010
Lactobacillus bavaricus	Starter culture	Sauerkraut	Stiles and Holzapfel, 1997
Lactobacillus helveticus	NSLAB culture, accelerate cheese ripening	Cheddar cheese	Johnson et al., 1995
Leuconostoc mesenteroides	CO_2 production, flavor	Roquefort cheese, fermented butter	Ogier et al., 2008
Oenococcus oeni, Lactobacillus spp.	Malolactic fermentation, flavor, aroma	Wine	Bintsis, 2018

Many these cultures used in food processing are commercialized in dried form which guarantees both reduction in transportation costs and space storage inside the

industry (Fu & Chen, 2011; Huang et al., 2017a). The dehydration of LAB involves the water removal by freeze-drying that basically consists in freezing the cells and promote the water removal by sublimation. The greater advantage of this technique in relation to others with same purpose lies in the fact that the fast freezing followed by sublimation promotes low mechanical damage to cellular structure (Peighambardoust et al., 2011). In this way, the cells suffer lesser injuries which justify the maintenance of cellular viability after the process (Fu & Chen, 2011).

On the other hand, the freeze-drying is a batch procedure that demands high times of operation and consumes a substantial amount of energy (Lievense & Van't Riet, 1993; Desobry et al., 1997; Ratti, 2001; Santivarangkna et al., 2007). In addition, the productivity is relatively low compared to other industrial drying methods (Schuck et al., 2014). Considering these operational drawbacks, several works have appointed the spray drying as being a promising alternative to replace the dehydration of LAB cultures by freeze-drying (Schuck et al., 2016; Huang et al., 2017a).

To get an idea, in 1996 only 386 scientific articles involving spray drying of bacteria were registered in the Science Direct platform while in 2018, 2575 articles are available. It means that in the last 22 years the number of works in this area increased approximately in 667 % consulting only this research platform. This ascendant interest can be explained by the fact that this technology consumes up to 10 times less energy than freeze-drying and can be conducted in continuous process (Boyaval & Schuck, 1994; Schuck et al., 2013). Moreover, some works have proposed that spray dried culture can be storage at room temperature reducing the cost with refrigeration (Schuck et al., 2013).

Despite all advantages, the spray drying promotes a series of injuries to cells causing viability loss and alterations of technological properties of culture (Santivarangkna et al., 2008). Depending of drying conditions, some spray dried cultures have their population substantially reduced; being this the main factor limiting the industrial application of spray dried bacteria (Huang et al., 2016a).

The aim of this review is expose the principles governing the spray drying technique and understood how the physical changes involved in drying procedure can affect the cellular viability of LAB.

2. Principle of spray drying

The drying of liquids by atomization, often called "spray drying", consists in dispersing the fluid in small droplets into a stream of hot air in order to quickly evaporate the water from droplets. The principle of technique is based on Fourier law which considers that the larger the exchange area, the faster the energy transfer in the heat form and therefore the greater the drying rate (Bimbenet, 1978; Santivarangkna et al., 2007). Spraying can significantly increase the exchange area between fluid and hot air: one liter of liquid in droplets of 100 μm in diameter has an area of 60 m^2, that is, the area is 1200 times higher than the area of a sphere of the same volume.

When cell suspension droplets are placed in a stream of air with low relative humidity and high temperature (150 °C to 300 °C), a difference in temperature and partial pressure of water is spontaneously formed between droplet and air. The result is a transfer of heat from the air to the droplet and a transfer of water from the droplet to the air (Bimbenet, 1978; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007; Schuck et al., 2009). In this stage the temperature of droplet surface is about 45 ° C (wet bulb temperature) and water is instantly evaporated at low temperature (Dittman and Cook, 1977).

The drying rate is linked to the evaporation surface, the difference of the partial water pressure between the droplet and the air and the rate of water migration in the droplet (Schuck, 1999). Thus, the adjustment of operational parameters (type of atomization, inlet air temperature and injection rate of cell suspension in the equipment) is a valid strategies to optimize the drying rate (Desmond, Stanton, Fitzgerald, Collins, & Paul Ross, 2002; Fu & Chen, 2011; Huang et al., 2017a).

The second stage of drying process is initiated when excedent water is removed and a dried shell is formed on the droplet. Under this condition, the evaporation of internal water molecules is depending of diffusion and the thermal energy provided by hot air promotes a gradual increasing in the particle temperature (Masters, 1991).

With the drying progress, the dried particles are carried out by air flow and recovered in the cyclone, where they stay in contact with outlet air and suffer additional heating. According to Písecký (1997) and Westergaard (2001), the particle temperature can reach values between 10 °C and 20 °C lower than the outlet air temperature. Therefore, considering temperatures used in industrial plants, it is predicted that the particles can assume up to 80 °C (Shokri et al., 2015).

The particles recovered in the end of spray dryer is normally refrigerated and packed in packing hermetically close and with modified atmospheric composition (Morgan, Herman, White, & Vesey, 2006; Silva, Freixo, Gibbs, & Teixeira, 2011; Barbosa et al., 2015a; Huang et al., 2017a).

3. Factors to be considered before spray drying of LAB

Several works have exposed a substantial range in the LAB viability that can vary from 4.71 to 100% survival, depend on species and strain (Table 2). The comparison of viability loss between the publications is quite complicated once the drying conditions and behavior of LAB strains are different from one study to another (Gharsallaoui et al., 2007). However, it is claimed that the operational parameters of spray dryer and the carrier material to suspend the culture, are crucial points to guarantee maximal cell survival after drying (Ghandi, Powell, Chen, & Adhikari, 2012b; Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fisk, 2013; Schuck, Dolivet, Méjean, Hervé, & Jeantet, 2013).

The inlet air temperature drives the drying rate of droplets besides to influence in the particle temperature in the end of process (Fu & Chen, 2011; Peighambardoust et al., 2011; Wang et al., 2016). The higher the inlet air temperature faster will be the cell dehydration and more intense will be heat treatment suffered by this (Ghandi et al., 2012b; Tee, Chuah, Rashih, & Yusof, 2012b).

On the other hand, the type and concentration of material carrier determine the size of droplets that will be dried (Lian, Hsiao, & Chou, 2002). When viscous fluids are atomized, bigger droplets tend to be formed hindering the water evaporation (Lievense and Van't Riet, 1994; Santivarangkna et al., 2007; Peighambardoust et al., 2011). This leads to an increase in the residence time in the drying chamber, resulting in greater thermal damage. By contrast, the formation of smaller droplets results in fine powders and higher energy cost. Therefore, it is necessary to find an optimal interval of solids content that, in general lines, is between 20-30% ($w.v^{-1}$) (Huang et al., 2017a).

In addition, the heat conduction inside the droplet to be dried is dependent to the thermal properties of carrier material (Lian et al., 2002). Materials with low thermal conductivity tends to limit the heat conduction (Guiné, 2018) and consequently protect the cells against thermal damages. Therefore, the choice of carrier material and its concentration is important to limit the cellular injury during the drying (De Castro-

Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012; Perdana, Fox, Siwei, Boom, & Schutyser, 2014).

The production of spray dried LAB cultures begins by growth of select strains in media and incubation conditions previously defined. Some LAB species may have very specific nutritional requirements which can make difficult or unfeasible the industrial cultivation (Fennema et al., 2004; Niel, 2006; Tsakalidou, Effie, Papadimitriou, 2011). However, most of LAB applied in the food industry can growth in easily obtained synthetic media or food matrices as milk or whey (Huang, Cauty, et al., 2016a; Huang, Méjean, et al., 2017b).

Besides the cultivation conditions, the growth phase is other factor should be considered before the spray drying. Although cells in log phase are in full metabolic activity, they are low adapted to adverse environment suffering higher death rate in the drying tower (Corcoran et al. 2005; Michida et al. 2006). Therefore, some authors have indicated cells in beginning of the stationary phase as being ideal once the reduced nutrients concentration or accumulation of metabolites as organic acids, for example, promote the activation of stress responses making the cells more resistant to subsequent stresses (Hurst & Collins,1974; Teixeira et al., 1994).

Finished the incubation period, the LAB culture can be directly inject into drying tower (Huang, Cauty, et al., 2016a; Huang, Rabah, et al., 2016b; Huang, Méjean, et al., 2017) or pass by additional steps to remove metabolites produced by cells. In the last case, the culture is centrifuged, the pellet rinsed and suspended into a new carrier material (De Man, Rogosa, & Sharpe, 1960; Golowczyc et al., 2010; Lavari, Páez, Cuatrin, Reinheimer, &Vinderola, 2014; Barbosa, Borges & Teixeira, 2015b). This step can be important since the metabolites are concentrated during drying and can assume harmful concentrations to microorganism.

Table 2 - Growth conditions, drying parameters and viability loss of spray dried LAB

LAB strain	**Growth conditions Medium/ T(°C)**	**$T_{inlet\ air}$ (°C)**	**Feed flow rate ($Kg.h^{-1}$)**	**Carrier material**	**Survival after drying (%)**	**Reference**
Lb.salivarius NRRL B-30514	MRS /37°C	170	n.a [a]	RSM [b] RSMST [c] RSMLT [d]	49.55 48.88 56.67	Zhang et al., 2016

Lb.plantarum 299v	MRS /37°C	150	n.a [a]	Orange juice + MD[g]	100	Barbosa, et al., 2015b
P.acidilactici HA-6111-2	MRS /37°C	150	n.a [a]	Orange juice + MD[g]	100	Barbosa, et al., 2015b
Lb. plantarum UFV-Lb26	MRS /30°C	180	1.33	RSM[b] RSW[e] RSWP[f]	100	Ferreira et al., 2017
Lc. lactis ssp. cremoris ASCC930119	M17 /30°C	130	n.a [a]	Lactose	4.71	Ghandi et al., 2012a
B.lactis BB12	MRS+0.05 % cysteine /43°C	80	0.85 - 1.00	SPI[f]+MD[g]	44	Chávez & Ledeboer, 2007
P. acidipropionici	YEL/30°C	130	65.06	Acid whey permeate	100	Schuck et al., 2013

[a] n.a:not avaible; [b] RSM: reconstituted skim milk; [c] RSMST: RSM with additional sucrose and trehalose; [d] RSMLT: RSM with lactose and trehalose; [g] MD: maltodextrin; [f] SPI: soy protein isolate; [h] YEL: Yeast Extract-Lactate medium.

4. Factors affecting LAB cell viability during and after spray drying

As before presented, the drying involves the pulverization of cell suspension into hot air flow promoting abrupt variations in the temperature and humidity of droplets. All events involved in the drying of bacteria pass in a short time and can occur simultaneously and synergistically. For this reason, a prediction and evaluation of real damages suffered by LAB is difficult to perform; which justify the existence of few studies dedicated to this theme. However, taking account the principles involved during the drying, it is possible to infer that the LAB undergo some thermal, osmotic and oxidative stresses.

In the first stage of spray drying, the cell suspension droplets are heated to approximately to 45 °C for a few seconds and it is supposed that this heat treatment is not sufficient to cause significant cell viability reduction. By contrast during this stage, the droplets are quickly dehydrated creating a water-poor matrix around the cells. The

immediate consequence is the formation an extracellular hypertonic media promoting a removal of water from the cytoplasm (Poirier et al. 1997; Mille et al. 2004). The abrupt outflow of water characterizes an osmotic stress causing damages in the intracellular proteins and compromising the organization of membrane lipids (Broeckx et al., 2016; Kavitake, Kandasamy, Devi, & Shetty, 2018).

Although the LAB show adaptation strategies to survive to osmotic stress, such as the compatible solute accumulation and recovery of damaged proteins by chaperones, the dehydration is quite fast which prejudice the activation of any survival strategy (Desmond et al., 2002;Tsakalidou et al., 2011; Huang et al. 2016b).

In the second stage of drying, the dried shell around the droplet limits the water evaporation rate and a gradual increasing in the temperature is expected. Under this optics, the LAB are submitted to higher temperatures (60-80°C) for some seconds which now, represent a significant heat treatment to induce the cellular viability loss (Peighambardoust et al., 2011). As example for probiotics, particle temperatures around 70 °C are considered lethal (Corcoran et al., 2004; Gardiner et al., 2000; Zamora et al., 2006).

