ANDREZA ANGÉLICA FERREIRA

BIODIVERSITY OF LACTIC ACID BACTERIA AND PRESERVING BY FREEZE AND SPRAY DRYING OF *LACTOBACILLUS PLANTARUM* FROM MARAJÓ CHEESE

Dissertation thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the PostGraduate Program in Food Science and Technology, to obtain the title of *Doctor Scientiae.*

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APROVED IN October, 20th 2016.

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Prof. Dr. Antônio Fernandes de Carvalho
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My parents, Moacir and Ana

I dedicate

“Talvez eu não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

(Marthin Luther King)

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Desenvolvimento de Fermento Lácteo por Tecnologia Alternativa. Vitrine de Inovação/InovaLácteos. 37ª Semana Nacional do Laticinista. July, 2016 – Juiz de Fora, Brazil.

Viabilidade de Lactobacillus plantarum e Lactococcus lactis Encapsulados por Spray Drying. Temáticas de Pesquisa em Microbiologia e Biologia Molecular Aplicada a Leite e Derivados,

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Caracterização da biodiversidade e secagem via spray dryer de bactérias láticas isoladas de fazendas produtoras do Queijo Marajó, Pará. Temáticas de Pesquisa em Microbiologia e Biologia Molecular Aplicada a Leite e Derivados, II Workshop de Ciência e Tecnologia de Leite e Derivados, INOVALEITE. December, 2015 – Viçosa, Brazil.

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SUMMARY

LIST OF ABBREVIATIONS

BAL: bactérias láticas

LAB: lactic acid bacteria

Rep: repetitive extragenic palindrome

PCR: Polymerase Chain Reaction

PDO: Protected Designation of Origin

GMP: Good Manufacturing Practices

ADEPARÁ: Agência de Defesa Agropecuária do Estado do Pará

MAPA: Ministério da Agricultura, Pecuária e Abastecimento

GRAS: Generally Recognized as Safe

NSLAB: non-starter lactic acid bacteria

MC: Marajó cream type cheese

MB: Marajó butter type cheese

MRS: Man Rogosa and Shape

PCA: Plate Count Agar

BHI: Brain Heart Infusion

KOH: potassium hydroxide

FD: Freeze drying

SD: Spray drying

RSM: reconstituted skimmed milk powder

RSW: reconstituted sweet whey powder

RSWP: reconstituted sweet whey permeate powder

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ABSTRACT

FERREIRA, Andreza Angélica, D.Sc., Universidade Federal de Viçosa, October, 2016. **Biodiversity of lactic acid bacteria and preserving by freeze and spray drying of *Lactobacillus plantarum* from Marajó cheese.** Advisor: Antônio Fernandes de Carvalho. Co-advisors: Rosangela de Freitas, Monique Renon Eller and Luis Augusto Nero.

Northern Brazil, in the Amazon region stands out in the production of an artisanal cheese on the Marajó Island (Pará, Brazil). It is defined as a fresh cheese obtained through natural coagulation of raw buffalo milk by the autochthonous microbiota and subsequent curd fusion. LAB from artisanal cheeses contribute to original sensory properties in these cheeses, characterizing unique and specific products of each region, preserving a tradition of cultural, economic and social importance. Thus, the knowledge of LAB diversity is fundamental to the characterization of this type of cheese. In this context, the aim of this thesis was to isolate and to identify the LAB biodiversity involved in the production of Marajó cheese and to evaluate the effects of freeze drying and spray drying in the preservation of LAB. Preliminary characterization as Gram positive cocci and/or bacilli and as negative catalase plus identification by using 16S rDNA sequence analysis and Rep-PCR were undertaken for 149 LAB isolates obtained from samples of raw buffalo milk, curd and utensils involved in Marajó Cheese making and 97 LAB cocci from Marajó Cheese. Eight species have been identified as follows: *Weissella confusa, Streptococcus infantarius, Lactococcus lactis, Leuconostoc pseudomesenteroides, Weissella paramesenteroides, Pediococcus pentosaceus, Pediococcus acidilactici, Lactobacillus brevis* and the *Enterococcus* genus. The Rep-PCR was able to identify different genetic profile showing a high diversity among the evaluated isolates. Among the isolates, the species *Lactobacillus plantarum* and *Lactobacillus paraplantarum* were also identified coming from Marajó cheese. And a strain of *L. plantarum* was selected for preservation of freeze drying and spray drying.The cells of *L. plantarum* subject to spray drying process at approximately 10^9 CFU.g^{-1}, while the freeze dried samples showed 10^7CFU.g^{-1} after 60 days of storage at 4°C and 20 °C. The spray drying was less damaging than freeze drying for *L. plantarum* cells. The phenotypic characteristics showed by LAB isolated in this study allow of directing further investigations to preserve LAB strains involved in production of Marajó cheese in order to produce starter and adjunct cultures by

spray drying with a high number of viable cells to be used for industrial application through a cost-effective method.

RESUMO

FERREIRA, Andreza Angélica, D.Sc., Universidade Federal de Viçosa, outubro de 2016. **Biodiversidade de bactérias láticas e preservação por liofilização e secagem por atomização de *Lactobacillus plantarum* oriundo do Queijo Marajó.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Rosângela de Freitas, Monique Renon Eller e Luís Augusto Nero.

No norte do Brasil, na região Amazônica se destaca a produção de um queijo artesanal na Ilha de Marajó, Pará Brasil. Este queijo é definido como um queijo não maturado obtido pela fermentação natural do leite de búfala pela sua microbiota autóctone e posterior fusão de sua massa. As bactérias láticas (BAL) oriundas de queijos artesanais contribuem para propriedades sensoriais originais nesses queijos, caracterizando os produtos como únicos e específicos de cada região e preservam uma produção tradicional de importância cultural, econômica e social. Sendo assim, o conhecimento da diversidade de BAL é fundamental para a caracterização deste tipo de queijo. Neste contexto, o objetivo desta tese foi isolar e identificar a diversidade de BAL envolvidas no processamento do Queijo Marajó, bem como avaliar os efeitos da liofilização e da secagem por atomização na preservação de BAL. A caracterização preliminar de cocos e bacilos Gram positivos e catalase negativo permitiu a identificação de 149 isolados de BAL obtidas a partir de amostras de leite de búfala cru, massa fermentada e utensílios envolvidos no processamento do queijo Marajó e 97 cocos de BAL oriundas de amostras do queijo Marajó. Por meio do sequenciamento do gene 16S rDNA oito espécies foram identificadas: *Weissella confusa*, *Streptococcus infantarius*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides*, *Weissella paramesenteroides*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Lactobacillus brevis* e o gênero *Enterococcus*. A Rep-PCR foi capaz de identificar diferentes perfis genéticos demonstrando uma grande diversidade entre os isolados avaliados. Dentre os isolados, as espécies *Lactobacilus plantarum* e *Lactobacillus paraplantarum* também foram identificadas oriundas do queijo Marajó. E uma cepa de *L. plantarum* foi selecionada para estudos de preservação em processos liofilização e secagem por atomização. As células de *L. plantarum* submetida à secagem por atomização mantiveram-se aproximadamente em 10^9 UFC·g^{-1}, enquanto que as amostras submetidas à liofilização reduziram a viabilidade para 10^7 UFC·g^{-1} durante 60 dias de estocagem a 4 °C e 20 °C. Os efeitos da secagem por atomização na viabilidade das células de *L. plantarum* foram menores que a liofilização. As características fenotípicas apresentadas

pelas BAL, neste estudo, permitem direcionar futuras pesquisas para preservação das cepas de BAL envolvidas na produção do Queijo Marajó, bem como desenvolver culturas starter/adjuntas com um elevado número de células viáveis para aplicação industrial por meio da secagem por atomização a um de baixo custo.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Marajó Cheese is an artisanal product derived from buffalo milk produced on Marajó Island-PA, Brazil (Seixas et al., 2014b). Marajó Island has the biggest buffalo herd of the country. However, government and cheesemakers have not provided the necessary investment for the adequate processing of dairy production (Seixas et al., 2015).

The lack of infrastructure, process standardization, training of people and adequate handling techniques do not facilitate the development of cheese making on Marajó Island (Blaskovsky et al., 2010). However, studies have shown that Marajó Cheese can be considered a product of good microbiological quality. The pathogens *Listeria monocytogenes* and *Salmonella* sp. have not been detected in the Marajó Cheese and it has been in accordance with standards established in the current legislation (Seixas et al., 2015, 2014b).

Additionally, cheeses produced with raw milk according to traditional methods have a great diversity in their autochthonous microbiota (Castro et al., 2016; Dal Bello et al., 2010; Franciosi et al., 2009; Perin et al., 2012; Serhan et al., 2009; Silva et al., 2015; Terzic-Vidojevic et al., 2014). The authoctonous lactic acid bacteria (LAB) from Marajó Cheese can represent the geographically specific microbiota of the area. Thus, identifying and characterizing the biodiversity of LAB from raw material, utensils, equipaments, environment processing and Marajó Cheese is fundamental to determine the species involved in the microbial ecosystem balance and characterization of the final product.

Milk, utensils and equipments used in the production of these artisanal cheeses are also important sources for the isolation of LAB strains with interesting characteristics for industrial application (Franciosi et al., 2009; Galinari et al., 2014; Licitra et al., 2007; Lortal et al., 2009; Zeppa et al., 2004).

The LAB produce compounds of interest in fermented milk products that are able to decrease the pH of the medium which in turn inhibit the multiplication of other microorganisms. They also contribute to the texture changes in food and biochemical conversions that produce different tastes and flavors (Azizan et al., 2012; Moraes et al., 2012).

The microbial diversity in artisanal cheeses at the species level can be characterized by different phenotypic and genotypic methods. Methods based on genetic information have been used with success in biodiversity studies (Arcuri et al., 2013; Dal Bello et al., 2010; Freitas et al., 2015; Silva et al., 2015; Terzic-Vidojevic et al., 2014; Terzić-Vidojević et al., 2015; Tormo et al., 2015). The amplification of repetitive DNA elements by Polymerase Chain Reaction (PCR) is a technique known as Rep-PCR. This technique has a high discriminatory power, easy execution and it is used in the study of the ecology of diverse microorganisms (Gevers et al., 2001; Mustopa and Fatimah, 2014; Perin and Nero, 2014; Pogačić et al., 2013).

Among the LAB, *L. plantarum* species stands out which can be found in artisanal cheeses (Georgieva et al., 2008; Santos et al., 2014). Considered a ubiquitous species, *L. plantarum* strains were found to survive to the exposure to gastrointestinal conditions and have shown probiotics properties (Pisano et al., 2014; Ramos et al., 2013; Vries et al., 2006; Zago et al., 2011). This species has also been frequently studied in preservation process of LAB (Dolly et al., 2011; Khem et al., 2016, 2015; Perdana et al., 2014, 2012; Utami et al., 2016).

Preserving LAB is important to maintain the biodiversity of authoctonous strains with attractive features for further industrial application. Thus, freeze drying and spray drying have been studied and applied in the preservation of several LAB species (Berner and Viernstein, 2006; Carvalho et al., 2004a; Jalali et al., 2012; Zamora et al., 2006), including probiotic

strains (Huang et al., 2016; Maciel et al., 2014; Madhu et al., 2011; Pinto et al., 2015; Schuck et al., 2013; Shokri et al., 2015).

The cost of spray drying is approximately more than 10 times lower than freeze drying, and installed capacity in large-scale production make it an ideal technique for producing large amounts of dried probiotics (Santivarangkna et al., 2007; Schuck et al., 2013). However, the high temperatures during the process can affect the viability of cultures (Ananta et al., 2005; Fu and Chen, 2011; To and Etzel, 1997).

Several studies have been carried out with the aim of improving the survival rate of the cells after spray drying and during subsequent storage. The strategies include process optimization, application of different agents of protectants and improving cellular heat-resistance (Desmond et al., 2001; Fu and Chen, 2011; Huang et al., 2016; Schuck et al., 2013).

In this context, the aim of this thesis was to isolate and to identify the LAB biodiversity involved in the production of Marajó cheese and to evaluate the effects of freeze drying and spray drying in the preservation of LAB.

To achieve this objective, we have i) isolated and identified strains of LAB from raw buffalo milk, curd and utensils used in the production of Marajó Cheese, in order to get to know the lactic microbiota present in the raw material and processing environment of Marajó Cheese; ii) isolated and identified LAB cocci from Marajó Cheese to evaluate the species involved from this microbial group in the production of Marajó Cheese; iii) evaluated the technological potential of LAB by phenotypical characteristics of acid production, diacetyl production, proteolytic activity and antimicrobial activity; iv) selected a strain of *L. plantarum* that presented good probiotic and technological potential for preservation on freeze drying and spray drying; v) investigated the effects of freeze drying and spray drying on survival

rate, acidifying activity and probiotic potential using dairy-based carrier media of different compositions.

This work was therefore divided into five chapters: a literature review showing the scientific context (Chapter 1), this chapter is followed by experimental part comprising the biodiversity of LAB isolated from raw buffalo milk, curd and utensils used in Marajó Cheese making (Chapter 2), the isolation of LAB cocci of Marajó Cheese (Chapter 3), and the preservation of *L. plantarum* on freeze drying and spray drying (Chapter 4). Finally, the last chapter comprises the Final considerations divided in General discussion, Conclusions and Perspectives of this thesis (Chapter 5), and a list of all the references cited are drawn together in the end of this manuscript.

CHAPTER 1

LITERATURE REVIEW

1. Artisanal cheeses

The production of artisanal cheese differs from industrial production in some respects, i.e: there is no mechanized production processes and milk pasteurization. The fermentation is carried out spontaneously by the natural microbiota present in milk and utensils and equipment that can eventually contaminate the raw material and the final product. In Europe, the PDO scheme (Protected Designation of Origin) highlights artisanal cheese production. According to the European regulation No 510/06, these cheeses are considered to be cultural references of the society. The unique microbial biodiversity found in these cheeses is considered by manufacturers and consumers as a special characteristic that relates the product to a specific region (Randazzo et al., 2009).

In Brazil, the artisanal production of cheese began in the 18th century in the state of Minas Gerais during the colonial period (Lima et al., 2009). Portuguese settlers have brought the "Serra da Estrela" cheese making process, and some manufacturing techniques remain unchanged despite adaptations that have been made according to the environmental conditions of each region (Lima et al., 2009). The importance of these cheeses is not only limited to their sensorial quality insofar that they also play an essential social, economic and cultural role (Arcuri et al., 2013).

In many cases, the artisanal cheese production is a familiar activity being a demonstration of sustainability in different types of community. The technologies involved are passed on to the younger generations and have become part of cultural richness of certain regions (Ferreira and Ferreira, 2011). The valorization of artisanal cheese is an important factor of development for a great number of rural properties in Brazil and a strategy to promote local resources and improve product quality (Nóbrega, 2012), ensuring recognition and financial return to small farmers.

The typical artisanal cheeses in Brazil are “Coalho” and “Manteiga” cheese produced in the northeastern region of the country (Guerra and Guerra, 2003; Nassu et al., 2003). In the state of Minas Gerais there are five regions that produce artisanal cheeses known as “Serro” (Brant et al., 2007), “Canastra” (Dores et al., 2013; Resende et al., 2011), “Araxá” (Sobral et al., 2013), “Salitre/Cerrado” (Lima et al., 2009) and “Campos das Vertentes” (Costa Júnior et al., 2014). The production of "Serrano" cheese and "Colonial" cheese stands out in the southern region (Delamare et al., 2012; Souza et al., 2003).

The brazilian typical artisanal “requeijão” has been produced mainly in the northern and northeastern regions of Brazil for over 150 years and lots of industries maintain a traditional system of production (Aquino, 2011). The Marajó cheese (Seixas et al., 2015, 2014a, 2014b) is produced with raw buffalo milk on Marajó island, in the state of Pará, while the “requeijão Sertão or Baiano” is from the northeastern region (Aquino, 2011).

The cheeses produced with raw milk according to traditional methods have a great diversity in their autochthonous microbiota. The presence of several microorganisms such as bacteria, filamentous fungi and yeasts constitutes a complex microbial ecosystem (Serhan et al., 2009). The diversity is influenced by such factors as practices conducted during milking, hygienic and sanitary conditions of the milking environment, processing, origin and treatment of milk. Steps such as acidification, heating, whey drainage, salting and ripening also have a great influence on the final characteristics of the cheese and have a significant role in the microbial composition (Randazzo et al., 2009). Many authors have conducted researches on the identification and on the characterization of autochthonous LAB from artisanal cheeses (Dal Bello et al., 2010; Franciosi et al., 2009; Perin et al., 2012; Serhan et al., 2009; Terzic-Vidojevic et al., 2014), and differences in the microbial composition of cheeses made with raw milk and cheeses made with pasteurized milk has been mentioned in the literature, demonstrating the importance of these studies to characterize the LAB biodiversity.

Arcuri et al. (2013) analyzed the microbial diversity from samples of "Minas" cheese made with raw milk in four regions of Minas Gerais state, Brazil (Cerrado, Araxá, Serro e Serra da Canastra) and "Minas" cheese samples made with pasteurized milk. The authors found a higher diversity of LAB in the cheeses made with raw milk, mainly bacterial species of *Streptococcus* genus.

Coppola et al. (2001) analyzed mozzarella cheese samples from artisanal and industrial production and they identified by a culture-independent method the prevalence of *Lactobacillus* sp., *Lactococcus* sp. and *Streptococcus* sp. genus. However, *Lactococcus lactis* species was only identified in artisanal mozzarella cheese samples. It demonstrates the higher variability of microbiota in cheeses made with raw milk.

Studies on the autochthonous LAB diversity are essential to understand and preserve the microbiota from artisanal cheeses (Al-kotami et al., 2015; Arcuri et al., 2013; Dal Bello et al., 2012, 2010; González et al., 2015; Perin and Nero, 2014; Silva et al., 2015). New bacterial strains can be isolated and used in the processing of traditional products or on an industrial scale to improve quality of existing dairy products, giving them distinct sensory properties (Terzic-Vidojevic et al., 2014).

The ability of wild LAB strains to produce different aroma compounds allows the development of cheeses with new or improved sensory properties. LAB microbiota from raw milk is heterogeneous thus suggesting that it plays a significant role in the ripening process and that its presence is beneficial for developing a full-flavoured cheese made of raw milk (Van Hoorde et al., 2010).

2. Marajó cheese

The state of Pará has the greatest number of buffalo herds in Brazil, representing approximately 40% of the national herd which is estimated at 1.15 million heads (MAPA,

2016). The cities of Soure and Cachoeira do Arari, localized on Marajó Island are considered the greatest producing centres of buffalo milk and cheese in Pará that in turn becomes an attractive investment for the local economy. In general, the total volume of bufallo milk produced in this region is approximately 50 L per day and directed to the Marajó cheese making. There are approximately 28 cheesemakers in Marajó Island with a capacity of production between 21 to 50 kg of cheese per day (Seixas et al., 2014a).

Marajó cheese is obtained through natural coagulation of raw buffalo milk by the autochthonous microbiota and subsequent curd fusion. According to cheese making process, Marajó cheese is classified as: (1) Marajó cream type cheese (MC-cheese), which raw buffalo skim milk is used and the cream obtained from skim milk is added during the curd fusion; (2) Marajó butter type cheese (MB-cheese), which raw buffalo whole milk is used and butter is added during the curd fusion. Raw cow milk can be mixed with buffalo in a maximum proportion of 40% of the total volume of milk (Adepará, 2013).

This cheese shows soft, compact and closed texture, with small and few pores, pleasant aroma, with a cylindrical or regular shape. It has a pleasant taste, being slightly salty and acid (Simões et al., 2014).The consumption of Marajó cheese is immediate, in pieces, slices or it is used in sandwiches, pastels and others. The cheese is packaged in waxed paper or plastic packs (250 g and 500 g), and sold in supermarkets, bakeries, snack bars, restaurants, street markets and hotels of the state of Pará, Brazil (Seixas et al., 2014b).

Several authors have conducted studies on the characteristics of Marajó cheese (Bittencourt et al., 2013; Figueiredo et al., 2011; Seixas et al., 2015, 2014a, 2014b; Simões et al., 2014) and they have not observed uniformity in the physico-chemical and microbial characteristics of the evaluated cheese, which shows the existence of a great variability in the processing of these cheeses among the cheesemakers. Bittencourt et al. (2013) determined the

existence of variation between the macronutrients and the physico-chemical characteristics in Marajó cheese samples collected in industries of Marajó Island.

In relation of microbial characteristics, Seixas et al. (2015) characterized the Marajó butter type cheese produced during two seasons of the year. These authors described that the counts of *Staphylococcus aureus* were very high, even though the cheeses are under national and state legislation. The data confirms the necessity of an immediate implementation of Good Manufacturing Practices (GMP) for product safety, ensuring the microbial counts to be in line with the current legislation. According to Bittencourt et al. (2013) the setting of processing standards and legal requirements is fundamental to ensure standardized products with higher added value.

However, the government and farmers are still ensuring the necessary investment to properly benefit from the dairy production. The lack of infrastructure, process standardization, training of people and adequate handling techniques do not facilitate the development of the cheese making on Marajó Island (Blaskovsky et al., 2010).

The Marajó cheese making is a fundamental economic element for the viability of families that depend on this activity. Approximately 20% of cheesemakers depend exclusivately on the production of Marajó cheese (Seixas et al., 2014a). Seixas et al. (2014a) have made a diagnosis to characterize the social and economic profile of Marajó cheese farmers. The great majority of interviewees (80% of manufacturers of cream type cheese and 100% of manufacturers of butter type cheese) had over 10 years of experience in manufacturing, being a familiar production. The cheesemakers have a long experience and perpetuate ancient methods which are a source of resistance to change into better manufacturing techniques and to comply with the GMP determined by legislation.

In this context, the recent modification of legislation in the state of Pará defining numerous requirements and recommendations that will allow the sale of the product locally

and in other states is a progress. Besides, for a greater development of this sector, it is necessary to create public policies that consider strategies to improve the organizational chain and competitiveness of it. The regularization of cheese factories that go on illegal and of training of cheesemakers is primordial, because it facilitates access to greater income generation for producers (Seixas et al., 2014a).