During heating, the LAB are submitted to a successive injuries including damages in intracellular and membrane proteins and DNA structure impairment (Santivarangkna, Kulozik, & Foerst, 2008). The denaturation of key enzymes to metabolism as well as the compromise of structures involved in the reproduction and protein synthesis are decisive to conduct the cellular death (Ananta et al., 2005; Gong et al., 2014; Fu & Chen, 2011).

Although the heat treatment is associated as the primordial cause of cell death, the heating effect is not only more drastic because of low water content and occluded air in the particle restricts the heat diffusion (Chen & Patel, 2007). Furthermore, dry products with considerable porosity (greater than 25%) and a low moisture content are expected to have a low thermal conductivity range (Carson, 2015) which contributes to the survival of LAB.

During all drying process, the powder particles are in contact with the air and eventually cells positioned in the periphery or surface of droplet to be dried are exposed to oxygen (Guchte et al., 2002; Ghandi et al., 2012a). The O_2 in turn can give rise to the chemical compounds highly reactive, known as reactive oxygen species (ROS), able to oxide lipids and proteins (Niel, Hofvendahl & Hahn-Hägerdal, 2002).

Some microorganisms have mechanisms to detoxify themselves of ROS by production superoxide dismutase, catalase and glutationa peroxidase; however, LAB have no or low synthesis capacity of these enzymes (Condon, 1987). This justifies why these microorganisms are sensible to environment containing high oxygen content and can lose their viability by oxidative stress (Chen, Shen, Solem, & Jensen, 2013, Ghandi et al., 2012a).

After drying, the powder particles are recovered in the cyclone where stay in contact with the outlet air. Although particles cool in this stage, the powder can keep temperatures around 50 °C for a considerable amount of time and its water activity (Aw) can influence on the LAB survival (Schuck, 2011; Zuidam & Nedovic, 2012; Broeckx et al., 2016). Some works claims that the ideal Aw to preserve cells is close to 0.25 (Adhikari et al., 2009; Nualkaekul et al., 2012). Below this value, the lack of a hydration layer on the cells structure can facilitates the membrane lipid oxidation intensifying the stress oxidative (França, Panek & Eleutherio, 2007; Ezraty, Gennaris, Barras & Collet, 2017). On the other side, in more elevated Aw values, some metabolic enzymes can be active while other not (Higl et al., 2007). Consequently, several intermediate metabolites are accumulated in the cytoplasm causing disorganization in the metabolic via.

To prevent the propagation of lipid oxidation during storage of dried LAB, it is advised stock the powder in packaging that protects from light and in modified atmospheric composition without oxygen (Morgan et al., 2006; Schuck, 2011). Several works demonstrated that the storage under refrigeration conditions is ideal to prevent the cellular viability loss over time although of other works propose the storage under room temperature conditions (Teixeira, Castro and Kirby, 1994; Gardiner et al., 2002; Wang, Yu and Chou, 2004; Corcoran et al., 2004; Huang et al., 2016a; Huang et al., 2016b; Schuck et al., 2013). This last one is advantageous from the point of view of commercialization of dried cultures once bane the cold chain during transportation and storage of powder in the industry.

Althrough storage at room temperature is more pratical and reduces some costs; the refrigeration decreases the chemical reactions rate in the powder prolonging the viability of LAB. Therefore, the choice of storage temperature should taking account the energetic cost of this and, in the same time, the degree of microorganism survival over time.

5. Conclusion

The spray drying has been approached by scientific literature as a suitable technique to produce dried cultures to industrial application. Although the high number of works claiming the benefits of this technique, what is actually perceived is a very low production of spray dried LAB by industry.

The reason to this resides in the fact that the maintenance of cellular viability is not easy to control and can exigence several adaptations on the drying process, which can be unfeasible in some industrial plants. In addition, LAB can have their technological characteristics compromised after drying increasing drastically the fermentation time and resulting in more expensive fabrications.

The scientific works have focused now in propose alternatives to solve the problems of viability loss during the spray drying. Among the strategies discussed in the literature, can be cited as more promising the selection of heat resistant strains, cell adaptation to subletal stress before drying, definition of ideal operational drying parameters, choice of carrier material with thermal protection and optimization of storage conditions.

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CHAPTER 2

Determination of Ideal Water Activity and Powder Temperature After Spray Drying to Reduce Lactococcus lactis Cell Viability Loss

ABSTRACT

Spray drying presents a promising technology for preserving bacteria despite a low survival rate of heat-sensitive cultures when subjected to the drying process. The aim of this study was to determine the ideal powder parameters (water activity (Aw) and temperature ($T°C_{powder}$) needed to produce dehydrated Lactococcus lactis subsp. lactis with a high viability after drying. Cell concentrates injected into a spray dryer using varying cell concentrate flow rates ($F_{concentrate}$ = 0.3 to 1.0 $Kg.h^{-1}$) and inlet air temperatures ($T°_{inlet\ air}$ = 115 to160 °C), resulted in powders with different values of Aw, $T°C_{powder}$, and different levels of cell viability loss. Lower cell viability reduction (~0.43 log cycles) was obtained in conditions of Aw = 0.198 and $T°C_{powder}$ = 52 °C which can be met by using $T°_{inlet\ air}$ ~126 °C regardless of $F_{concentrate}$ values. After 60 days of storage at room temperature, cell population varied from 7.0 x 10^5 to 1.1 x 10^8 $UFC.g^{-1}$. The initial powder Aw had no influence on cell death rate, but $T°C_{powder}$ influence was observed. The approach adopted in this study can be applied to other bacteria or spray dryer equipment to determine optimal drying conditions.

Keywords: Starter culture, Water activity, Powder temperature, Drying conditions, Storage, Cell viability.

1. Introduction

In dairy technology, lactic acid bacteria (LAB) are added to food to obtain desirable sensory characteristics through the production of lactic acid, flavor compounds, thickening agents and enzymes with lipolytic or proteolytic activities (Vlieg et al., 2006). Furthermore, LAB as starter culture helps standardize food production as well as aiding biopreservation/bioprotection via the synthesis of antimicrobial molecules which protect against deteriorating and pathogenic bacteria (Frantzen, Kleppen, & Holo, 2017).

In most cases, industrial use of LAB as starter culture relies on dehydration preservation methods that ensure high viability and cell metabolic activity during drying, storage and industrial applications.

Freeze-drying or lyophilization is commonly used to preserve LAB because causes less stress to the cells and results in ferments with higher viability. However, industrial production and commercialization of freeze-dried bacteria can be limited by factors which include the high energy costs of batch drying and refrigerated powder storage (Ghandi, Powell, Chen, & Adhikari, 2012).

Alternately, the energy costs for spray drying are 10 times lower than freeze drying. Spray drying also permits continuous production and possible room temperature powder storage (Schuck et al., 2013). Despite these considerable advantages, this technique remains underused due to a low survival rate of heat-sensitive LAB cultures (Huang et al., 2016).

Among LAB food production applications, Lactococcus lactis strains stand out due to their high acidification potential, caseinolytic and lipolytic activities, and their ability to both produce compounds with organoleptic qualities and inhibit pathogenic microorganisms via bacteriocins such as nisin, lacticin and lactococcin (Attar et al., 2018). Nevertheless, the thermal-sensitivity of these bacteria remains a drawback to production of spray-dried ferments (Ghandi et al., 2012).

The inactivation of L. lactis strain during spray drying procedures is generally only linked to thermal damage suffered by the cells. Nevertheless, the effect of water activity on cell death has not previously been studied. Certain studies claim that water activity presents an important parameter for bacterial heat inactivation in dried food

matrices, although this element has often been overlooked in research on LAB drying techniques (Lian et al., 2015; Smith et al., 2016; Syamaladevi et al., 2016).

Based on the current lack of information, the objective of this work has been to determine the ideal water activity and temperature required during LAB powder production in order to produce dehydrated L. lactis cells with low viability loss during the drying process. The effect of water activity and powder temperature on the cell viability loss during storage was also investigated.

2. Material and methods

2.1 Drying medium preparation

200 g of whole milk powder were dispersed in 250 g of sterile distilled water in order to obtain concentrated reconstituted milk with a final solid content of 40 °Brix when measured with a refractometer (Biobrix, Brazil). The concentrate was heated to 85 °C for 1 h to eliminate any possible microorganism contaminants as proposed, with modifications, by Ananta, Volkert, &, Knorr, 2005. The drying medium was then refrigerated until the temperature reached 40 °C.

2.2 Preparation of cell concentrate

Lactococcus lactis subsp. lactis (ATCC® 7962™), a nisin-producing LAB with potential applications in the dairy industry, was used as model for thermal-sensitive bacteria in this study. 100 µL of the strains kept at -60 °C in Eppendorf tubes containing Man, Rogosa and Sharpe broth (MRS, Difco, France) and 30 % ($v.v^{-1}$) glycerol, were transferred to a tube containing 10 mL of MRS broth and incubated at 30 °C/ 18 h. For a second activation, all tube contents were inoculated into 500 mL of MRS broth which was incubated at 30 °C during 18 h. The cell culture was centrifuged (Eppendorf, German) at 5000 x g for 10 min and the supernatant was discarded. The pellet was washed twice in saline solution 0.85% ($w.v^{-1}$) and resuspended in a drying medium to obtain a cell concentration of approximately 10^9 CFU per gram of dry matter. The cell concentrate was kept in a water bath at 40 °C until drying began.

2.3 Drying conditions and storage of dried cells

The cell concentrate was injected into a pilot scale spray dryer model MSDi 1.0 (Labmaq do Brasil, Brazil) at cell concentrate flow rates ($F_{concentrate}$) varying from 0.3 to 1.0 $Kg.h^{-1}$ while the inlet air temperature ($T°C_{inlet\ air}$) varied from 115 to 160 °C (Figure 1).

At the end of the cycle, the powders were transferred to sterile glass bottles equipped with thermometers to monitor the powder temperature ($T°C_{powder}$, in °C) (Figure 1).

Different combinations of $F_{concentrate}$: $T°C_{inlet\ air}$, as shown in Figure 1, were made in order to produce powders with various water activity values (aw) and $T°C_{powder}$.

The powders were placed in hermetically-sealed sterile glass bottles and stored at room temperature (24.4 ± 1.3 ºC) for 60 days.

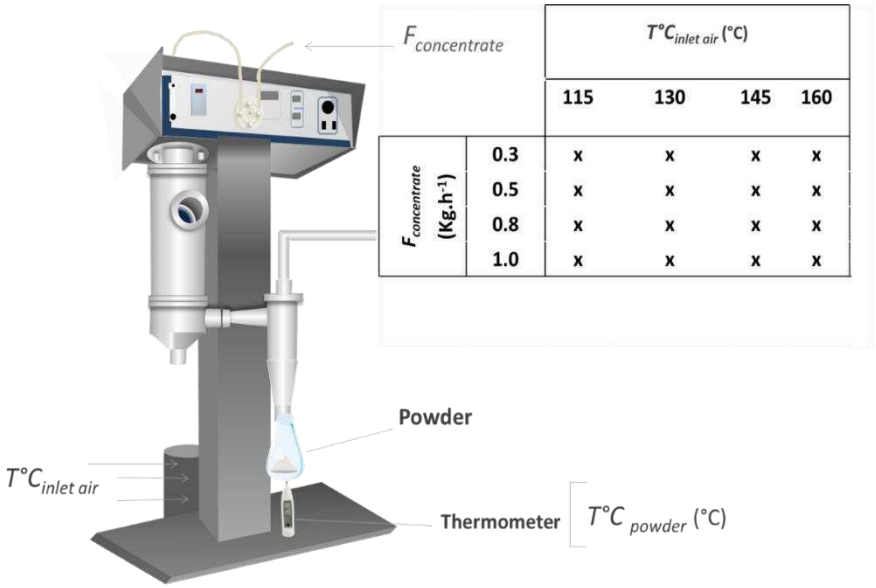

Figure 1 - Schematic representation of the experiment setup.

2.4 Physical analysis of powders

2.4.1 Water activity

The water activity (Aw) of the samples obtained was determined at 25 ºC using a water activity meter (Aqualab, Decagon 3TE, Decagon Devices Inc., USA). All measurements were performed in duplicate.