In 2013, the Agricultural and Livestock Defense Agency of the State of Pará (ADEPARÁ) approved the Technical Regulation of production of Marajó cheese. The legislation describes a number of recommendations and requirements for farmers on the quality of the used water, the milking process, the hygiene conditions of the manufacturing site, transport, and storage, among other topics such as health condition and training of the manufacturers in GMP. The farmers interested in certifying a product should formally request to ADEPARÁ provided that they work within the norms of the current legislation.

The manufacturer of the Marajó cheese will then receive a certification of origin, called Geographical Identity Certification of Marajó Cheese and will be authorized to commercialize the product in the entire state of Pará. After taking on the directives of the Ministry of Agriculture, Livestock, and Supply (MAPA) of Brazil and with the requested adjustments in place, the commercialization of the Marajó cheese throughout Brazil will be authorized. This certification is an important progress that will bring social and economic benefits and will ensure safety for consumers.

Thus, more studies are a must to better characterize this product, mainly from a microbiological perspective in order to determine the LAB biodiversity with potential of biopreservation. Consequently, to contribute to a better understanding of the microbial interactions in the physico-chemical and sensorial characteristics of the product.

2.1. Marajó cheese processing

Marajó cheese is handcrafted in small farms of Marajó Island, which show technical variations during the manufacturing process. However, Figure 1.1 shows the general flowchart for the production process of artisanal Marajó cream and butter type cheese.

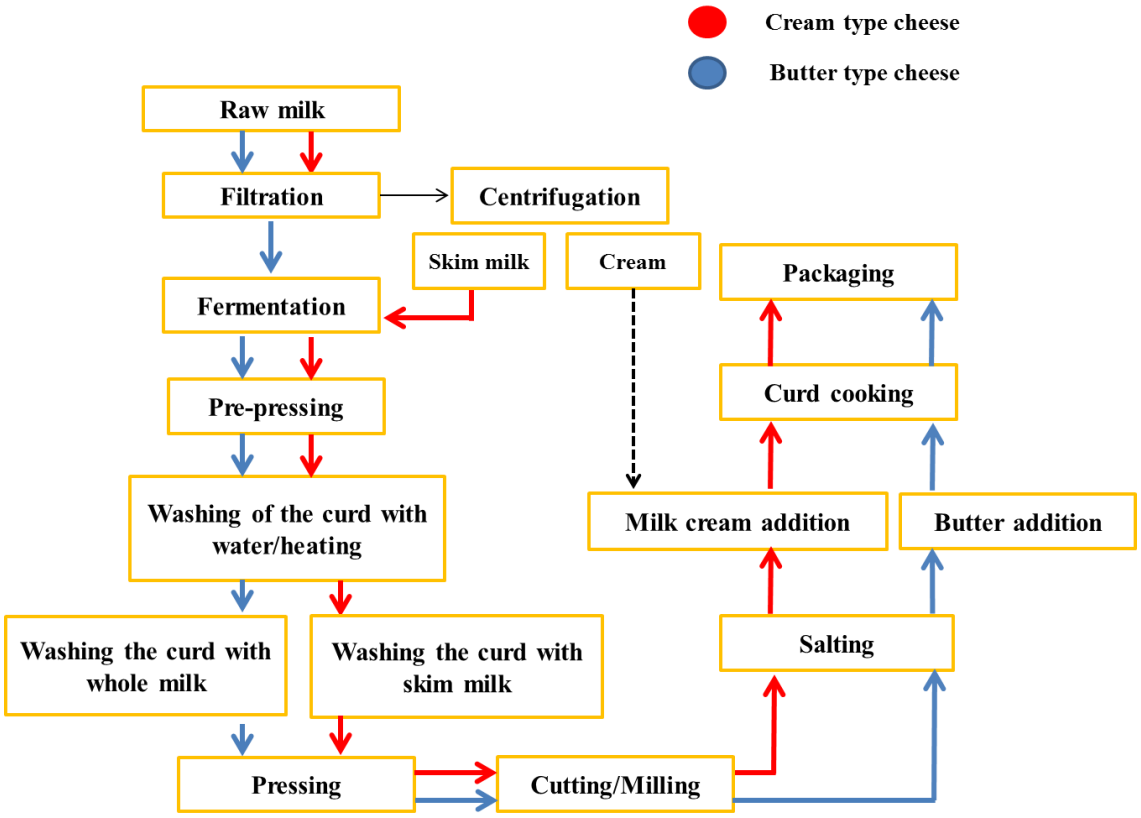

Figure 1.1 – Flowchart for the production process of artisanal Marajó cream and butter type cheese.

In general, the manufacturing process is described as follows:

1) Milking process: the raw milk used for Marajó cheese comes from female buffalos and is sometimes mixed with cow milk. The utilization of cow milk is employed in the manufacturing of cheese and it is allowed by the current legislation up to 40% of the total milk volume (Adepará, 2013).

2) Raw milk transport: Right after milking, the milk is transported to the farms in plastic milking jug.

3) Filtration: to remove undesirable particles of sediment. In this process, a thin plastic cloth is normally used.

4) Centrifugation: only milk used in the manufacturing of the cream type cheese is skimmed in an electric or manual centrifugal cream separator, separating the fat or the cream from milk.

5) Fermentation: the skim or whole milk (butter type cheese) is left to rest for 24 hours to coagulate. In this case, the commercial microbial culture is not used. The product is fermented by autochthonous microbiota. The autochthonous culture is obtained from whey drainage from cheese made on the previous day, which is collected and used for cheesemaking on the next day and from milk.

6) Pre-pressing: the curd is put in a nylon bag to remove whey.

7) Curd washing with water/heating: Curd is first washed with water. Then, the curd is put in a cooker and heated for approximately five minutes at 50 °C. After heating, the curd is pressed and subject to a second wash with water.

8) Curd washing with skim or whole milk/heating: The curd is washed with skim milk (cream type) or whole milk (butter type). The curd is heated for 15 minutes at 80 °C.

9) Curd pressing: the curd is pressed until whey is drained off.

10) Curd cutting/milling: the cold curd is cut into small pieces and subject to manual or mechanical milling.

11) Salting: addition of sodium chloride in variable amounts.

12) Addition of cream or butter: Addition of fresh cream (cream type) or butter (butter type), little by little as the curd heats in variable amounts depending on curd texture.

13) Curd cooking: The curd is stirred until it pulls away from the bottom of the pan and smells of fried butter. The curd becomes brilliant, forming long strings that are suspending on the spoon.

14) Packing: After cooling, the product is wrapped in waxed paper and in plastic packaging.

3. LAB cultures in food fermentation

LAB are widely distributed in nature, especially in milk, but also in vegetables (Nomura et al., 2006) and in the digestory tract, upper respiratory tract and urogenital tract of humans and animals (Dalié et al., 2010), such as fish (Poffo and Silva, 2011). This group is characterized by rods and cocci, gram-positive, negative catalase, non spore-forming, low GC content (lower 50%), acid tolerance, anaerobic state, aerotolerance (Liu et al., 2011). The LAB group includes *Aerococcus, Enterococcus, Carnobacterium, Vagococcus, Tetragenococcus, Lactococcus, Lactobacillus, Pediococcus, Leuconostoc, Oenococcus, Streptococcus* and *Weissella* genus (Khalid, 2011).

The fundamental importance of LAB is mainly associated with their physiological characteristics such as substrate use, metabolic capacity and probiotic properties. These

bacteria, known as fastidious, are nutritionally demanding and they frequently need specific aminoacids and vitamins of B-complex as multiplication factors (Khalid, 2011).

Some LAB genus are homofermentative and produce lactic acid which is the main product of lactose fermentation, while other genus are heterofermentative and produce carbon dioxide and ethanol, besides lactic acid. These compounds are responsible for the sensorial characteristics of fermented food (Liu et al., 2011).

The dairy industry is a major food industry that uses LAB in the processing of various fermented dairy products such as: cheese, yogurt, milk beverages among others. In cheese making also is commonly used lactic cultures that include mesophilic species of the genus *Lactococcus* and *Leuconostoc* and thermophilic species of the genus *Lactobacillus* and *Streptococcus* (Dal Bello et al., 2012, 2010; Ennahar et al., 2000; Moraes et al., 2012).

The lactobacilli constitute a major group of LAB. They are generally found in environments with high levels of carbohydrates, such as food products (dairy products, fermented meat, sourdoughs) and plant-derived substrates. Lactobacilli can be present in the human body including the respiratory, gastrointestinal and urogenital tract (Siezen and Vlieg, 2011). Consequently, lactobacilli have been studied initially because of their importance for food production and their probiotics characteristic, defined as ‘‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’’ (FAO/WHO, 2001).

The probiotics strains from food have been studied by several authors, including *L. plantarum* (Pisano et al., 2014; Ramos et al., 2013; Zago et al., 2011). The research of LAB strains from food, mainly spontaneously fermented food that shows resistance to biological barriers of the human gastrointestinal tract with physiological characteristics as probiotic may lead to the finding of new probiotic strains for functional food (Zago et al., 2011) and production of differentiated product in the food industry.

L. plantarum is a widely distributed species in much fermented products of animal or plant origin and can be used in controlled fermentation or is derived from the environment and emerges after manufacture (Parente et al., 2010). Initially, *L. plantarum* was proposed as *Streptobacterium plantarum* by Orla-Jensen in 1919. This specie is a facultative heterofermentative organism that is closely related to *L. paraplantarum, Lactobacillus pentosus* and species *Lactobacillus fabifermentans* (De Bruyne et al., 2009). According to Siezen and Vlieg (2011), *L. plantarum* has become one of the model microorganisms in LAB research, especially after the establishment of its molecular characteristics to genomics level.

L. plantarum helps in certain types of cheese during ripening as an adjunct starter. In many fermentations it dominates especially in later stages , presumably because of its high acid tolerance, being considered as NSLAB (non-starter lactic acid bacteria) (Parente et al., 2010; Siezen and Vlieg, 2011).

The group of NSLAB is particularly heterogeneous with lactobacilli being mostly represented: *Lactobacillus farciminis* among obligately homofermentative species, *Lactobacillus casei*, *Lactobacillus paracasei*, *L. plantarum*, *Lactobacillus pentosus*, *Lactobacillus curvatus* and *Lactobacillus rhamnosus* among facultatively heterofermentative species and *Lactobacillus fermentum*, *Lactobacillus buchneri*, *Lactobacillus parabuchneri* and *Lactobacillus brevis* among obligately heterofermentative species. The non-*Lactobacillus* species of NSLAB commonly isolated during cheese ripening are *Pediococcus acidilactici, Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium* and also *Leuconostoc* with the same species that act as starter cultures (Settanni and Moschetti, 2010).

LAB are responsible for giving sensory characteristics and technological attributes to cheese. They also contribute to the biopreservation of the final product due to production of organic acids (lactic and acetic acids), with a consequent reduction in pH or production of

antimicrobial substances such as hydrogen peroxide, diacetyl or bacteriocins (Dal Bello et al., 2012, 2010; Ennahar et al., 2000; Moraes et al., 2012).

Understanding the importance of the lactic microbiota in the production of cheeses requires the study of the biodiversity of microbiota of a region to better understand the role of microbiota in defining the characteristics of cheeses. Classic methods of identification or LAB such as physiologic and biochemical tests have not been efficient to differentiate species and sub-species (Buyukyoruk et al., 2010), even if these methods are reasonably sensitive, it is not possible to detect genetic differences among certain bacterial groups (Randazzo et al., 2009).

The introduction of molecular technique of Polymerase Chain Reaction (PCR) in microbial identification determined a viable alternative to the traditional methods. This technique shows various advantages in comparison with traditional techniques, as a higher typification and discrimination power, is quicker to execute and obtain results, has a good detection limit, a higher selectivity and specificity (Gandra et al., 2008). The PCR is a versatile technique is also used for epidemiological studies. Among the epidemiological type analyses, variations were developed such as Rep-PCR, being the latter considered the PCR amplification of repetitive extragenic palindromic elements (Gevers et al., 2001).

The repetitive extragenic palindrome (Rep) was firstly described and identified in *Salmonella* Typhimurium and *Escherichia coli* (Gilson et al., 1984; Higgins et al., 1982). The family of Rep elements have generally between 33 to 40 bp, 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* or *Salmonella* (Gilson et al., 1984; Higgins et al., 1982).

This method shows the following characteristics: a high discrimination power, low cost, appropriate for a high number of samples and reliable on typifying and classifying a large range of gram-positive and gram-negative bacteria (Gevers et al., 2001). However, the

sensitivity of this technique may limit the genetic differentiation among different species. Several studies have shown the application of Rep-PCR in LAB (Gevers et al., 2001; Mustopa and Fatimah, 2014; Perin and Nero, 2014; Pogačić et al., 2013; Terzic-Vidojevic et al., 2014; Tormo et al., 2015).

The Rep-PCR uses primers of complementary bases in DNA of natural occurrence, highly conserved and interspersed with repetitive consensus sequences that allow amplification of diverse-sized DNA fragments (Gevers et al., 2001). The genomic identities resulting from the technique allow the differentiation in intra- species, sub-species and inter-species (Lupski and Weinstock, 1992). The resulting DNA fingerprint patterns and specific for individual bacterial can be compared by Rep-PCR after fragmentation by electrophoresis (Spigaglia and Mastrantonio, 2003).

The associated of Rep-PCR with the 16S rDNA gene sequencing that has become an important technique to identify microorganisms (Janda and Abbott, 2007), allows of identifying the dominant species of LAB in artisanal foods. It can help in the characterization and selection of strains with distinct properties that may be used in the dairy industry to develop products with different sensory characteristics.

Generally, LAB used as starter culture in dairy industry are frozen. However, there are many commercial disadvantages in using frozen cultures, especially high transportation costs that may limit the use of frozen starter cultures in distant areas or countries not to mention storage at low temperature. The freeze drying has commonly been used (Carvalho et al., 2004b), because it eliminates the step of sub-culture, reduces the costs associated to bulk culture preparation and lowers the risk of bacteriophage infection. However, this process has high manufacturing costs and high energy consumption. For this reason, increasing attention has been paid to alternative drying processes such as spray drying.

Some studies have focussed on the preservation of LAB probiotics strains by alternative low cost methods like drying by atomization via spray dryer for industrial application (Dolly et al., 2011; Golowczyc et al., 2011; Maciel et al., 2014; Perdana et al., 2014, 2012; Reddy et al., 2009; Shokri et al., 2015b).

The use of LAB cells as starter culture requires a high cell density and the retention of biological activity before their incorporation into the food formulation in order to ensure the desired fermentation (Berner and Viernstein, 2006; Peighambardoust et al., 2011). Thus, all these preservation methods for industrial applications require the maintenance of a high viability of bacterial populations during preservation and storage, therefore one of the main challenges in the commercialization of LAB cultures is the development of storable formulated products that ensure the viability and the activity of the initial population (Strasser et al., 2009).

4. Drying methods of preservation of lactic acid bacteria

4.1. Freeze drying

In food industry, the cultures can be made available through concentration/preservation: in the frozen form after centrifugation for concentration of the cells and in the dried form like freeze drying, for example. The major disadvantages of the use of frozen cultures are cost of transport, storage and manipulation (Carvalho et al., 2004b; Santivarangkna et al., 2007). Dried preparations have the double advantage of long-term preservation and convenience in handling and storage. Freeze drying is a widespread technique and a number of freeze dried cultures are commercially available (Santivarangkna et al., 2007) being widely used in production of LAB cultures (Kandil and Soda, 2015; Madhu et al, 2011; Strasser et al., 2009). The degradation reactions are sufficiently inhibited not to mention usefulness for handling (Kasper and Friess, 2011).

There are three steps that define the traditional freeze drying: freezing, primary drying and secondary drying. The first step is freezing by vacuum, during which ice starts to nucleate, following by ice growth. This results in a matrix of glassy and/or crystalline solutes due to the separation of most of the water into ice crystals. Subsequently, during primary drying the crystalline ice formed during freezing is removed by sublimation. The chamber pressure is reduced well below the vapor pressure of ice and temperature is raised to have the heat remove the ice by sublimation. In this step, the product can still contain approximately 15–20% of unfrozen water, which is eliminated during the secondary drying, usually at elevated temperature and low pressure to allow a product with a low moisture content (Kasper and Friess, 2011).

There is no precise definition of the optimal cells concentration for freeze drying. According to Morgan et al. (2006) the cell concentration ($>1\times10^8$ cells ml^{-1}) has been decided as the highest initial cell concentration that allows the longest cell viability to survive during storage. However, Costa et al. (2000) found that the optimum initial cell concentration is related to the protective medium used for drying. When sucrose was used, a high initial cell concentration of 10^{10} cfu·ml^{-1} was desired as optimal for the highest freeze dried recovery; on the other hand, when skim milk was the protective medium, an initial cell concentration of 10^8 cfu ml^{-1} was enough for a highest freeze dried recovery (Costa et al., 2000).

According to Carvalho et al. (2004b) the composition of the growth and drying media can exert influence in the protection during storage of freeze dried cells. Studies have shown that the growth media can have a significant effect on the freeze dried survival of LAB (Berner and Viernstein, 2006; Carvalho et al., 2003, 2004a; Costa et al., 2000).

The skim milk can be considered as the best protection media for the cells during the drying process (Fu and Chen, 2011). Although the protective mechanism of skim milk has not been fully understood, it has been suggested that lactose in skim milk interacts with the cell

membrane and helps to maintain membrane integrity in a similar way as to other sugars including trehalose (Corcoran et al., 2004). Another major constituent of skim milk is protein and whether or not it exerts a significant protective effect remains to be elucidated (Fu and Chen, 2011).

Berner and Viernstein (2006) studied the effect of different protectants and the impact of the initial cells density on the viability of *L. lactis* and obtained maximum viability of the cells after freeze drying with sucrose and skim milk mixtures as protective agents (78% viability). In the same study, the authors determined that freeze drying with protectants based on skim milk or MRS-broth were more effective than other agents of protection tested (Berner and Viernstein, 2006).

Rathnayaka (2013) found a good survival in *Lactobacillus rhamnosus* and *L. plantarum* freeze drying with UHT milk as protection media during six month storage at 4°C. Addition of other cryoprotectant such as sucrose, sorbitol and trehalose did not improve the microbial survival more than UHT milk alone. According to the author, the probiotic properties of microorganisms tested were not affected by the freeze drying process or long term storage (Rathnayaka, 2013).

Jalali et al. (2012) evaluated the effect of various formulations of cryoprotectant media containing skim milk, trehalose and sodium ascorbate on the survival rate of probiotic bacteria during freeze drying at storage temperatures of 4 °C and 23 °C. The survival rate was only 2-3% and the population of the bacteria decreased in a significant manner ($P<0.05$) when the microorganisms were freeze dried in water alone and in the absence of any cryoprotective substance. The addition of 6% skim milk to the medium increased the viability of bacteria up to 20%. A survival rate of about 76% was found in capsules containing 6% skim milk, 4% sodium ascorbate and 8% trehalose in the case of *Lactobacillus paracasei* subsp. *tolerance* and 72% in the case of *L. delbrueckii* subsp. *bulgaricus*. The reduction rate of the initial

population of probiotics was significant in all media when stored at 23 °C ($P<0.05$). These studies also highlighted that the effects can be strain and protectant dependent.

During freeze drying the bacterial cells are also subject to stress conditions including extreme levels of pH, low temperature, formation of ice crystals and removal of water from the cell. The effects of freezing on cell viability might damage the cell membrane, cause protein and DNA denaturation, and decrease cell survival (Zhao and Zhang, 2005).

Lievense et al. (1994) considered the plasmatic membrane to be the principal site of lethal damage after freeze drying, which might affect DNA and provoke lipid oxidation on the membrane. Bacterial cells are also subject to osmotic stress as a result of low water activity of the medium and internal accumulation of compatible solutes (Kandil and Soda, 2015), affecting the viability during storage time.

Castro et al. (1996) reported that there was a change in the lipid profile during storage of *Lactobacillus bulgaricus* lyophilized in skim milk powder. This change expressed by a decrease in the proportion of the unsaturated to saturated fatty acids affects the membrane fluidity in low water activity conditions. Furthermore, the decrease of this proportion was correlated with a decrease in cell viability. A decrease in the ATPase activity was also observed during the study probably as a consequence of alterations in lipid composition (Castro et al., 1996).

Castro et al. (1997) suggest that the absence of oxygen during freeze drying can be important to avoid the lipid oxidation and consequently affect the permeability of the membrane and also affect enzymatic activities associated with the membrane. It is important to understand the mechanisms responsible for causing cell death, because it is mandatory, at industrial level, an appropriate concentration of viable cells to allow for a successful direct inoculation in fermentation tanks (Castro et al., 1997).

Nevertheless, the high costs and complexity of the process itself that can sometimes take days to complete for large product loads justify for the quest to develop alternative drying techniques (Carvalho et al., 2004b; Morgan et al., 2006; Santivarangkna et al., 2007; Silva et al., 2011). The high cost is due to the time needed for the growth of these microorganisms, to the slow energy transfer rate needed to dry the material during the process itself and to the storage in low temperatures to keep the cells alive for some cultures (Carvalho et al., 2004b; Morgan et al., 2006; Santivarangkna et al., 2007; Schuck et al., 2013).

Considering that freeze drying is an expensive process with low yields whereas spray drying higher yields , the latter offers an inexpensive alternative approach (Silva et al., 2011) that can be better studied and optimized for industrial application of spray dried cultures (Huang et al., 2016).

4.2. Spray drying

The development of spray drying equipment and techniques started in the 1870s. The concept of spray drying was first patented by Samuel Percy in 1872, and its industrial application in milk and detergent production began in the 1920s (Peighambardoust et al., 2011). However, this technique was largely used during the Second World War because of the need to transport large quantities of food with long shelf lives and reduced weight and volume (Silva et al., 2011).

Briefly, spray drying process consists of dispersing the liquid product in small droplets onto a current of hot air, resulting in a powder. Food placed in a current of air with a low relative humidity (pressure 1554 Pa) and a high temperature (150 ºC – 200 ºC), creates a spontaneous difference of temperature and partial pressure of water between food and air. It results in an energy transfer in the form of heat from the air to the product and a water transfer from product to the air (Schuck et al., 2005).

The spray drying process can produce a good final product with low water activity and weight reduction, resulting in easy storage and transportation. The physicochemical properties of the final product mainly depend on inlet air temperature, air flow rate, feed flow rate, atomizer speed, types and concentration of carrier agent (Singh and Dixit, 2014).