2.4.2 Moisture and dry extract analysis

3 g of cell concentrate, or 1.5 g of powder, were mixed with sand and heated at 105 °C until they attained a constant weight (~5 h) (Schuck et al., 2005). Moisture (M%) was calculated by determining weight loss after heating expressed in percentage, while the dry extract (DE, in %) values were determined by the follow equation:

$$DE = 100 - M\% \quad (1)$$

2.5 Determination of cell viability in concentrate and powders

Cell viability was evaluated in the cell concentrate and the powders immediately after drying (time: 0 days) and during storage. 1 mL of concentrate or 1 g of powder was dispersed in 9 mL of sterile saline solution 0.85% ($w.v^{-1}$) and serial dilutions were performed in the same diluent.

The enumeration of viable cells was performed by microdroplet technique. An aliquot of 20 μL of appropriate dilution was deposited on the surface of MRS agar and incubated at 30 °C/ 72 h in anaerobic jars. The enumeration of colony-forming units was performed in triplicate and conducted in droplets containing between 8 and 80 colonies.

The number of viable cells (N) was calculated in relation to dry material content ($CFU.g^{-1}$) of concentrate or powder using equation 2:

$$N = n \times DF / aliq \times DE \quad (2)$$

Where n represents the number of colonies counted, DF is the dilution factor, aliq denotes the aliquot plated (0.02 mL for concentrate or 0.02 g for powder) and DE is the dry extract given in $g.mL^{-1}$ or $g.g^{-1}$ for the cell concentrate and powder, respectively.

The cell viability loss was determined by the following equation:

$$\text{Viability loss} = \log N_0 - \log N' \quad (3)$$

Where N_0 represents the number of $CFU.g^{-1}$ of surviving L. lactis in the cell concentrate while N' indicates the number of viable cells ($CFU.g^{-1}$) just after drying.

2.6 Calculation of inlet energy

The thermal energy used by the equipment to dehydrate the L. lactis cells (E_{inlet}) was estimated according the following equation proposed by Silva et al. (2017):

$$E_{inlet} = [T°_{inlet\ air}\ (1.01+1.89\ AH_{inlet}) + 2500\ AH_{outlet}]\ [54.35\ (1+AH_{out})^{-1}] +129.933\ F_{concentrate} \quad (4)$$

Where $T°_{inlet\ air}$ corresponds to inlet air temperature; AH_{inlet} and AH_{outlet} is the absolute humidity of air (kg water·(kg dried air)$^{-1}$) in the inlet and outlet of equipment, respectively; and $F_{concentrate}$ denotes the cell concentrate flow rate.

2.7 Statistical analysis

The drying processes were carried out with three repetitions and the results were compared using the Student's t-test statistical method with a significant difference at p-value < 0.05. In addition, the results were fitted by regression analysis. The statistical analysis was made using Excel® software.

3. Results and discussion

3.1 Influence of input parameters on Aw and powder temperatures

Several food matrixes have been considered for use as carrier material for spray drying the bacteria; among them, reconstituted milk was chosen to dry L. lactis cells due to recognized protective properties of dairy proteins, calcium and lactose when subjected to drying conditions (Rudolph and Crowe, 1985; Zheng et al., 2015; Huang et al., 2016).

The water activity (Aw) and the temperature of powders ($T°C_{powder}$), measured just after drying, were regulated by varying the operational spray dryer input parameters (Figure 2).

When the inlet air temperature was increased ($T°C_{inlet\ air}$), the powder water activity went down (Figure 2A) which is in keeping with previous studies (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fisk, 2013; A-Sun, Thumthanaruk, Lekhavat, & Jumnongpon, 2016; Dantas et al., 2018). This behavior can be explained by the increased rate of heat transfer into the particles at higher temperatures, which boosts the driving force for moisture evaporation and causes higher water removal from food matrix (Solvol, Sundarajan, Alfaro & Sathivel, 2012).

However, the observed quadratic relation probably occurs due to the greater difficulty there is to remove water molecules that are contained inside macromolecules or immobilized by hydrogen binding (Lewicki, 2004). Thus, the variation of Aw in function of $T°C_{inlet\ air}$ (Figure 2A) is obtained using the following equation:

$$Aw = 10^{-4}(T°C_{inlet\ air})^2 - 0.029(T°C_{inlet\ air}) + 2.31 \qquad R^2 = 0.98 \qquad (5)$$

By increasing the inlet air temperature, the powder temperature increased proportionally as shown in Figure 2B and obtained using equation 6:

$$T°C_{powder} = 0.288(T°C_{inlet\ air}) + 15.4 \qquad R^2 = 0.98 \qquad (6)$$

According to Bimbenet (1978), a low pressure system promotes water evaporation without high heating of the particle during initial contact between a particle and inlet air. During this step, which the particle surface temperature is around 45 °C (wet bulb temperature), low cell inactivation is expected (Dittman & Cook, 1977). However, after free water molecules had been completely evaporated, the thermal energy from the air gradually increased the dried particle temperature, thus promoting progressive thermal damages to the bacteria.

It can therefore be concluded that increasing the inlet air temperature proportionally increases the drying energy (Figure 3A), which results in powders with higher temperatures once processing has been completed.

By contrast, cell concentrate flow rates ($F_{concentrate}$) had no significant influence on the Aw and powder temperatures in the tested range (Figures 2C and D).

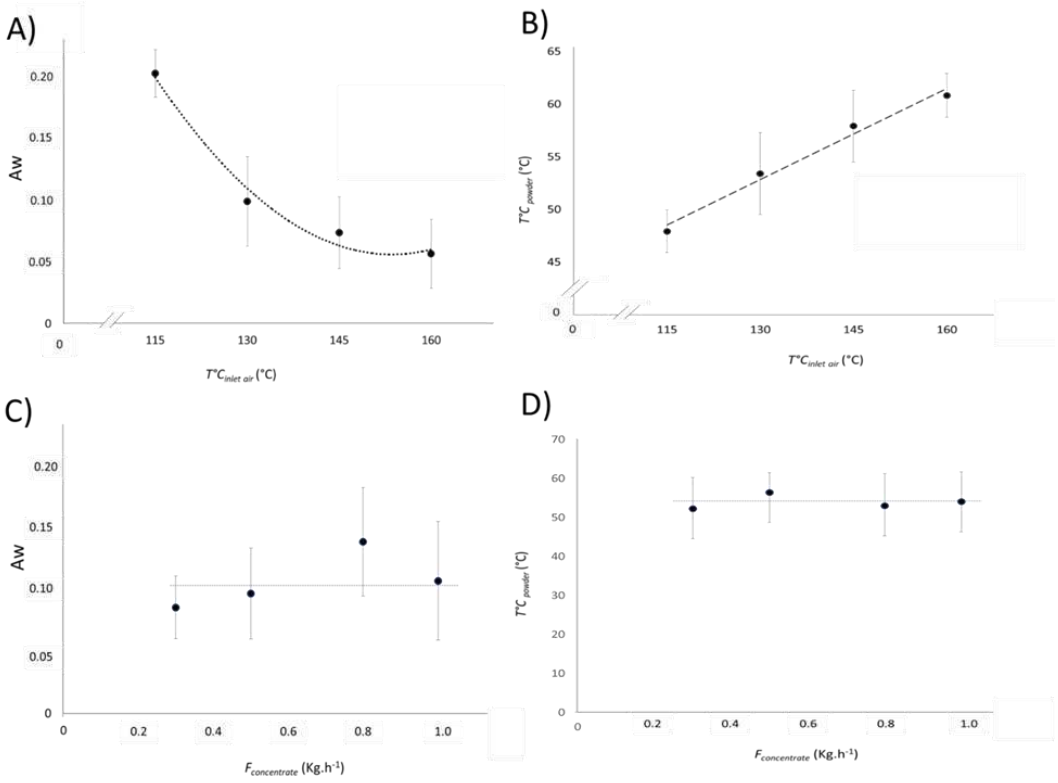

Figure 2 - Relation between inlet air temperature ($T°C_{inlet\ air}$) and cell concentrate flow rate ($F_{concentrate}$) for Aw (A and C) and powder temperature ($T°C_{\ powder}$) (B and D).

Although the increase in cell concentrate flow rate contributes positively to the thermal energy increase applied to drying (E_{inlet}), as proposed by Silva et al. (2017) (see equation 4), the flow rate ranges tested showed low impact on the inlet energy (between 39 and 130 $kJ.kg^{-1}$). A low variation of E_{inlet} in function of $F_{concentrate}$ was shown to be the case (Figure 3B), which help justify the results observed in Figures 2C and D.

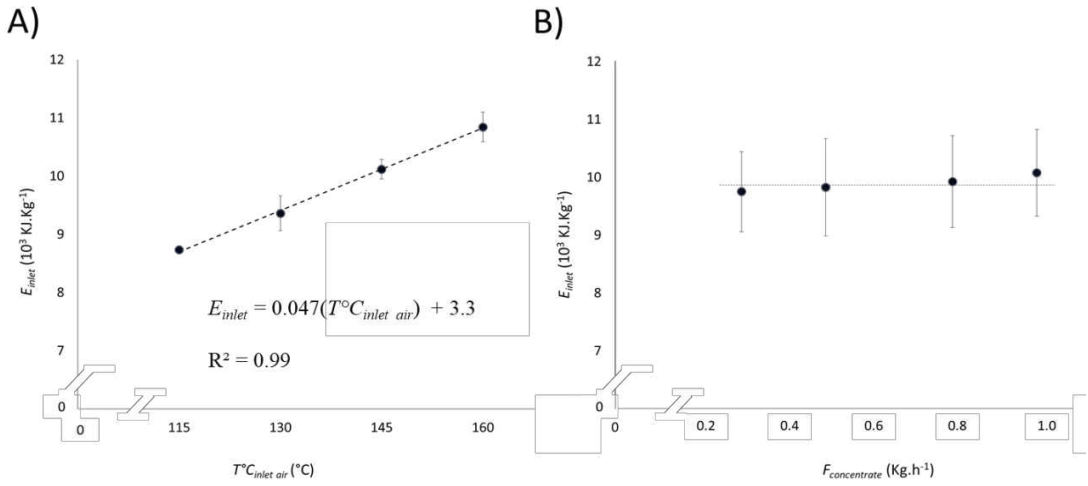

Figure 3 - Relation between inlet air temperature ($T°C_{inlet\ air}$) (A) and cell concentrate flow rate ($F_{concentrate}$) (B) for inlet thermal energy (E_{inlet}).

3.2 Effect of Aw and T°C $_{powder}$ on viability loss just after drying

Cell viability was significantly influenced by water activity variations and powder temperatures after drying (Figures 4A and B). The relation between loss of cell viability and powder water activity followed a quadratic function pattern (Figure 4A) represented by this adjusted equation:

$$\text{Viability loss} = 59.65\ (Aw)^2 - 23.66\ Aw + 2.76 \qquad R^2 = 0.99 \quad (7)$$

This type of behavior suggests that when Aw < 0.10 or Aw > 0.20, higher cell viability loss can be expected (Figure 4A). For low Aw values, the cell inactivation mechanism can be associated with lipid oxidation which promotes the release of free radicals that are harmful to cells (França et al., 2007). Furthermore, lipid oxidation can occur on the cell membrane, thus causing irreversible damage to its structure and resulting in loss of viability (Ezraty et al., 2017).

Alternately, the inactivation of cells in the powders with elevated Aw values could be explained by the higher thermal conductivity of water molecules which transfer heat more efficiently to cells while the powder is in in the drying chamber.

In addition, Higl et al. (2007) suggest that in higher Aw situations, cells might enter a partially active state in which some enzyme systems are active, but others are not. Consequently, the intracellular accumulation of intermediate metabolites that may occur can causing detrimental effects on the microorganisms.

In concordance with our findings, Vesterlund and collaborators (2012) demonstrated that the viability of Lactobacillus rhamnosus GG in dry food matrices during storage was reduced in powders containing high Aw values (0.43).

To estimate the ideal water activity for preserving the cells in a given powder, a derivative of equation 7 should equal zero. With this equation, it is possible estimate that an Aw = 0.198 guarantees the highest L. lactis survival rate after drying.