Spray dryers can dry a product very quickly compared to other methods of drying (Afoakwah et al., 2012). The quick process of spray drying keeps flavor loss to a minimum. Dairy products, such as milk, whey, cheese, buttermilk, butter and dry creamer are common items that are made by using the spray drying technique. Instant coffee, dry creamer and instant soups can also be spray dried, and previously spray dried food often serve as baby food (Afoakwah et al., 2012).

Spray drying is also considered a good preservation method for LAB and probiotic cultures (Ghandi et al., 2013; Huang et al., 2016; Perdana et al., 2014; Schuck et al., 2013). The spray drying of microorganisms dates back to 1914 and to the studies of Rogers on dried lactic acid cultures (Rogers, 1914). The speed of drying and the continuous production capability is very useful in drying large amounts of starter cultures. The low production cost of spray drying makes it more energy efficient compared with freeze drying and other techniques (Table 1.1). (Peighambardoust et al., 2011).

Table 1.1- Costs of drying process referenced to that of freeze drying

Drying process	Fixed costs (%)	Manufacturing costs (%)
Freeze drying	100	100
Vacuum drying	52.2	51.6
Spray drying	12.0	20
Drum drying	9.3	24.1
Fluidized bed drying	8.8	17.9
Air drying	5.3	17.9

Adopted from Santivarangkna et al., 2007

Schuck et al. (2013) showed that it is possible to produce a spray dried bacteria powder using a less expensive process (about 10 times less) than freeze drying with a high level of cells (>10^{10} CFU·g^{-1}) and a high viability during process and storage. Moreover, the authors have shown that spray drying may be an alternative to freezing or freeze drying to produce powders with varying viable levels of bacteria (viability > 95%) over a long time (several months) at room temperature and even longer at 4°C. Improvements in bacteria culture (type, resistance to stress, temperature, etc.), and in process (air treatment, sterilization of the support and introduction of a belt in the place of the crystallizer, before the vibrofluidizer) should improve the drying efficiency of dairy bacteria.

Nevertheless, spray drying of microbial cultures has been less developed commercially. Some authors explain that it is mainly due to low survival rates during culture drying, low stability under storage, initial concentration of microorganisms, growth conditions, growth medium, drying medium, rehydration conditions (Carvalho et al., 2003, 2004a, 2004b; Peighambardoust et al., 2011) and possibility of cross-contamination during the process.

The inactivation of the cells during the spray drying process can occur by high temperatures in addition to inactivation by dehydration. At the beginning of spray drying, the temperature of atomized cell suspension is limited to the wet bulb temperature by the evaporative cooling effect. At the next stage the temperature and thermal inactivation of the cells increases depending on drying parameters such as inlet/outlet air temperature, residence time and feed rate. The outlet air temperature or the temperature at which the product leaves the drying chamber is believed to be the major drying parameter affecting viability of spray dried starter cultures (Santivarangkna et al., 2008b).

Silva et al. (2002) found that relatively small changes in the outlet temperature appear to have significant effects on the survival of *Lactobacillus* strains, indicating that spray drying temperatures need to be optimized individually for every new application and microorganism.

The rehydration solution itself, as well as the rehydration conditions may also affect the rate of survival and viability of bacteria (Carvalho et al., 2004b; Muller et al., 2010). According to Teixeira et al. (1995), it is recommended to dry the cells at the stationary phase of growth and to use slow rehydration procedures.

During the initial process of rehydration the cells are exposed to high moisture, in this case, slow rehydration rate is recommended for adaptation of the cells to increased water activity and then a full dissolution of the product could be carried out to achieve the most cell recovery. Another critical parameter is the temperature of rehydration medium. Very cold or very hot temperatures reduce the recovery rate of the cells (Fu and Chen, 2011). Wang et al. (2004) reported that the optimum rehydration temperature was around 20 °C for LAB spray dried.

Storage method, packaging material and methodology also influence shelf life of the dried product. The cell viability and fermentation activity of spray dried bacterial starter cultures can be directly affected by storage conditions (Peighambardoust et al., 2011). The storage conditions have an important effect on the survival of probiotics in dried powders and the correct storage conditions are essential to maintain viable populations of dried probiotic bacteria. Survival decreases over storage time, especially when storage temperatures are higher (Ghandi et al., 2013). Therefore, Hubálek (2003) suggests that the packaging materials would be different types of barriers to oxygen, moisture, light, microbial contamination and elevated temperatures.

Packaging methods such as storage under vacuum or in nitrogen may be a more cost-effective way to resolve the problems of storage and to avoid the oxidation of the fatty acids

due to the absence of the oxygen (Morgan et al., 2006). According to Teixeira et al. (1996) the lipid composition of the bacterial cell membrane changes due to lipid oxidation during storage. Thus the oxidation of the fatty acids of the membrane lipids is the most probable cause of death of microbial cells during storage.

Otero et al. (2007) compared the viability of *Lb. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* between storage in air and storage in nitrogen and under vacuum. Storage within sealed glass vials under vacuum or nitrogen gas were more efficient. The dried cells remained possibly permeable to oxygen during storage in air, making it difficult to recover the cells once the accumulation of free radicals such as oxygen inside a cell that cannot metabolize them and take them out may result in irreversible damage inside of it hence making it unviable.

Several studies have been developed with the aim of improving the viability and biological activity of the cells during and after spray drying and during subsequent storage and rehydration of the powder The strategies include preparation of the cells, process optimization, application of different agents of protectants and improving cellular heat-resistance (Desmond et al., 2001; Fu and Chen, 2011; Huang et al., 2016; Schuck et al., 2013).

Maximizing the survival of LAB cultures during drying and ensuring a subsequent storage for long periods is essential to maintain technological characteristics and economic viability. The biological activity of a lactic acid starter that includes cell viability and physiological state is necessary to evaluate the quality of the starter. Biological activity is defined as the ability of a lactic acid starter to acidify a certain medium (Peighambardoust et al., 2011).

The use of carrier media of dairy-based can improve the viability and biological activity of the cells during the spray drying process. Skim milk is one of the most effective

protectants reported in literature (Fu and Chen, 2011). However, the mechanism by which skim milk promote protection of the bacteria in drying methods is unclear It is necessary more scientific research to evaluate the impact and the mechanisms of this protection (Fu and Chen, 2011; Huang and Chen, 2013). There are just some hypotheses (Ananta et al., 2005b; Corcoran et al., 2004; Santivarangkna et al., 2008a, 2007).

This protection can be explained by the fact that during water removal in the spray drying, the lactose interacts with polar groups of phospholipids and proteins of the cell membrane, thereby minimizing damage to the membrane (Ananta et al., 2005b; Santivarangkna et al., 2008a, 2007). Two hypotheses, the so-called water replacement and vitrification, were proposed to explain the mechanism of membrane stabilization by sugars (Santivarangkna et al., 2008a). Vitrification hypothesis is based on glass formation in a dry state by sugars (Crowe et al., 1998). Another hypothesis refers to water replacement, where specific and particular interactions between phospholipids and sugars are required to ensure the protective effect. The interactions occur via the hydrogen bond between hydroxyl groups of the sugars and the phosphate group at the surface of the bilayer (Santivarangkna et al., 2008a).

Researchers suggest that other milk components as calcium (Ca^{2+})can also help to maintain the viability of the cells during the drying process and to avoid damages by thermal stress (Ananta et al., 2005b; Fu and Chen, 2011; Huang and Chen, 2013; Huang et al., 2016, 2014).

Huang and Chen (2013) reported that certain Ca^{2+} concentrations enhanced the heat resistance of the LAB strains to different extents. Huang et al. (2014) evaluated improving the viability of LAB under heat stress in Ca-aggregated milk. According to the authors the presence of Ca^{2+} in the milk induced the highest microbial heat stability among the bacteria tested due to the formation of milk capsules around LAB cells that neutralize the surface

charge and increase the adhesiveness of bacterial exopolysaccarides. Moreover, the casein micelles interaction with untreated milk and whey proteins and denatured whey proteins under heat can explain heat stability of the Ca-aggregated milk (Huang et al., 2014). The Ca^{2+} could allow the adhesion of bacterial cells to milk proteins (Tian et al., 2012).

Huang et al. (2016) reported that the supplementation of casein peptone in sweet whey in different concentrations and the use of sweet whey in large solids concentrations increased the final population in probiotic strains.

Carrier media in large solids concentrations allow the induction of osmotic stress conditions where the cells can adapt and accumulate intracellular coumpounds. This adaptation to osmotic stress can promote a better protection after the drying process by heat (Huang et al., 2016). According to Desmond et al. (2001) the accumulation of the compatible solutes exhibits cross-protection effects on microbial stresses as heat stress.

The synthesis of stress response proteins during growth of bacterial cells can also furnish the cells with essential proteins during the recovery stage after drying. Work by Teixeira et al. (1995) demonstrates that protein synthesis was not involved in the repair process of sublethal damage by *Lb. delbrueckii* ssp. *bulgaricus*. This indicates that the cells do not need to synthesize new proteins to repair the damage caused by drying in the cells or that the cells become unable to synthesize proteins due to drying damage. Thus, these proteins of stress response must be synthesized prior to drying in order to assist cell recovery after drying (Morgan et al., 2006).

The definition of an optimal growth phase for drying survival depends on the organism, the growth phases of bacterial in batch cultures, i.e. lag, log, stationary and death phases can also affect the responses of bacterial cultures to stress (Carvalho et al., 2004b; Fu and Chen, 2011; Zhang et al., 2012). For example, due to carbon starvation and exhaustion of available food sources in stationary phase, generally, bacteria develop a stress resistance and

therefore they would be more resistant to various types of stresses when compared with bacteria in another growth phase. These survival responses can protect the cell in other adverse conditions, such as inactivation by dehydration and inactivation for exposition in high or low temperatures during the process (Morgan et al., 2006).

The knowledge of the impact and the nature of the injury produced by a variety of stressful conditions (e.g. freezing, drying, storage or rehydration) on survival cells is important for the production of dried starter cultures. On the basis of information on the protection mechanisms related to media components used in drying, the growth phase, the induction of stress proteins and the synthesis of compatible solutes, the cultures will be characterized by high survival rates and biological activity (Keivani et al., 2014).

Normally, the processing conditions of freeze drying are milder than those of spray drying and higher survival rates can be achieved in freeze dried powders (To and Etzel, 1997; Wang et al., 2004). However, various factors need to be considered, such as the species of microorganism, storage conditions, drying media, etc in both processes. Indeed, the amount of information comparing both techniques is scarce and controversial. While some researchers reported a higher survival with FD (Berner and Viernstein, 2006; Corcoran et al., 2006; Costa et al., 2000; To and Etzel, 1997; Wang et al., 2004), others did not find differences between these two drying methods (Teixeira et al., 1995; Zamora et al., 2006).

To and Etzel (1997) tested the survival and lactic acid production of *L. lactis* ssp. *cremoris* D11, *Lactobacillus casei* ssp. *pseudoplantarum* UL137, and *St. thermophilus* CH3TH, separately frozen, freeze dried or spray dried before and after processing. After spray-drying, survival was greatest for *S. thermophilus* and lowest for *L. lactis*. Survival after spray-drying was much lower than after freeze drying and frozen.