A direct relation between cell viability loss and powder temperature was found (Figure 4B) as determined by equation:

$$\text{Viability loss} = 0.045\ (T°C_{\text{powder}}) - 1.67 \qquad R^2 = 0.93 \quad (8)$$

According to Silva et al. (2018), the temperature is the most important process variable in the microbial inactivation and it is a direct measurement of the energy transferred by a substance in the form of heat.

In this case, the drying conditions should be regulated in order to reduce the maximum as possible the temperature of powder after drying. This result suggests that powder temperature is associated with thermal damage suffered by cells that results in a more pronounced death rate with the increasing in the $T°C_{powder}$.

In accordance with this result, Perdana et al. (2012) and Khem et al. (2015) also verified that the viability loss of Lactobacillus plantarum cells increased in powders subjected to higher temperatures after drying. Ananta et al. (2004) also concluded that the viability loss of Lactobacillus rhamnosus in skim milk powder was directly proportional to powder temperature.

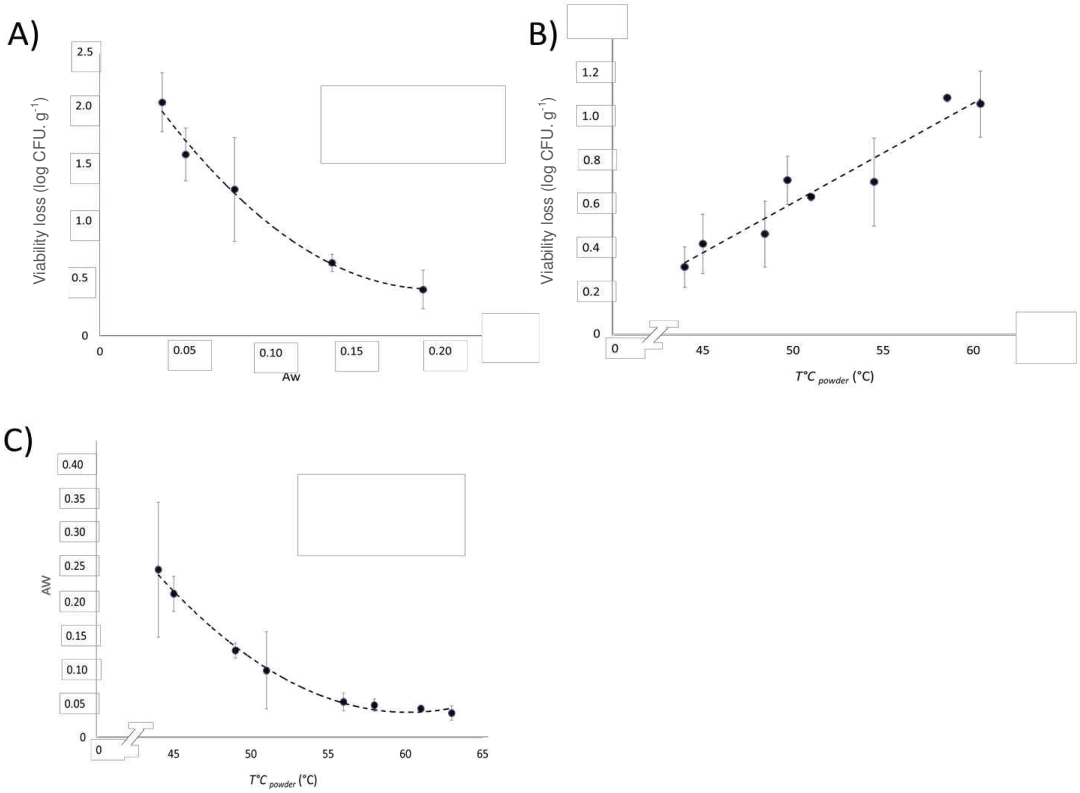

Figure 4 - Effect of Aw (A) and $T°C_{powder}$ (B) on cell viability loss. Relation between Aw and $T°C_{powder}$ of powders (C).

It has been shown that the combined effects of heat and mechanical stress result in cellular damage that lead to a loss of viability of microorganisms (Chávez & Ledeboer, 2007). These cellular injuries include DNA and RNA denaturation, ribosomal damage, dehydration and destabilization of plasma membrane due to water removal (Silva et al., 2018).

The results showed in Figures 4A and 4B indicate that of L. lactis survival depends both on water activity and powder temperatures at the end of processing. Thus, a compromise between these two parameters must be made to determine optimal drying conditions.

By correlating Aw values and powder temperatures obtained from the experiment results, a second-order polynomial curve can be obtained with the following equation (Figure 4C):

$$Aw = 0.0008\ (T°C_{\ powder})^2 - 0.092\ (T°C_{\ powder}) + 2.82 \qquad R^2 = 0.99 \quad (9)$$

With an ideal value of Aw (0.198) in equation 9, 52 °C appears to be the ideal temperature for the powder after drying.

When Aw = 0.198 in equation 5, it can be estimated that inlet air temperature should be adjusted to 125 °C for minimal cell viability loss. Conversely, replacing $T°C_{powder}$= 52 °C in equation 6 would set the inlet air temperature estimate at 127 °C. By determining the average of these two calculations, higher cell survival can be expected in conditions of inlet air temperature at around 126 °C regardless of cell concentrate flow rate.

Although the inlet air temperatures determined by equations 5 and 6 are close, the difference in the calculated values can be explained by mathematical rounding and measurement error.

In our experiments, drying L. lactis at $T°C_{inlet\ air}$ = 130 °C resulted in lower mean viability loss (0.43 log cycles) compared to other inlet air temperatures used in this study (viability loss between 0.60 and 1.22 log cycles).

3.3 Effect of Aw and T°C$_{powder}$ on viability loss during storage

In the first part of our experiments, it was demonstrated that the Aw and T°C$_{powder}$ have an important effect on the viability loss of L. lactis just after the drying. In this second part, the influence of initial Aw and T°C$_{powder}$ values on cell survival during storage was investigated.

The dried L. lactis cells were placed in hermetically-sealed bottles under ambient conditions for 60 days and a progressive reduction in the cell population was observed over time (Figure 5).

The powders' water activity had no significant influence on the death rate of cells during storage, probably due to the fact that powders conserve low available water after drying (Figure 5A). According to Ying and collaborators (2016), water would be not available for degradative reactions or solubilization/component mobility in the formulation because the powders maintained low water activity values. This helps explain why the cell death rate remains constant even with Aw variations in the powders.

Our findings are corroborated by Farakos et al. (2013) who observed that Salmonella cells maintained a constant death rate when stored in dairy powders with Aw between 0.16 and 0.26. By contrast, other studies involving pathogens and probiotics demonstrated an increase in cell death rate with an Aw increase in the powders (Lian et al., 2015; Romano et al., 2018). It should be noted that the cited works used a broader range of Aw values (from 0.11 to 0.75) than were tested in this study.

Considering that water availability in the interval is low, cell death can be explained in part by lipid oxidation when storage conditions were not kept free of oxygen and ambient light.

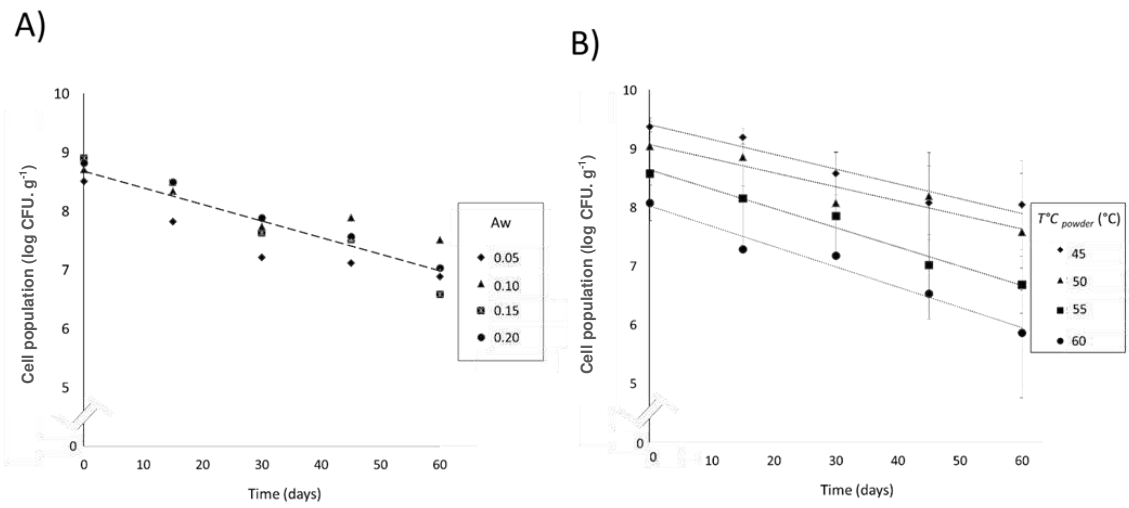

Figure 5 - Effect of Aw (A) and T°C $_{powder}$ (B) on the viability loss during storage time

By contrast, the higher the $T°C_{powder}$ after drying, the higher the cell death rate during storage (Figure 5B). For example, when $T°C_{powder}$ = 45 °C, the cell population went down by 0.025 log cycles.day^{-1} whereas when $T°C_{powder}$ = 60 °C the reduction was around 0.035 log cycles.day^{-1}.

According to Farakos et al. (2013), heating cells to temperatures above their maximum growth temperature causes damage to the cytoplasmic membrane and ribosome degradation, which can provoke injuries to the microorganisms. In these cases, rigorous thermal treatments probably made the strains more susceptible to cellular component oxidation over time (Niel, Hofvendahl, & Hahn-Hägerdal, 2002; Santivarangkna, Kulozik, & Foerst, 2008).

After 60 days of storage, the cell population went down from 1.32 to 2.15 log cycles depending on the powders' initial temperatures (Figure 5B). In conditions where $T°C_{powder}$ = 45 °C, the viable cell concentration after 60 days was approximately 1.1.x10^{8} CFU.g^{-1} while a lower population (~7.0 x10^{5} CFU.g^{-1}) was found when $T°C_{powders}$ = 60 °C (Figure 5B).

The viability of L. lactis in the powders after storage was still relatively low compared to a sample of commercially-available freeze-dried cultures (~10^{9} to 10^{11} CFU/g) (Ishibashi and Shimamura, 1993; Huang et al., 2016). Nevertheless, the results are promising when it is considered that a thermal-sensitive bacterium was tested and that the storage conditions were not optimized to guarantee high cell survival.

4. Conclusion

Drying heat-sensitive LAB remains a challenge in the dairy industry because of low cell survival rates after drying. Low cell survival occurs especially when elevated inlet air temperatures are used in the process.

In this study, it was demonstrated that the survival rate of spray-dried cells is determined by powder parameters such as water activity and end-of-process temperatures. This approach has been used in studies involving pathogens in dried food matrices but evaluating cell viability in drying procedures had not previously been investigated.

Under the experimental conditions, the powder should reach an ideal Aw value of 0.198 and temperature of 52 °C to guarantee maximal cell survival after drying. By means of mathematical tools, it was predicted that the inlet air temperature should be set around 126 °C, regardless of cell concentrate flow rate, in order to obtain the ideal parameters of Aw and $T°C_{powders}$.

In future research, other carrier materials and optimized storage conditions will be tested in order to improve the LAB viability during prolonged storage.

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CHAPTER 3

Effect of lactose and fat contents on the microencapsulation of Lactococcus lactis in dairy matrices

ABSTRACT

Adverse conditions during the spray-drying process can be minimized by the application of good carriers. Milk matrices stand out as carrier agents since contribute to increase the cell survival after the drying process. The objective of the study was to evaluate the effect of encapsulating dairy matrices containing different lactose and fat contents on the survival of Lactococcus lactis during drying and storage. The encapsulating with lactose hydrolysis resulted in more pronounced cell viability loss (3.90 log cycles). However, encapsulating medium supplemented with lactose ($EM_{19\%}$ and $EM_{20\%}$) or without fat ($EM_{19\%}^{no\ fat}$) showed protection effect against the adverse drying conditions besides guarantee the higher cell survival when storage at room temperature. In contrast, the fat content has low contribution on the L. lactis preservation. The results revealed that the supplementation of milk with lactose seems to be ideal to drying of heat-sensitive bacteria.