However, Zamora et al. (2006) reported that loss of viability of freeze dried cells were observed immediately after drying whereas cellular damage due to spray drying did not

become evident until the storage. And Teixeira et al. (1995) did not find significant differences between these two drying methods.

PREAMBLE

PREAMBLE

The isolation of autochthonous LAB from artisanal cheeses allows identifying the species involved in the characterization of product. Certain compounds as lactic acid, bacteriocins, diacetyl among other produce by LAB can determine the selection of potential strains for industrial applications. In this context, the aim of this thesis was to isolate and to identify the LAB biodiversity involved in the production of Marajó cheese and to evaluate the effects of freeze drying and spray drying in the preservation of LAB. This thesis was divided in two steps. The Chapter 2 and Chapter 3 consists the first step and the Chapter 4, the second step. Our questions in the first step were:

- Which LAB strains are involved in the production of Marajó cheese?
- Have the LAB isolated interesting phenotypic characteristics for industrial application?

In the first step, the LAB were isolated in different samples (raw buffalo milk, curd, utensils and Marajó cheese) in appropriate culture media (M17 and MRS) for this microbial group. The isolates were selected based on compatible characteristics with LAB group (cocci and bacilli Gram-positive and negative-catalase). Posteriorly, the identification was performed by 16S rDNA gene sequencing and the different genetic profiles were determined by Rep-PCR. The phenotypical characteristics of strains for potential industrial application also were evaluated.

Some strains identified in the first step showed interesting phenotypical characteristics for potential industrial application. Among the isolates, we selected a strain of *L. plantarum* from Marajó cheese isolated in MRS agar as a model of study for preservation of LAB by freeze drying and spray drying in the second step of this thesis. This strain showed good

acidifying activity in skim milk 10%, diacetyl production, proteolitic activity in milk ágar, antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, and potential probiotic (acid and bili salts conditions). Our questions in the second step were:

- What are the effects on the survival rate of LAB after freeze and spray drying?
- Can the carrier media minimize the effects of freeze and spray drying?

The cells of *L. plantarum* were concentrated by centrifugation and added in three different dairy-based carrier media (skim milk, sweet whey and sweet whey permeate). The effects of freeze drying and spray drying were evaluated immediately after the processes (Time 0), in relation to survival rate, acidifying activity and probiotic potential. The survival rate was also evaluated during 60 days of product storage at 4 °C (freeze and spray dried samples) and 20 °C (spray dried samples). All these analyses were performed in triplicate.

CHAPTER 2

Biodiversity of autochthonous lactic acid bacteria involved in the Marajó cheese making from Amazon region, Brazil

Abstract

Northern Brazil, in the Amazon region stands out in the production of an artisanal cheese on the Marajó Island (Pará, Brazil). Marajó artisanal cheese is made from raw buffalo milk and fermented by its autochthonous microbiota. The knowledge of LAB diversity is fundamental in the characterization of this type of cheese. The aim of this study was to isolate and to identify the LAB present in raw buffalo milk, curd and utensils used in the Marajó Cheese Making and to evaluate phenotypical characteristics of strains for potential industrial application. Preliminary characterization as Gram positive cocci and bacilli and as negative catalase and identification using 16S rDNA sequence analysis and Rep-PCR was undertaken for 149 LAB cocci and bacilli obtained from all the samples evaluated to identify and characterize the LAB species involved in Marajó cheese making. Seven species were identified as follows: *Weissella confusa, Streptococcus infantarius, Lactococcus lactis, Leuconostoc pseudomesenteroides, Weissella paramesenteroides, Pediococcus pentosaceus, Lactobacillus brevis* and *Enterococcus* genus. Different species of LAB were identified in all the evaluated samples. The Rep-PCR was able to identify different genetic profile showing a high diversity among the evaluated isolates. It also was possible to observe different phenotypic characteristics among the isolates considered as clone by Rep-PCR. The genetic and phenotypic variability shown by strains allow of directing the definition of properties that contribute to the characteristics of the Marajó cheese in order to facilitate its standardization and commercialization to meet consumer demands for a good quality product.

Keywords: artisanal cheese, Rep-PCR, 16S rDNA sequencing, *Weissella* sp., *Streptococcus infantarius*

1. Introduction

The artisanal cheeses traditionally produced with raw milk have a great diversity in their autochthonous microbiota. The diversity of lactic acid bacteria (LAB), a fundamental group in the characterization of this type of cheese, depends mainly on the microbiota present in the raw milk used (Feutry et al., 2012) and is influenced by factors such as practices conducted during the cheese making. Steps such as acidification, heating, salting and ripening have a great influence on the final characteristics of the cheese and have a significant role in the microbial composition (Randazzo et al., 2009). Additionally, LAB is also commonly found in biofilms of equipment or utensils used in the cheese making. These biofilms can come off the surface of utensils, becoming part of the microbiota that will contribute to the curd fermentation and cheese ripening (Galinari et al., 2014; Licitra et al., 2007; Lortal et al., 2009).

LAB from artisanal cheeses contribute to original sensory properties, being then featured as unique and specific of each region, preserving a traditional production of cultural, economic and social importance (Arcuri et al., 2013). Cheese flavor and texture are the result of a complex mixture of compounds, originating mainly from the enzymatic degradation of curd components by cheese microbiota. Additionally, LAB can produce compounds of interest in fermented milk products which are able to decrease the pH of the medium that inhibits the multiplication of other microorganisms (Azizan et al., 2012; Moraes et al., 2012).

The study of biodiversity of the involved microbiota in artisanal cheese making unfolds the knowledge of LAB associated with features and quality of the product as well as

new strains identification (Franciosi et al., 2009). They are closely related with environmental conditions such as temperature, origin and milk quality, hygiene conditions and specificities of the cheese production process (Baruzzi et al., 2000; Morea et al., 1999; Randazzo et al., 2007, 2002; Terzic-Vidojevic et al., 2014). Many authors have conducted researches on the identification and on the characterization of LAB from artisanal cheeses (Dal Bello et al., 2012, 2010; Franciosi et al., 2009; González et al., 2015; Perin et al., 2012; Serhan et al., 2009; Terzic-Vidojevic et al., 2014; Terzić-Vidojević et al., 2015). However, considering that milk is an important source of bacteria, the isolation of LAB from raw milk, rather than cheese, may have the advantage of giving a higher choice of selection of species and strains which may have interesting features for application in cheese that could otherwise be accidentally lost in several steps or practices conducted during the cheese making (Franciosi et al., 2009).

The biodiversity of LAB from artisanal cheeses can be determined by using genetic fingerprinting techniques such as amplification of repetitive DNA elements by Polymerase Chain Reaction (Rep-PCR). This technique has a high discriminatory power and it is used in the study of the ecology of diverse microorganisms (Gevers et al., 2001; Mustopa and Fatimah, 2014; Perin and Nero, 2014; Pogačić et al., 2013; Terzic-Vidojevic et al., 2014; Tormo et al., 2015). Moreover, 16S rDNA sequencing has been used for investigating the microbial populations in milk and dairy products (Feutry et al., 2012; Franciosi et al., 2009; Terzic-Vidojevic et al., 2014).

In Brazil, there are traditionally producing regions of artisanal cheeses such as the state of Minas Gerais (Castro et al., 2016; Freitas et al., 2015; Galinari et al., 2014; Luiz et al., 2016; Martins et al., 2015) and, cheese making itself is considered as Brazilian Immaterial Cultural Patrimony (Castro et al., 2016). The production of this type of cheese is familiar and

the technologies involved are passed on from generation to generation, constituting part of cultural richness of certain regions.

In Northern Brazil, in the Amazon region, what stands out is the production of an artisanal cheese on the Marajó Island (Pará, Brazil). Marajó artisanal cheese is made from raw buffalo milk and fermented by its autochthonous microbiota. Although there is legislation that determines the quality parameters of Marajó Cheese (Adepará, 2013), there is still few information on autochthonous LAB microbiota responsible for natural fermentation of milk that contributes to the definition of the characteristics of this typical cheese.

The aim of this study was to isolate and to identify the LAB present in raw buffalo milk, curd and utensils used in the Marajó Cheese making and to evaluate phenotypical characteristics of strains for potential industrial application.

2. Material and methods

2.1. Lactic Acid Bacteria Isolation

Samples of raw buffalo milk (n = 3), curd (n = 3), aluminum pan (n = 4), milking jug (n = 3) and wooden utensil (n = 1) were aseptically collected in two dairy cheesemakers, located on Marajó Island (Pará, Brazil). The cheesemakers selected are considered the major producers of Marajó Cheese in the region. All collected samples were kept under refrigeration until microbiological analysis.

The raw buffalo milk samples were diluted in 0.85% NaCl (w/v). For curd samples, 25 g of each sample was obtained and transferred to individual sterile bags containing 225 mL of 0.85% NaCl (w/v), homogenized in stomacher for 1 min, and diluted in the same solution. In addition, surface samples (50 cm^2) delimited by plastic molds were obtained using individual and sterile swabs and put in tubes containing 10 mL peptone water (peptone 10 $g \cdot L^{-1}$; sodium chloride 5 $g \cdot L^{-1}$) according to modified method propose by Wehr and Frank (2004) from the

following points: aluminum pan, milking jug, wooden utensil and mechanical press. The swab was realized five times for each sample, totaling an area of 250 cm^2. The dilutions were prepared with the same solution described above.

From each sample, the dilutions were pour-plated in duplicate in Man, Rogosa and Sharpe (MRS, Difco, France) agar, incubated at 30 °C for 48 h (Lactobacilli) under aerobic conditions and M17 (Difco, France) agar, incubated at 30 °C for 48 h (Lactococci) and 42 °C for 48 h (Streptococci) under aerobic conditions.

After incubation, 10% of colonies of each sample were randomly picked from the highest dilutions and were classified according to Gram staining and catalase. Isolates were then purified by streaking, in duplicate on MRS agar plates incubated at 30 °C for 48 h under aerobic conditions. Subsequently, purified isolates were stored at -20 °C in MRS broth media supplemented with 30% (v/v) glycerol. Prior to molecular analysis, the stored colonies were transferred to MRS broth and incubated at 30 °C for 24 h.

2.2. Molecular identification

2.2.1 16S rDNA sequencing

The isolates characterized as Gram positive cocci and bacilli and as negative catalase were identified by 16S rDNA gene at species level. The genomic DNA of the isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). For the amplification reaction the primers P027 (GAGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGCTT), specifically for V9 and V6 regions of the 16S rDNA gene, were used. The PCR amplification products were subject to sequencing of the 16S rDNA gene for identification of the bacterial species (Macrogen, Seoul, Korea). Subsequently, the resulting nucleotide sequences were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) to verify the similarity with strain references.

2.2.2 Rep-PCR analysis

From the 16S rDNA sequencing results the biodiversity at the strain level for the isolated LAB was assessed by $(GTG)_5$ Rep-PCR. Rep-PCR fingerprinting was performed with a single oligonucleotide primer $(GTG)_5$(5'-GTGGTGGTGGTGGTG-3') (Gevers et al., 2001). PCR reactions were performed according to Dal Bello et al. (2010); Perin and Nero, (2014) containing 12.5 µL of Go Taq Green Master Mix 2x (Promega), 100 pMol of the primer, 2 µL of DNA (50 $ng \cdot \mu L^{-1}$) and ultra-pure PCR water (Promega) to a final volume of 25 µL. PCR amplification was carried out under the following conditions: 5 min at 95°C as initial step, 30 cycles of 30 s at 95°C; 30 s at 40°C and 8 min at 65°C for anneling, and final extension of 16 min at 65°C.

Rep-PCR products were electrophoresed in a 2% agarose gel for 3h at a constant voltage of 90 V in 0,5X TBE buffer (10 $mmol \cdot L^{-1}$ Trisborate, 1 $mmol \cdot L^{-1}$ EDTA, pH 8.0). A 1 Kb DNA ladder (Invitrogen) was used as molecular size marker. Gels were stained using GelRed (Biotium Inc., Hayward, CA, USA), and the Rep-PCR profiles were visualized and recorded under ultraviolet light using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil). The resulting fingerprints were analyzed by the BioNumerics 6.0 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation with 1% optimization and average linkage dendrograms (UPGMA) were obtained from the isolates.

2.3. Phenotypic characteristics

Fourteen strains of LAB most frequently isolated in this study were selected for evaluation of technological potential. The aim was to determine the technological potential of species for application in dairy products.

2.3.1 Acidifying activity

The selected strains were activated in MRS broth and grown at 30 °C for 20h. For the acidifying activity test, the strains (1% v/v) were inoculated in 10 mL of sterile skim milk (RSM 10% w/v) and incubated at 30 °C. The pH was measured after 6 h and after 24 h with a pH meter. The data is expressed on the average of duplicate analyses.

2.3.2 Proteolytic activity

Extracellular proteolytic activity was determined following the modified method of Franciosi et al. (2009). Briefly, ten microliters of activated strains were placed on the surface of a Plate Count Agar (PCA, Difco, France) medium supplemented of 10% (w/v) skim milk powder and incubated at 30 °C for 4 days. Proteolytic activity was indicated by a clear zone forming around the colonies.

2.3.3 Diacetyl production

Diacetyl production was determined according to the adapted method (Dal Bello et al., 2012). Revitalized strains (1% v/v) were inoculated in 10 mL of skim milk and incubated at 30 °C for 24 h. One mL of each cell suspension was combined with 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) and incubated at 30 °C for 30 min. Diacetyl production was indicated by the formation of a red ring on the top of the tubes.

2.3.4 Potential of antimicrobial activity

The antimicrobial activity was tested by agar diffusion assay according to the adapted method of Hungaro et al. (2013). A top agar layer was prepared containing 1 mL of pathogenic bacteria in 5 mL of soft-agar (0.7% w/v) and applied to the surface of BHI (Himedia, India) agar plates. After solidification of the top layer , micro droplets of 20 µL of the selected LAB were placed on and incubated at 37 °C for 24 h. A clear zone of inhibition around the droplets was taken as a positive signal for possible antimicrobial activity. The top layer was prepared containing *Escherichia coli* O157:H7, *Salmonella* Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 7644 or *Staphylococcus aureus* ATCC 25923 separately.

3. Results

3.1. Identification of microbial isolates

For this study, two cheesemakers were selected based on their importance in Marajó cheese production. Among the different isolates from all the samples, 149 isolates were chosen based on compatible characteristics with LAB group (Gram-positive and negative-catalase). The isolates were obtained from raw buffalo milk (n = 41), curd (n = 44), aluminum pan (n = 37), milking jug (n = 21) and wooden utensils (n = 6). Isolates from raw buffalo milk, curd, aluminum pan, milking jug and wooden utensil were submitted to 16S rDNA and the isolates were identified as *Weissella confusa* (n = 73), *Streptococcus infantarius* (n = 32), *Enterococcus* (n = 22), *Lactococcus lactis* (n = 15), *Leuconostoc pseudomesenteroides* (n = 2), *Weissella paramesenteroides* (n = 2), *P. pentosaceus* (n = 2) and *Lactobacillus brevis* (n = 1). The specie *Staphylococcus saprophyticus* (n = 4) was also identified from M17 medium. And the specie *W. confusa* was the most frequently isolated and present in all the evaluated

samples in the two cheesemakers, followed by *S. infantarius*, *Enterococcus* genus. The table 2.1 shows the isolates identified among the evaluated samples.

Table 2.1 – Isolates involved in Marajó cheese making

Isolates	Milk		Curd		Milking jug		Aluminium pan		Wood utensil	
	A	B	A	B	A	B	A	B	A	B
W. confusa	6	7	11	14	4	5	8	15	3	-
S. infantarius	13	4	-	4	-	-	-	9	2	-
Enterococcus	3		6	5	3	1	1	3	-	-
L. lactis	6	1	-	3	5	-	-	-	-	-
W. paramesenteroides	-	-	-	-	-	-	-	-	2	-
P. pentosaceus	-	-	-	-	2	-	-	-	-	-
Le. pseudomesenteroides	1	-	-	1	-	-	-	-	-	-
Lb. brevis	-	-	-	-	1	-	-	-	-	-

A: cheesemaker A; B: cheesemaker B

3.1. LAB characterization by Rep-PCR

The analysis of the Rep-PCR profiles allowed differentiation of 65 different profiles among the 149 isolates analyzed, identifying 21 distinct profiles for *W. confusa* isolates, 18 for the *S. infantarius* isolates, 12 for the *Enterococcus*, 9 for the *L. lactis* isolates and 2 profiles for *Le. pseudomesenteroides* isolates; for the other genera only one strain was identified, presented each a distinct profile. Among the isolates from cheesemaker A, the Rep-PCR has defined 37 distinct genetic profiles and 28 distinct genetic profiles and among the isolates from cheesemaker B with 90% of similarity. From this typing method, the bacteria

with the same profile could be identified as coming from different biotopes and cheesemakers.

Among the strains from cheesemaker A were identified eleven distinct profiles for the *S. infantarius* strains, 9 profiles for the *W. confusa* strains, 7 profiles for the *Enterococcus* strains, 6 for the *L. lactis* strains and 1 profile for the *Lb. brevis, Le. pseudomesenteroides, P. pentosaceus* and *W. paramesenteroides* strains. Some strains from different samples showed the same Rep-PCR profile. The strains of the same genetic profile of *W. confusa* and *Enterococcus* were identified and distributed among the samples of milk, curd, milking jug, aluminum pan and wooden utensil whereas all the strains of *S. infantarius* that showed the same genetic profile are from milk. And among the strains of *L. lactis* no genetic identical strain was identified in different samples.

From cheesemakers B were identified 12 distinct profiles for the species *W. confusa*, 7 profiles for *S. infantarius* strains, 5 profiles for *Enterococcus* strains, 3 profiles for *L. lactis* strains and 1 profile for *Le. pseudomesenteroides* strains. The strains of the same genetic profile of *W. confusa* and *S. infantarius* were identified and distributed among the samples of milk, curd, milking jug and aluminum pan. While all the strains of *Enterococcus* that showed the same genetic profile are from curd. And among the strains of *L. lactis* just one clone was identified in milk and curd samples.

Considering the two cheesemakers it was possible to identify 21 distinct profiles of *W. confusa* (Fig. 2.1) and 20 distinct profiles of *S. infantarius* (Fig 2.2). The same genetic profile was detected in some strains of *W. confusa* between the two cheesemakers and in different samples of milk, curd, milking jug, aluminum pan and wooden utensils evaluated in this study whereas *S. infantarius* strains from different cheesemakers were grouped separately.

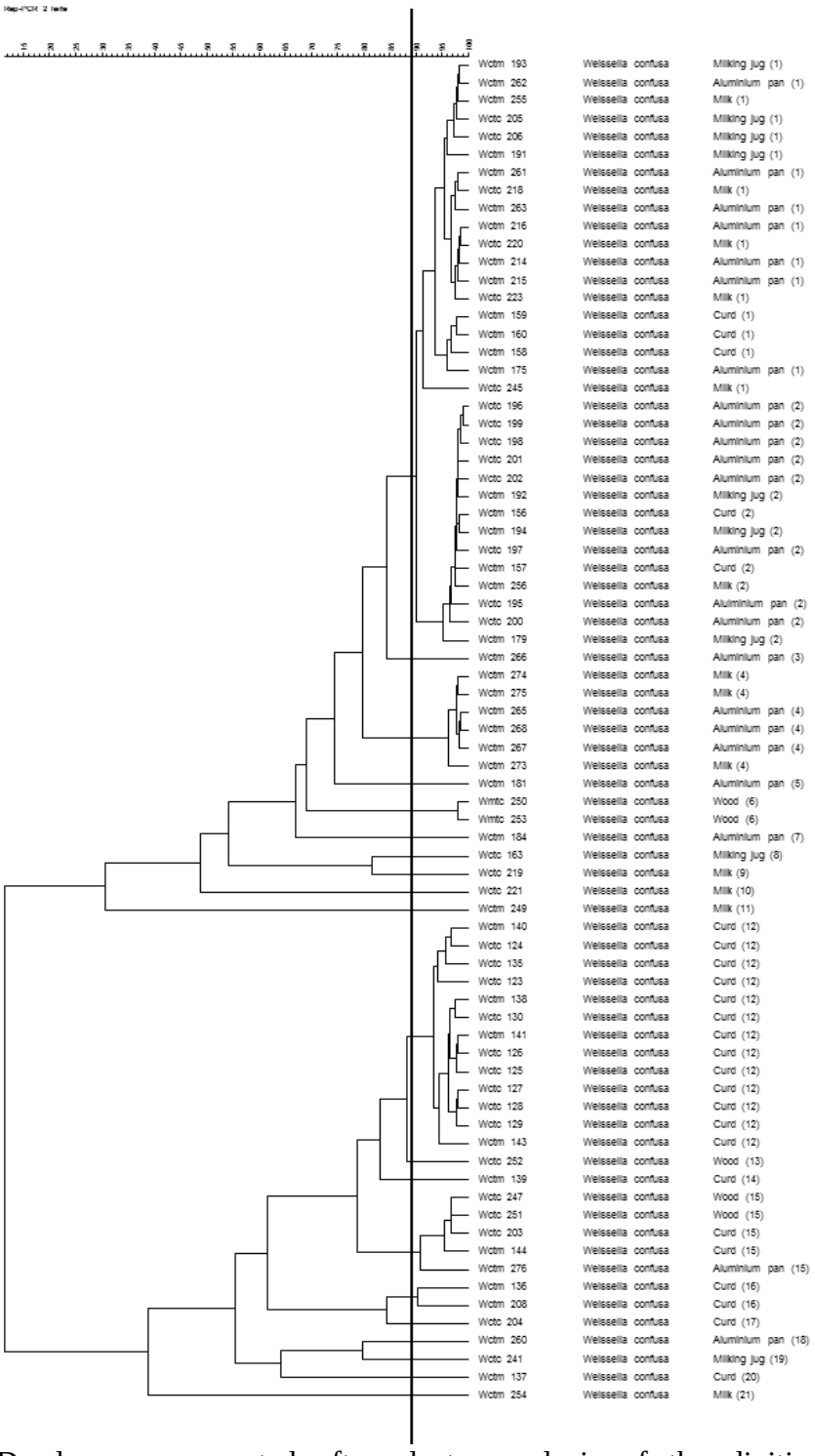

Figure 2.1 – Dendrogram generated after cluster analysis of the digitized Rep-PCR fingerprints of *Weissella confusa* strains