Keywords: spray dryer, Lactococcus lactis, viability, lactose, fat, dairy matrice

1. Introduction

Among the latic acid bacteria (LAB) applied in food industry, Lactococcus lactis strains stand out due to its high acidification potential, caseinolytic and lipolytic activities, production of compounds with organoleptic qualities and inhibition of pathogenic bacteria by bacteriocins such as nisin, lacticin and lactococcin (Harris, Fleming & Klaenhammer, 1992; Sahraoui et al., 2015; Attar et al., 2018). In most of cases, the exploitation of L. lactis as starter culture depends on its preservation by dehydration technologies which must ensure a high viability and metabolic activity of cells during the application in industrial processes.

Spray-drying is a promising technology to preserve LAB, once it has energetic cost 10 times lesser compared to freeze-drying besides the possibilities to operate in continuous process and powder storage at room temperature (Schuck et al., 2013). However, during the drying, L. lactis is submitted to adverse conditions responsible to cause sequential injuries to cells, being these, the responsible factors by pronounced cell viability loss (Santivarangkna et al., 2008).

In order to overcome this drawback, several works has indicate milk or dairy matrices as encapsulating materials responsible to protect LAB against the harmful drying spray conditions (Maciel et al., 2014; Tavares, Croguennec, Carvalho, & Bouhallab, 2014; Zheng et al., 2015). Although of the protecting mechanism of milk is not well defined, some works appoint that calcium stabilizes cellular structures besides to combine with milk proteins to form agglomerates with cells, which cushions the stresses caused by dehydration and high temperature (Huang et al., 2016). The heat protection effect of milk is usually associated to its proteins but the role played by the other dairy components is little known and exploited by literature (Soukoulis et al., 2014). In special, the contribution of the lactose and dairy fat to maintenance of L. lactis viability has not yet been reported.

The aim of this study was evaluate the effect of encapsulating dairy matrices containing different lactose and fat contents on the survival of spray dried L. lactis. In addition, the protective behavior of encapsulating matrices during storage was also investigated.

2. Material and methods

2.1 Culture conditions and preparation of cell concentrate

Lactococcus lactis subsp. lactis, isolated from artisanal cheese produced in the Amazonian region (Martins et al., 2018) was selected due to pronounced thermosensitivity demonstrated in preliminary studies.

One hundred microliter of the strain kept at -60 °C in Eppendorf tubes containing Man, Rogosa and Sharpe broth (MRS, Difco, France) and 30% $v.v^{-1}$ of glycerol, was transferred to a tube containing 10 mL of MRS broth and incubated at 30 °C/ 18 h. For a second activation, 1% $v.v^{-1}$ of pre-activated culture was inoculated into 250 mL of MRS broth which was incubated at 30 °C during 18 h.

After incubation, the cell suspension was centrifuged at 5000 x g for 10 min at 4 °C (Eppendorf, Germany) and the supernatant discarded. The pellet (cell concentrate) was washed twice in saline solution 0.85 % $w.v^{-1}$.

2.2 Preparation of encapsulating dairy matrices

The encapsulating matrices were produced from whole milk powder or skimmed milk powder containing 37.5 and 51% $w.w^{-1}$ of lactose, respectively (Itambé, Brazil).

Encapsulating matrix containing 0% $w.w^{-1}$ of lactose was produced by dilution of whole milk powder in distillated water followed by hydrolysis of lactose by enzyme β-galactosidase (Prozyn, Brazil) as described by Fialho et al. (2017) with modifications (Table 1). Briefly, 0.6% $w.w^{-1}$ of β-galactosidase was added to the reconstituted milk and the lactose hydrolysis carried out over 4 h.

Encapsulating matrix with 17% $w.w^{-1}$ of lactose was prepared by dissolution of 100 g of whole milk powder in 125 g of distillated water (Table 1). Matrices containing 19 or 20% $w.w^{-1}$ of lactose were similarly prepared from whole milk powder only differing by addition of 5 and 10 g of lactose crystals (Cromoline, Brazil), respectively (Table 1).

Encapsulating matrix without fat was produced by dissolution of 80 g of skimmed milk powder in 140 g of distilled water. All formulations and their compositions are displayed in the Table 1.

The encapsulating matrices were heat treated at 85 °C / 1 h as proposed by Ananta et al., 2005, with modifications, and refrigerated to 40°C to inoculation of cell concentrate.

Table 1 - Composition of drying medium (encapsulating matrices)

Encapsulating matrix	Formulation	Composition (% w.w^{-1})		
		Lactose	Fat	Protein
EM$_{0\%}$	100 g whole milk powder + 1.35 g β-galactosidase + 125 g water	0	12	11
EM$_{17\%}$	100 g whole milk powder + 125 g water	17	12	11
EM$_{19\%}$	100 g whole milk powder + 5 g lactose crystals + 125 g water	19	11	10
EM$_{20\%}$	100 g whole milk powder + 10 g lactose crystals + 125 g water	20	11	10
EM$_{19\%}^{\text{no fat}}$	80 g skimmed milk powder + 140 g water	19	0	12

2.3 Spray drying of L. lactis and storage of dried cells

Cell suspensions (cell concentrate + encapsulating matrix) containing approximately 10^9 CFU.g^{-1} were dried in spray dryer model MSD 1.0 (Labmaq, Brazil), by using drying parameters previously determined: inlet air temperature = 130 °C and cell suspension flow rate = 0.5 Kg.h^{-1}. The powder was recovered in sterile glass bottles and storage at 4 or 25 °C.

2.4 Physical analysis of powders

2.4.1 Water activity (Aw)

The water activity of powders was determined at 25 ºC using a water activity meter (Aqualab, Decagon 3TE, Decagon Devices Inc., USA). All measurements were performed in duplicate.

2.4.2 Moisture and dry extract analysis

3 g of cell suspension, or 1.5 g of powder, were mixed with sand and heated at 105 °C until they attained a constant weight (~5 h) (Schuck et al., 2005). Moisture (M%) was calculated by determining weight loss after heating expressed in percentage, while the dry extract (DE, in %) values were determined by the follow equation:

$$DE = 100 - M\% \quad (1)$$

2.4.3 Powder microstructure

The morphology of powder particles containing L. lactis cells was evaluated by scanning electron microscopy (Hitachi TM 3000, Hitachi Ltd., Tokyo, Japan). The size of particles was determined by image analysis of 100 particles by using imageJ® software. The measurements were performed in triplicate totalizing 300 particles analyzed by sample.

2.5 Determination of cell viability

Cell viability was evaluated in the cell suspension or in the powders immediately after drying (time: 0 days) and during storage. 1 mL of concentrate or 1 g of powder was dispersed in 9 mL of sterile saline solution 0.85% $w.v^{-1}$ and serial dilutions were performed in the same diluent.

The enumeration of viable cells was performed by microdroplet technique which an aliquot of 20 µL of appropriate dilution was deposited on the surface of MRS agar and incubated at 30 °C/ 72 h in anaerobic jars. The enumeration of colony-forming units was performed in triplicate and conducted in droplets containing between 8 and 80 colonies.

The number of viable cells (N) was calculated in relation to dry material content ($CFU.g^{-1}$) of concentrate or powder using equation 2:

$$N = n \times DF / aliq \times DE \quad (2)$$

Where n represents the number of colonies counted, DF is the dilution factor, aliq denotes the aliquot plated (0.02 mL for cell suspension or 0.02 g for powder) and DE is the dry extract given in $g.mL^{-1}$ or $g.g^{-1}$ for the cell concentrate and powder, respectively.

The cell viability loss was determined by the following equation:

$$\text{Viability loss} = \log N_0 - \log N' \quad (3)$$

Where N_0 represents the number of $CFU.g^{-1}$ of surviving L. lactis in the cell concentrate while N' indicates the number of viable cells ($CFU.g^{-1}$) just after drying.

Cell viability after storage was evaluated by the difference between initial population in the powder immediately after drying (log N') and final population after 45 days of storage.

2.6 Statistical analysis

The drying processes were carried out with three repetitions and the results were evaluated by ANOVA and Tukey test ($p < 0.05$) by means of the comparison between means. Statistical analysis was performed using the PAST software.

3. Results and discussion

3.1 Microcapsules characterization

The microscopic analysis of powders revealed the majority presence of microcapsules with spherical shape varying between 3.0 and 22.6 μm in mean diameter (Figure 1). Microcapsules produced from lactose hydrolyzed milk (Figure 1A) show higher mean diameter compared to other encapsulating materials ($p<0.05$), what in part is explained by presence of bigger particles and aggregation between them.

Microcapsules produced with 19 and 20% $w.w^{-1}$ of lactose showed average size of 5.4±2.1 μm (Figures 1C and 1D). However, 17% $w.w^{-1}$ of lactose resulted in larger microcapsules (10.8±4.0 μm) ($p<0.05$) (Figure 1B). By contrast, the fat removal of milk resulted in smaller microcapsules (3.0±1.0 μm) ($p<0.05$) with a shrink surface.

In concordance with our observations, Fialho et al. (2017) also found that microcapsules formed from milk without lactose have higher tendency to suffer agglomeration. According to Schuck et al. (2005), the lactose hydrolysis promotes the lowering of glass transition temperature of powder which in microscopic scale is characterized by aggregation between the microcapsules. Bigger microcapsules produced from lactose hydrolyzed milk were also reported by Roos (2002), Fernández et al., (2003) and Torres et al., (2017). The lactose hydrolysis increases the apparent viscosity of milk resulting in the formation of bigger droplets by atomizer and, consequently, bigger particles after drying (Schuck et al., 2005; Paramita, Iida, Yoshii, & Furuta, 2010).

Some aggregation between microcapsules can be observed by microscopy in the formulations containing lactose (Figures 1B, C and D). Contrary to found to formulation without lactose (Figure 1A), the aggregation in this case is caused by presence of fat in the particle surface making them stick to each other. In concordance, other authors also reported the sticking of microcapsules by presence of superficial fat (McKenna, 1997; Peleg, 1977).

Microcapsules produced by matrix without fat ($EM_{19\%}^{\text{no fat}}$; figure 1E) showed wrinkled surface characterizing an irregular shaped microcapsule as proposed by Martins et al., 2017. Etzel et al., (1996) indicate that the shrunk surfaces are formed when the material is overheated and posteriorly cooled. During heating the air bubbles inside the microcapsule expand; however these ones shrunk during cooling and the microcapsule withers.

From this approach, the results suggest that the fat have a heat protection effect considering that the microcapsules containing this component (Figure 1) showed spherical morphology.

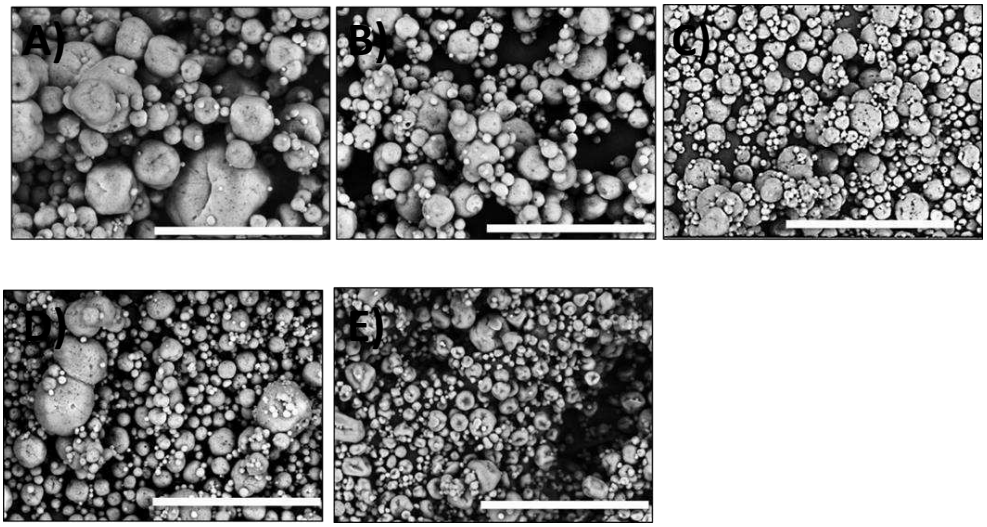

Figure 1 - Micrography of encapsulated L. lactis in dairy matrices with varied lactose and fat content. A) EM0%; B) EM17%; C) EM19%; D) EM20%; E) EM19%no fat. White bar: 100 μm.

3.2 Protective effect of encapsulating medium on the L. lactis cells

The encapsulating medium showed different degrees of cell protection depending on its composition in terms of lactose content (Figure 2).

The lactose hydrolysis resulted in more pronounced cell viability loss (3.90 log cycles) followed by medium containing 17% $w.w^{-1}$ of lactose (1.41 log cycles) (Figure 2). Encapsulating medium supplemented with lactose ($EM_{19\%}$ and $EM_{20\%}$) or without fat showed similar cell viability loss ($EM_{19\%}^{no\ fat}$) with no statistical difference (~ 0.26 log cycles) (Figure 2).

The lactose hydrolysis results in the conversion of one disaccharide in two others molecules (glucose and galactose) and thus, the osmotic pressure is also increased as predicted by van't Hoff law:

$$\pi = RT\left(\frac{n}{v}\right)i$$

where R = 8.314 J mol^{-1} K^{-1}; T = 293.15 K; n/V is the molarity of the solute in $mol.m^{-3}$ and i is the number of soluble particles.

In this way, it is believed that the increase in osmolarity difference between the cells and the drying medium intensifies the osmotic stress suffered by L. lactis during drying causing more pronounced cell viability loss. An increased osmotic pressure leads to cell death by loss of functional membrane structure (Korber et al., 1996; Pagán and Mackey, 2000; Laroche et al., 2001).

Furthermore, the milk-based microcapsules are constituted by a continuous mass of amorphous lactose, in which fat globules, proteins and cells are incorporated (Walstra et al., 1999). Therefore, the lactose hydrolysis reduces the encapsulating action of the dairy matrix leaving the cells more susceptible to heat, dehydration and oxidative stress during drying.

By the contrary, higher L. lactis survival was observed in medium containing lactose (Figure 2) suggesting a possible protective effect of this constituent against the drying conditions. As previously discussed, amorphous lactose is responsible to recover the milk constituents (proteins and fat) and the cells avoiding excessive thermal damages. As can be observed, increasing the concentration of lactose from 17 to 20 % $w.w^{-1}$ the cell viability loss reduced from 1.41 to 0.26 log $CFU.g^{-1}$ (Figure 2). Thus, may be suggested that 17% $w.w^{-1}$ of lactose was not sufficient to recover all cells.

On the other hand, no statistical difference was found between the treatments with 19% ($EM_{19\%}$) and 20% $w.w^{-1}$ ($EM_{20\%}$) of lactose (Figure 2). In this case, it is supposed that lactose should reach a critical concentration (~19% $w.w^{-1}$) to recover the cells and, above which, additional recovering not prevent the damages caused by drying.

In concordance, Perdana, Fox, Siwei, Boom, & Schutyser (2014) also verified that the increasing of trehalose concentration in the encapsulating matrix not resulted in reduction of Lactobacillus plantarum viability loss during drying.

The treatment without fat ($EM_{19\%}^{no\ fat}$) did not show statistical difference in relation to encapsulating matrix containing equivalent lactose concentration ($EM_{19\%}$) (Figure 2). Although to heat protection aspect of fat, as suggested by microcapsule morphology (Figure 1), an evident thermal protection of the cells was not verified.

Studies involving the effect of lactose on the survival of spray dried bacteria are explored, but still there is no consensus on the effect of lactose on cell survival during the drying process. In general, the protective effect of lactose is related to the ability to stabilize the membrane and cell macromolecules during the dehydration process by hydrogen bonding at previously occupied sites of water molecules (Rudolph and Crowe, 1985). Nevertheless, our findings suggest another protective mechanism of lactose based on its recovering property of cells.

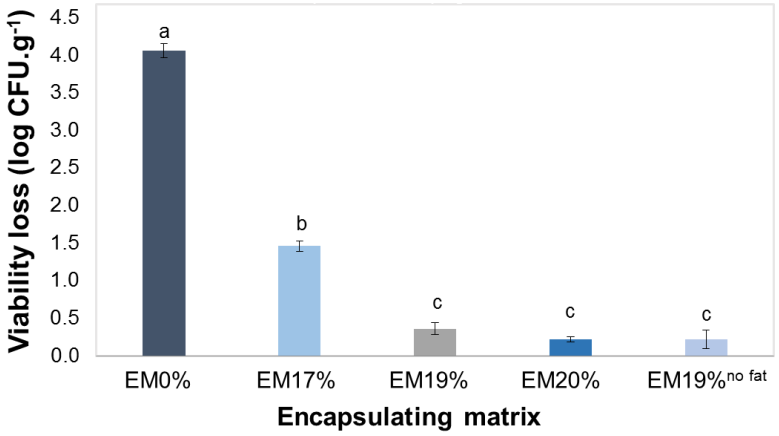

Figure 2 - Cell viability loss of L. lactis after spray drying. Different letters on the bars indicate statistical difference among the treatments ($p<0.05$).

3.3 Stability and survival of L. lactis during storage

The powders containing L. lactis cells were storage under refrigeration (4 °C) and room conditions (25°C) in transparent glass bottles hermetically sealed.

The storage temperature had no influence on the cell viability loss over time except to powder produced from lactose hydrolyzed milk ($EM_{0\%}$) (Figure 3A). In this case, the viability loss was 2.7 times greater at 25 °C than the 4 °C.

In relation the encapsulating matrices, lower viability loss (~ 0.45 $logCFU.g^{-1}$ cycles) was observed in the medium supplemented with lactose ($EM_{19\%}$ and $EM_{20\%}$) or without fat ($EM_{19\%}^{no\ fat}$) (Figure 3A).

By analysis of L. lactis survival over time, it is possible to infer that depending of the encapsulating material the cells can keep their viability no matter the storage temperature. In comparison with the freeze-dried microbial cultures available in the market, it can be considered a positive point once it dispenses the cold chain from transportation to storage in the industry.

Although the storage of dried culture at room condition is not usual, Schuck et al., 2013 also demonstrated the conservation of spray dried Propionibacterium acidipropioniciin in sweet whey permeate powder at room temperature. Likewise was observed high survival of freeze-dried Lactobacillus paracasei ssp. paracasei in lactose matrix during storage at 20°C (Higl et al., 2007).

On the other hand, a high loss of viability was observed of spray dried Lactobacillus plantarum 299v in maltodextrin stored at room temperature (Barbosa et al., 2015). In agreement, Soukoulis et al., 2014 related that increase in storage temperature was accompanied by increase of the inactivation rates of spray dried Lactobacillus acidophilus in sodium caseinate suggesting that the matrices used in these studies were not effective in cell encapsulation.

The viability loss in $EM_{0\%}$ was strongly influenced by storage temperature which is contrary to others matrices (Figure 3A). Works using lactose hydrolyzed milk as encapsulating material for bacteria are not available in the literature; however, a possible explication to the behavior displayed in the Figure 3A can be found in studies involving glass transition of powders (Roos, 2002; Schuck et al., 2005; Higl et al., 2007).

The glass transition consists of a change of the system from the vitreous state (high viscosity fluid) to the gummy state. This last physical state is characterized by a low viscosity solution conducting to important structural changes of material (Roos, 2002; Schuck et al., 2005).

The glassy transition occurs at a certain temperature, called the glass transition temperature (T_{Tg}), which is variable with the specific composition of product (Couchman & Karasz, 1978). The T_{Tg} of lactose (98°C) is approximately 3 times the T_{Tg} of glucose (31°C) and galactose (30°C) and, in this way, microcapsules produced from lactose hydrolyzed milk tends to present lower T_{Tg} (Roos, 1993). From a practical point of view, this means that the lactose hydrolyzed milk powder suffer glass transition in lower temperatures, which probably occurred in the microcapsules storage at 25 °C.

The change of the powder amorphous state during the storage leads to a decrease of the viscosity resulting in a greater mobility of the molecules, thus favoring the enzymatic reactions (Meste et al., 2002). Consequently, the recovering properties of milk constituents are lost and the bacteria are exposed to environmental conditions (oxygen, light).

In addition to galactose and glucose, water shows a very low T_{Tg} (-135 °C) and, for this reason, the water content should be kept reduced, mainly in lactose hydrolyzed milk powders due to higher susceptibility to glass transition.

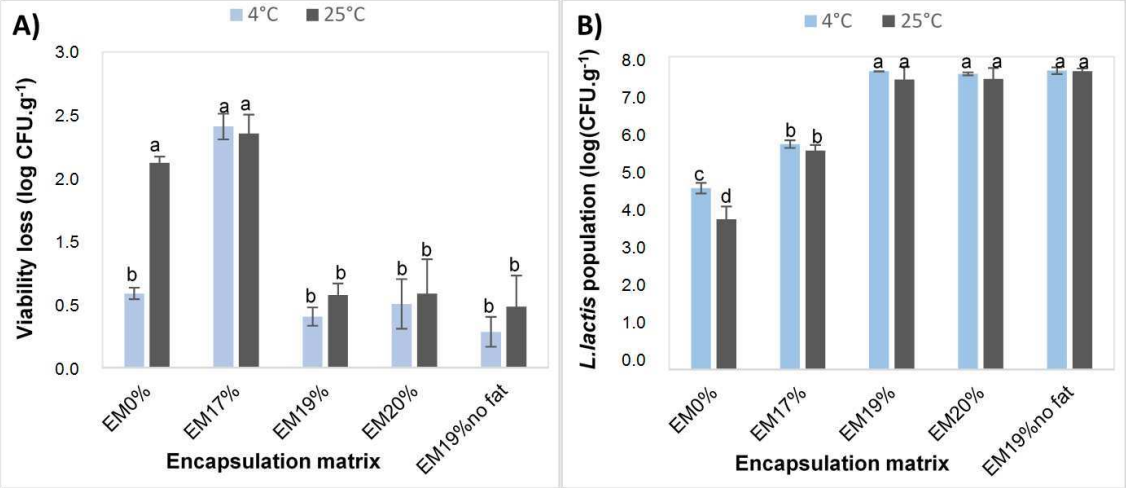

Figure 3 - Stability of encapsulated L. lactis cells after 45 days of storage at 4 °C or 25 °C. A) Cell viability loss during storage; B) Viable population of L. lactis after 45 days of storage. Different letters on the bars indicate statistical difference among the treatments ($p<0.05$).

The moisture analysis of powders just after drying (Table 2), revealed that the microcapsules produced with the matrix without lactose ($EM_{0\%}$) showed higher water content in relation to other matrices. This reinforces the idea that microcapsules produced with $EM_{0\%}$ probably suffered glass transition at room temperature. The results corroborate with Roos & Karel (1991) and Schuck et al., (2005) which observed a reduction in $T_{Tg}$with increasing moisture content.

Table 2 - Moisture content of the powders produced form different dairy matrices

	Sample				
	$EM_{0\%}$	**$EM_{17\%}$**	**$EM_{19\%}$**	**$EM_{20\%}$**	**$EM_{19\%}^{no\ fat}$**
Moisture (%, w.w^{-1})	5.55 ± 0.00^{a}	2.85 ± 0.00^{b}	2.81 ± 0.00^{b}	2.83 ± 0.00^{b}	3.36 ± 0.00^{b}

Different letters indicate a significant difference between the values of moisture ($p < 0.05$)

In terms of viable cells in the powder, a population of approximately log 10^8 CFU.g^{-1} was obtained in microcapsules produced with larger lactose content ($EM_{19\%}$, $EM_{20\%}$ and $EM_{19\%}^{no\ fat}$) after 45 days of storage (Figure 3B).

L. lactis is known to be sensitive to the presence of oxygen, by the absence of the catalase enzyme responsible for the neutralization of reactive oxygen species (H_2O_2) (Rochat et al., 2005). Therefore, this found demonstrates that lactose not only protects the cells against drying damages, but also protects them against the oxidative stress over time.

According to Zhang, Lin and Zhong (2016), the protective effect of lactose is based on the capacity to promote chemical bonds with cell structures during drying providng benefits during the storage of powders. The cell encapsulation by lactose technique limits the oxygen access and prevents oxidative stress in LAB during the storage (Corona-Hernandez et al., 2013).

Considering powders with larger lactose content ($EM_{19\%}$, $EM_{20\%}$ and $EM_{20\%}^{no\ fat}$), the cell population reduced on average 0.2 and 0.5 log cycles after drying and storage, respectively (Figures 2 and 3A). Taking account the thermosensivity of the strain used in this study, the application of lactose in encapsulating matrices can be a promising approach to make feasible the industrial production of spray dried bacteria.

4. Conclusion

The presence of lactose in the encapsulating dairy matrices shows be important to protect the cells against the adverse drying conditions besides guarantee the higher cell survival over time even when storage at room temperature. By contrast, no influence of fat content was observed which indicates low contribution of this constituent on the L. lactis preservation.

The supplementation of milk with lactose seems to be ideal to drying of heat-sensitive bacteria, such as L. lactis. By contrary, lactose hydrolyzed dairy matrices are not efficient to preserve the cells during drying and storage probably due to low glass transition temperature of material.

In this study, the production of spray dried L. lactis cells with low viability loss was demonstrated by adjusting of lactose content in the encapsulating matrix. This promising and relative simple approach can be applied to other heat-sensitive bacteria.

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CHAPTER 4

Influence of adaptive stresses on the survival of spray dried Lactococcus lactis cells

ABSTRACT

The influence of different sublethal conditions, acid, osmotic and oxidative stress, on the survival of Lactococcus lactis subsp. lactis during the drying process and subsequent storage was evaluated in this study. The survival of L. lactis during the spray drying process increased after progressive application of oxidative stress. However, progressive applications of acid stress and osmotic stress did not influence survival. After 45 days of storage, at both temperatures of 4 and 25 ° C, the final L.lactis population was higher (112.5%) when submitted to the sublethal condition of oxidative stress. The acid stress condition conferred on lower survival after 45 days of storage and application of osmotic stress did not influence the survival. The previous application of cellular pre-adaptation to oxidative stress conditions is shown as a potential strategy for producing dehydrated cultures of strains sensitive to the presence of oxygen.

Keywords: Lactococcus lactis, acidic stress, osmotic stress, oxidative stress, dairy matrix

1. Introduction

The lactic acid bacteria (LAB) is a group of microorganisms that shows as general characteristic the production of lactic acid from the fermentation of carbohydrates (Cesselin et al., 2011). In food industry, LAB strains are used as starter or coadjunct cultures acting on the sensorial characteristics of products or inhibiting the growth of spoilage and pathogen microorganism by bacteriocins production (Bintsis, 2018).

In the last years, the interest in the preservation of LAB by spray drying technique has been increasing which can be explained by the fact that this technology consumes up to 10 times less energy than freeze-drying and can be conducted in continuous process (Boyaval & Schuck, 1994; Schuck et al., 2016). Moreover, some works has proposed that spray dried cultures can be storage at room temperature reducing the cost associated to maintenance of cold chain (Schuck, Dolivet, Méjean, Hervé, & Jeantet, 2013).

Despite all advantages, the spray drying promotes sequential injuries to cells causing viability loss and alterations of technological properties of culture (Santivarangkna et al., 2008). Depending of drying conditions, some cultures have their viability reduced; being this the main factor limiting the industrial application of spray dried bacteria (Huang et al., 2016).

The dehydration of cultures by atomization consists in dispersing of a cell suspension in small droplets into a stream of hot air in order to quickly evaporate the water (Schuck et al., 2009). The instantaneous water removal associated with exposure to high temperatures and oxygen promote osmotic, thermal and oxidative stresses to LAB causing cell death to lead to a survival of only 4.71% survival (Ghandi Powell, Chen, & Adhikari, 2012a; Ghandi, Powell, Chen, & Adhikari, 2012b).

To overcome this drawback, studies have appointed the control of operational drying parameters and the using of encapsulating materials with protective thermal properties as strategies to improve the cell survival in spray drying procedures (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fisk, 2013). However, the application of these strategies alone or combined can involve temptations and error methods and not always results in increasing of survival rate.

Another possibility consists in pre adapt the microorganism by application of sublethal stresses which result in increasing of LAB tolerance during and after spray

drying by acquisition of adaptative responses (Peighambardoust, Golshan Tafti, & Hesari, 2011; Alonso, 2016). Nevertheless, to this end, a pre-disposition of the genome is required making the adaptation process specific for each strain (Bokhorst et al., 2012).

A strategy still unexplored and that it can improve the cell resistance to drying, consists in promote cell adaptation by application progressive sublethal stresses with increasing intensities. To evaluate this new approach, the aim of study was investigating the effect of progressive acid, osmotic and oxidative stress on the survival of Lactococcus lactis subps. lactis during spray drying and storage of dried culture.

2. Material and methods

2.1 Microorganism and culture conditions

L. lactis subsp. lactis isolated from artisanal cheese from the Amazon region (Martins et al., 2018) was selected to this study due to its proven low resistance to thermal treatments. The lactic culture activation was carried out by addition of 1 % $v.v^{-1}$ of the culture in 10 mL of Man, Rogosa and Sharpe medium (MRS, Difco, France) with incubation at 30 °C during 18 h.

2.2 Preparation of the drying medium

100 g whole milk powder (Itambé, Brazil) and 5g of lactose crystals (Cromoline, Brazil) were dispersed in 125 g of distilled water. The the drying medium was subjected to a heat treatment at 85 ° C for 1 h as proposed by Ananta, Volkert, & Knorr (2005) with modifications, and subsequently kept at 40 ° C in water bath until the inoculation of L. lactis cells.

2.3 Application of progressive sublethal stress

In order to improve the L. lactis tolerance to drying conditions, activated cells were submitted to progressive conditions of acid, osmotic and oxidative stress.

To progressive acid condition, MRS with pH adjusted with acetic acid 10% $v.v^{-1}$ (Cap-Lab, Brazil) was used to cultivate the L. lactis cells. 1 % $v.v^{-1}$ of active culture was added to 0.9 mL of MRS with pH 6.5 and incubated at 30 °C during 24 h. After incubation, 1 % $v.v^{-1}$ of the culture was transferred to 0.9 mL of MRS with pH 6.0 and

incubated again at 30 °C/24 h. The pH of MRS was reduced in 0.5 units in each new cultivation until reach a final pH of 4.5.

Osmotic stress was performed from the incubation of the active culture in MRS containing increasing concentrations of NaCl of 1.5, 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 7.0, 7.5 and 8.0% $w.v^{-1}$ (Dinâmica, Brazil) while the oxidative stress was performed in MRS containing concentrations of 0.5 and 1.0 mM of hydrogen peroxide (Dinâmica, Brazil). The progression of osmotic and oxidative stresses was done in a similar way as described to acid condition.

2.4 Preparation of cell suspensions

After adaptation to stresses conditions, 1% $v.v^{-1}$ of culture was transferred to 250 mL of MRS with pH 4.5 or containing NaCl 8.0% $w.v^{-1}$ or added of 1.0 mM of H_2O_2. The cultures were incubated at 30 ° C during 18 h, centrifuged (Eppendorf, German) at 5000 x g for 10 min and the supernatant discarded. The pellets were washed twice in saline solution 0.85% $w.v^{-1}$ and each one was resuspended in the drying medium obtaining cell suspensions with approximately 10^9 $CFU.g^{-1}$.

2.5 Spray drying of L. lactis

Cell suspensions were dried in spray dryer model MSD 1.0 (Labmaq, Brazil), by using drying parameters previously determined: inlet air temperature = 130 °C and cell suspension flow rate = 0.5 $Kg.h^{-1}$. The powder was recovered in sterile glass bottles and storage at 4 or 25 °C.

2.6 Physical analysis of powders

2.6.1 Water activity (Aw)

The water activity of powders was determined at 25 ºC using a water activity meter (Aqualab, Decagon 3TE, Decagon Devices Inc., USA). All measurements were performed in duplicate.

2.6.2 Moisture and dry extract analysis

3 g of cell suspension, or 1.5 g of powder, were mixed with sand and heated at 105 °C until they attained a constant weight (~5 h) (Schuck et al., 2005). Moisture (M%)

was calculated by determining weight loss after heating expressed in percentage, while the dry extract (DE, in %) values were determined by the follow equation:

$$DE = 100 - M\% \quad (1)$$

2.6.3 Powder microstructure

The morphology of powder particles containing L. lactis cells was evaluated by scanning electron microscopy (Hitachi TM 3000, Hitachi Ltd., Tokyo, Japan). The size of particles was determined by image analysis of 100 particles by using imageJ® software. The measurements were performed totalizing 300 particles measured by sample.

2.7 Determination of cell viability

Cell viability was evaluated in the cell suspension or in the powders immediately after drying (time: 0 days) and during storage (45 days). 1 mL of concentrate or 1 g of powder was dispersed in 9 mL of sterile saline solution 0.85% $w.v^{-1}$ and serial dilutions were performed in the same diluent.

The enumeration of viable cells was performed by microdroplet technique which an aliquot of 20 µL of appropriate dilution was deposited on the surface of MRS agar and incubated at 30 °C/ 72 h in anaerobic jars. The enumeration of colony-forming units was performed in triplicate and conducted in droplets containing between 8 and 80 colonies.

The number of viable cells (N) was calculated in relation to dry material content ($CFU.g^{-1}$) of concentrate or powder using equation 2:

$$N = n \times DF / aliq \times DE \quad (2)$$

Where n represents the number of colonies counted, DF is the dilution factor, aliq denotes the aliquot plated (0.02 mL for cell suspension or 0.02 g for powder) and DE is the dry extract given in $g.mL^{-1}$ or $g.g^{-1}$ for the cell concentrate and powder, respectively.

The cell viability loss was determined by the following equation:

$$\text{Viability loss} = \log N_0 - \log N' \quad (3)$$

Where N_0 represents the number of $CFU.g^{-1}$ of surviving L. lactis in the cell concentrate while N' indicates the number of viable cells ($CFU.g^{-1}$) just after drying.

Cell viability after storage was evaluated by the difference between initial population in the powder immediately after drying (log N') and final population after 45 days of storage.

2.8 Statistical analysis

The drying processes were carried out with three repetitions and the results were evaluated by ANOVA and Tukey test ($p < 0.05$) by means of the comparison between means. Statistical analysis was performed using the PAST software.

3. Results and discussion

3.1 Influence of adaptive stresses on the survival of L. lactis

The progressive stresses (acid, osmotic and oxidative) were conducted until the cell population had a maximum reduction of 1 log cycle in relation to the control (L. lactis growth in MRS with pH 7.0 without addition of NaCl or H_2O_2). By using this criterion, the final culture condition was defined as shown in the Table 1.

Table 1 - Final culture condition to L. lactis under progressive acid, osmotic and oxidative stresses

Stress	Final condition
Acidic (adjusted with acetic acid)	pH 4.5
Osmotic (NaCl)	8.0% $w.v^{-1}$
Oxidative (H_20_2)	1.0 mM

After drying, it was observed that L. lactis adapted to acid or osmotic conditions not presented significant different in terms of viability loss in relation to control (~0.38 log cycles) (Figure 1). On the other hand, cells adapted to oxidative conditions had their viability reduced in only 0.01 log cycles; reduction 35 times lesser then control (Figure 1).

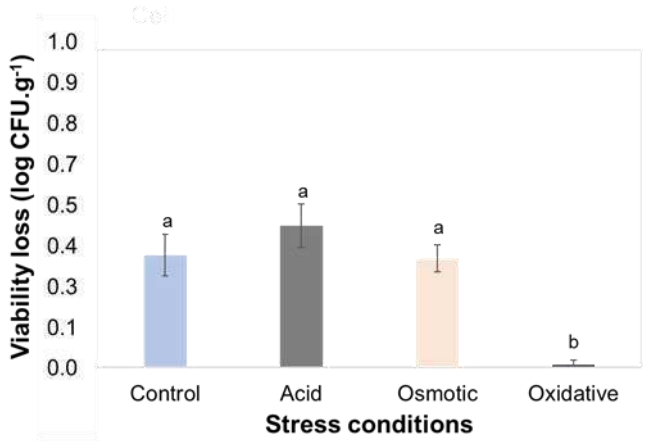

Figure 1 – Cell viability loss of L. lactis after spray drying. Different letters on the bars indicate significant difference between the treatments ($p<0.05$).

During spray drying, the microorganisms are constantly exposed to air flow, thus the cell inactivation due the oxidative during spray drying may be significant for some LAB (Huang et al., 2017). In concordance, Ghandi et al. (2012) reported that more than 80% of L. lactis cell death occurred during the spray drying process, which was associated to the adverse conditions of oxidative stress and shear stress delivered to the cells in this step.

The exposition to oxygen can promote the formation of reactive oxygen species, or ROS, which are highly reactive, can damage proteins and nucleic acids (Miyoshi, Rochat, & Gratadoux, 2003). L. lactis is characterized to be extremely sensible to oxidative damages since it lacks the production of catalase enzyme responsible by degradation of hydrogen peroxide; toxic to cell (Chen, Shen, Solem, & Jensen, 2013). However, the excessive accumulation of ROS in the intracellular environment can induce oxidative stress response mechanisms (Rochat et al., 2005). In this way, our results suggest that the increase in the cellular survival of L. lactis after progressive adaptation to sublethal doses of oxygen peroxide is effective to guarantee higher survival after spray drying.

The mechanisms of response to oxidative stress frequently found in L. lactis are based on the NADH oxidase / NADH peroxidase system and on the superoxide dismutase (SOD) enzyme (Condon, 1987; Miyoshi et al., 2003). Other resistance type is associated with expression of the recA gene, which is related to induction of synthesis of proteins that repair DNA and macromolecules, i.e., the chaperones (Miyoshi et al., 2003).

The chaperones synthesis is a general response mechanism equivalent to thermal stress (Lim et al., 2000). Thus, the exposition of cells to H_2O_2 can also have increased their resistance to heat, through the cross-response acquisition. The results is in concordance with the study that reported that some strains of L. lactis have tolerance in both oxidative and thermal stress conditions, indicating a correlation between the adaptive responses of these stress conditions (Dijkstra et al., 2014).

Some works has claimed that the inactivation mechanism of microorganisms during spray drying is primordially associated to effects of temperature and dehydration on the cells (Fu, Suen and Etzel, 1995; Santivarangkna, Kulozik, & Foerst, 2008). During spray drying, L. lactis was exposed to hot air with temperature of 130 °C causing thermal injuries to cellular components including denaturation of proteins and DNA structure impairment (Santivarangkna et al., 2008). In the same time, the fast water removal from cells can compromising the cellular membrane structure by lipid disorganization or besides of destabilization of proteins (Farakos, Frank, & Schaffner 2013).

The acid stress as applied in this study provokes several adverse conditions to L. lactis affecting the homeostatic system, disrupting substrate transport system and inhibiting metabolic pathways (Beales, 2004). In order to reestablish the functional conformation of proteins and enzymes essential to metabolism, some microorganisms are able to produce chaperones, responsible to repair macromolecular damages (Kajfasz & Quivey, 2011). This adaptative response to acid stress is similar to found for heat stress, thus the cells adapted to acid conditions are recognized to show increased tolerance to heat treatments (Lim et al., 2000). The chaperones synthesis in L. lactis, induced by expression of the groEL gene, was demonstrated by Venema & Kok, (1999) indicating that this microorganism is able to created mechanism to survival to drying.

Contrary to supposed, that is, cells adapted to acid conditions is more resistant to drying, no difference was found between adapted and not-adapted L. lactis (Figure 1).

In the same way, it is demonstrated that the osmotic sublethal stress allows the microorganism to created strategies to avoid the rapid and excessive dehydration by compatible solute accumulation (Desmond et al., 2002; Huang et al. 2016). In L. lactis strains, mechanisms of adaptation to osmotic stress such as synthesis of compatible solutes (betaine) and also chaperones synthesis had already been proven in previous research indicating the potential adaption of the cells against dehydration (Venema &

Kok, 1999). As observed to acid condition, the osmotic adapted cells not shown improvements in survival after drying.

A reasonable explication to the observed can be related to origin of the bacteria used in this study which was isolated from an Amazonian artisanal cheese produced by a singular method. The artisanal cheese is produced from raw cow milk ,which is fermented by own microbiota and pass by heating and whey removal steps (Randazzo et al., 2009; Martins et al., 2018). Thus, it is believed that the manufacture proceeding, especially at the end of the fermentation, creates a hostile environment of low pH, nutrient restriction, unfavorable osmotic conditions and low temperature triggering the resistance mechanism of L. lactis (Duwat et al., 2000; Simões et al., 2014). In other words, the stressful environment of origin of the strain could have triggered its adaptive responses justifying the equivalent viability loss between the control and the culture submitted to acid and osmotic conditions.

Although the drying parameters have high influence on the maintenance of L. lactis, the physicochemical properties of powder (milk powder + viable cells) can also be determinant (Broeckx et al., 2016). In general, powders with values of water activity distant to 0.20 - 0.25 are not ideal to survival of spray dried bacteria (Adhikari et al., 2009; Nualkaekul et al., 2012).

To certify that the viability loss observed in the Figure 1 was an effect of the adaptive stress and not by water content variation in the powders, all dried cultures were investigated (Table 2).

Table 2 - Water activity (aw) of powders.

Control	Acidic	Osmotic	Oxidative
0.204 ± 0.033^{a}	0.207 ± 0.000^{a}	0.217 ± 0.020^{a}	0.204 ± 0.003^{a}

Different letters in the same line indicate significant difference between the means.

The analysis of water activity revealed that all samples not differs among them (Table 2) indicating the viability loss variation is only explicated by progressive stresses suffered by L. lactis. Furthermore, the values exposed in the Table 2 are in concordance with those applied by dairy industry and are also indicated to preserve dehydrated cultures (Abe et al., 2009; Adhikari et al.,2009; Vesterlund et al., 2012).

3.2 Stability of culture during storage

In order to evaluate the effect of the storage temperature on the survival of microorganism, the powders were storage in sealed glass bottles and kept at 4 and 25 °C during 45 days. Vacuum conditions were not used to pack the powders simulating an abusive storage which the cells are in contact with atmospheric air.

Taking account the storage temperatures, the population reduction over time had not statistical difference between 4 and 25 °C indicating that refrigeration is not necessary to maintenance of viability (Figure 2). By contrast, considering the stress conditions, approximately 0.58, 0.82 and 0.68 log cycles in the population were observed to control, acid and osmotic adapted cells, respectively (Figure 2A). Again, lower viability loss was found in L. lactis conditioning to oxidative cultivation (Figure 2A).

The final population in powders varied from 6.17 to 9.26 log CFU.g^{-1} being the treatment in acid conditions the one with lowest population (Figure 2B).

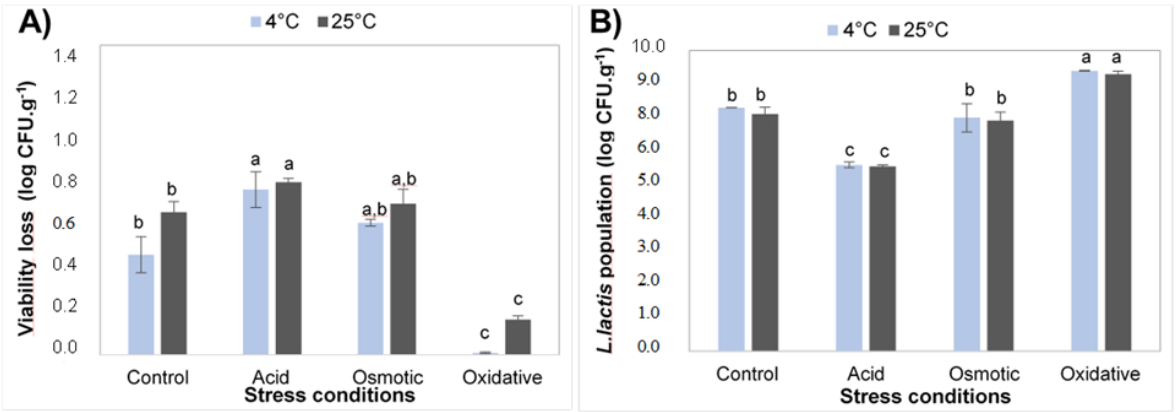

Figure 2 - Effect of progressive stresses on the survival of spray dried L. lactis at 4 and 25 °C. A) Viability loss after 45 days of storage; b) Viable cell population the powders after 45 days of storage. Different letters on the bars indicate significant difference between the treatments.

Under the storage conditions imposed, L. lactis was kept in contact with atmospheric air, which is prejudicial to microorganism considering its deficiency in production of catalase enzyme (Rochat et al., 2005). However, the stress caused by previous H_2O_2 exposition was efficient to avoid the cell viability loss after drying and during storage of powder, resulting in a 48-fold increase in cell survival when compared to the control.

Nevertheless, similar results are found involving other LAB, for example, a strain of Lactobacillus plantarum showed lesser loss of cell viability after 180 days of storage at room temperature when previously exposed to the sublethal condition of oxidative stress (45 mM H_2O_2) (Barbosa , Borges, & Teixeira, 2015). In addition, Desmond et al. (2002) observed a resultant increase of only 13% in the survival of Lactobacillus paracasei NFBC 338 to the heat treatment when previously exposed to a condition to H_2O_2 (3 mM / 30 min). This may suggests that the progressive application of oxidative stress may result in a more pronounced increase in the adaptative response to the drying process.

Considering that the variation on physicochemical properties of powders during storage can also have influenced on the results (Schuck, 2011), analysis of morphology of powder and moisture were performed. By scanning electron microscopy (SEM), it was found that the agglomeration state of particles of powders, the particles shape and mean diameter (5.3±2.0 µm) not varied over time. Water activity analysis revealed that powders have not their values altered conserving the same properties shown the Table 2, and the water content obtained moisture content of less than 4% $w.w^{-1}$. According to Schuck, (2011), in these conditions of water content (<4% $w.w^{-1}$) and Aw (~ 0.20), the glass transition temperature (T_{Tg}) will be close to 50 °C, what can be inferred that there were no modifications in the amorphous structure of the particles what can be confirmed by images SEM.

The microcopy together with the analysis of water content demonstrate that the glass bottles were hermetically sealed, and any change was carried out with ambient. Therefore, the viability loss found is recurrent of the progressive stresses given to L. lactis before the drying.

4. Conclusion

The low survival of bacteria in spray drying procedures is frequently associated to thermal and osmotic damages suffered by microorganism. However, it was demonstrated that to L. lactis, deficient in the synthesis of catalyze enzyme, the oxidative stresses seems to be the key factor to its death. By progressive adaptation of these microorganism in increasing concentrations of oxygen peroxide, it was possible produce

a dried culture with a very low cell reduction (<1 log cycle) even after storage. In addition, it was demonstrated that spray dried L. lactis can be stable over time at room temperature which represent an advantage compared to other industrial ferments that necessities of cold chain until the final application.

The new approach open opportunities to the production of spray dried microorganism of industrial interest comprising cells with low resistance to oxygen presence, as is the case of LAB. In perspective to this study, the genetic and molecular mechanisms developed by L. lactis cells to keep viable during drying and storage will be investigated in details.

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GENERAL CONCLUSION

GENERAL CONCLUSION

The application of strategies to increase L.lactis tolerance to the spray drying process have proved to be promising methods for the production of dehydrated lactic acid cultures of thermosensitive strains. The preliminary characterization of the drying process allowed defining the ideal parameters for L. lactis drying. Furthermore, the evaluation of the influence of dairy components on the matrices provided a more comprehensive knowledge of the lactose encapsulation property. The application of progressive stresses, in particular oxidative stress, allowed a pronounced increase in the survival of L. lactis after the drying process and during the storage period.

Additionally, the use of combined strategies allowed the reduction from 2.30 to 0.07 log cycles in the loss of cell viability in spray dried cultures stored at temperatures of 4 and 25 °C. Thus, the obtained results contribute to enable the spray drying of other LAB's sensible to the drying process and/or to the storage period.

Future studies are still needed to evaluate the effect of spray drying on the techno-functional properties of L. lactis, since they are indispensable and of great importance for industrial application. As well, it is necessary to investigate the possibility of producing dried cultures in conjunction with the use of this strategies studied, on an industrial scale.