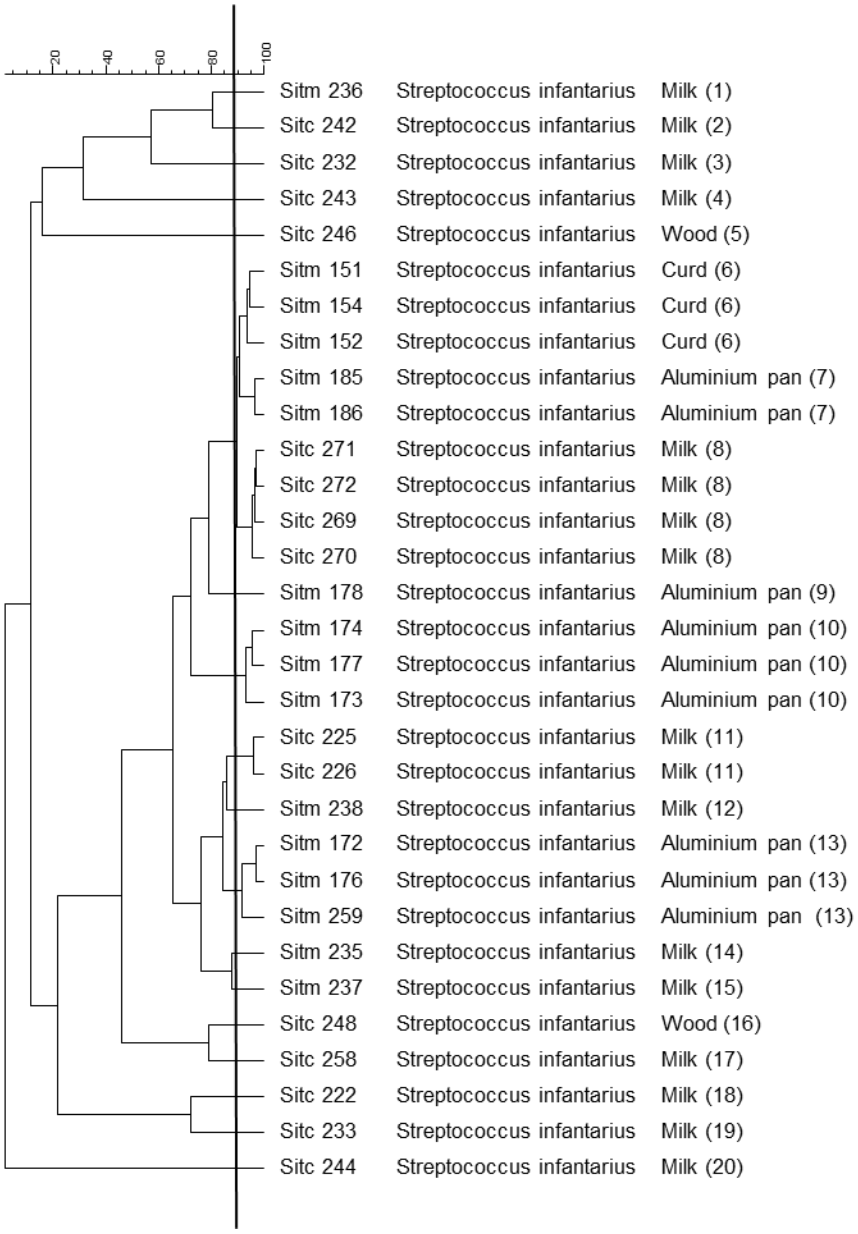

Figure 2.2 – Dendrogram generated after cluster analysis of the digitized Rep-PCR fingerprints of *Streptococcus infantarius* strains

3.2. Phenotypic characteristics

Strains of *W. confusa*, *W. paramesenteroides*, *S. infantarius* and *Enterococcus* were selected for evaluation of phenotypic characteristics. The selection was based on the presence of these species in all evaluated samples, especially, in milk and curd samples. Additionally, there are no frequent reports of isolation of the species *W. confusa, W. paramesenteroides* and *S. infantarius* in artisanal cheeses, though *Enterococcus* genus is frequently isolated in these types of cheeses. The results are shown in the Table 2.2. The majority of strains evaluated showed a good acidifying and proteolytic activity. Diacetyl have been produced by different species evaluated. The strains of *W. confusa*, considered genetically identical (Wctc 247 and Wctm 276) by Rep-PCR, showed different phenotypic profiles. Two strains of *Weissella* sp. that showed the same genetic profile by Rep-PCR showed different phenotypes. The strain (Wctm 276) coming from aluminum pan of cheesemaker B presented acidifying activity, proteolytic activity and diacetyl production. In contrast, the strain Wctc 247 had antimicrobial activity against *Salmonella* and *S. aureus* and the other strain Wctm 276 showed no antimicrobial activity against any of the tested pathogens in this study. On the other hand, *S. infantarius* strains showed distinct genetic profiles from different cheesemakers and samples. Among the strains of *S. infantarius* (Sitm 178 and Sitm 235) coming from milk and aluminum pan of cheesemaker B were considered more acidifying, proteolytic and better producers of diacetyl than the other.

Table 2.2 – Phenotypic characteristics of strains isolated from raw milk, curd and utensils

Strain	Origin	Acidification			Diacetyl production	Proteolytic activity	Antimicrobial activity
		pH t_0	pH t_{6h}	pH t_{24h}			
W. confusa 218	Milk (A)	6,67	6,53±0.02	6,0±0.04	-	+	SA; LM; EC
W. confusa 221	Milk (A)	6,67	6,35±0.02	5,80±0.03	-	+	SA; LM; SE
W. confusa 247	Wood (B)	6.67	6,46±0.08	6,15±0.02	-	-	SA; SE
W. confusa 249	Milk (B)	6.67	6,34±0.02	6,30±0.03	-	+	SA; LM; EC
W. confusa 266	Aluminium pan (B)	6.67	6,35±0.05	6,41±0.03	-	+	SA; LM; SE
W. confusa 276	Aluminium pan (B)	6.67	5,86±0.05	4,80±0.02	+	+	LM
S. infantarius 178	Aluminium pan (B)	6.67	5,90±0.03	4,84±0.05	+	+	-
S. infantarius 233	Milk (A)	6.67	6,65±0.06	6,60±0.05	-	-	SA
S. infantarius 235	Milk (B)	6.67	5,71±0.04	5,02±0.03	+	+	-
S. infantarius 242	Milk (A)	6.67	6,63±0.02	5,50±0.03	+	-	-
Enterococcus 132	Curd (A)	6.67	6,28±0.03	5,60±0.04	+	+	SA
Enterococcus 180	Aluminium pan (B)	6.67	6,23±0.04	5,70±0.02	+	+	SA; LM
Enterococcus 227	Milk (A)	6.67	6,24±0.03	5,56±0.05	+	+	-
W. paramesenteroides 250	Wood (B)	6.67	6,35±0.02	5,11±0.02	-	+	SA; SE

(+) positive ; (-) negative. A: Cheesemaker A; B: Cheesemaker B

EC = *E. coli*; SA = *S. aureus*; LM = *Listeria monocytogenes*; SE = *Salmonella* Enteritidis.

In general, the strains evaluated have also shown a good potential of antimicrobial activity against the pathogens tested in this study. From all 14 selected strains, nine showed antimicrobial potential activity for at least one of the pathogens. All *W. confusa* strains, except Wctm 276, two *Enterococcus* (Ettc 180 and Ettm 132), *S. infantarius* (Sitc 233) and *W. paramesenteroides* (Wptm 250) showed zone of inhibition around *S. aureus*. The strain Ettc 180 and the strains of *W. confusa*, except Wctc 247 showed zone of inhibition around *L. monocytogenes*. However, the strain Wctc 247 showed zone of inhibition around *Salmonella*, additionally other *W. confusa* (Wctc 221 and Wctm 266) and Wptm 250 also inhibited

Salmonella. While only *W. confusa* strains (Wctc 218 and Wctm 249) inhibited *E. coli*. In general, *W. confusa* strains demonstrated the best antimicrobial potential activity.

4. Discussion

Identifying microbiota from raw milk for artisanal cheese making is important, but just this identification cannot represent the reality of the all the species involved in the product. Therefore, it is also important to evaluate the utensils and equipment used in artisanal cheeses fabrication that also play a fundamental role in the determination of cheese characteristics during the different stages of production due to the possibility of cross-contamination (Galinari et al., 2014; Licitra et al., 2007; Somers et al., 2001) and to allow of a better understanding of the role of each species that can contribute to development of taste, flavor and texture of cheese.

The characterization of LAB from different origins is important in order to understand their diversity in different niches, thus allowing to relate the isolates with the characteristics that define the artisanal cheese as unique in a specified region. Moreover, research in this area facilitates the selection of strains for industrial application, especially as starter cultures in the production of cheese. According to Freitas et al. (2015) a good knowledge of the strains from artisanal cheeses can also help preventing the presence of defect-causing strains in certain products.

For isolation of LAB from different samples of milk, curd and utensils, the MRS and M17 were used as culture media. These media widely used in the isolation and enumeration of this microbial group were able to select seven different LAB genera. Among the isolates, *L. lactis, P. pentosaceus, Le. pseudomesenteroides, Lb. brevis* and the *Enterococcus* genus are normally isolated from milk, utensils, equipment and artisanal cheeses in other studies (Castro et al., 2016; Feutry et al., 2012; Perin et al., 2012; Terzic-Vidojevic et al., 2014b; Van Hoorde

et al., 2008). However, in this work, strains that are not present in the LAB group as *S. saprophyticus* were isolated in M17 medium. The low selectivity of this medium has been described by other authors (Dasen et al., 2003; Gonçalves et al., 2009).

In this study, *Weissella sp.* was the LAB genus most frequently identified. Though, there are few publications in the literature at present on the isolating of this genus in cheese (Ayeni et al., 2011; Fusco et al., 2015; Masoud et al., 2012). This genus has been identified in traditional fermented vegetables in Asian and Africa (Ono et al., 2014; Ouoba et al., 2010; Patel et al., 2013) and in European sourdoughs (Moroni et al., 2011; Robert et al., 2009). The interest for technological application of this genus, exploitation of specific strains as starter cultures for vegetables, sorghum and wheat sourdoughs have been considered (Galle et al., 2010; Park et al., 2012) or specific strains have been investigated for use as probiotics (Ayeni et al., 2011; Lee et al., 2012; Patel et al., 2013). Several studies also indicate the potential of antimicrobial activity of *Weissella sp.*in different environments (Ayeni et al., 2011; Nam et al., 2002; Ndagano et al., 2011). Additionally, *S. infantarius* is also not often associated with artisanal cheese, but this specie is normally present in African fermented milk products (Abdelgadir et al., 2008; Jans et al., 2013).

Considering the molecular characterization of the isolates, the generated dendrograms by Rep-PCR demonstrated that this molecular typing method is able to identify genetic differences among the studied strains. However, the data revealed that strains of *Weissella* sp. from different cheesemakers and samples showed the same genetic profile (Fig. 2.1). Although there is no genetic difference among the strains, according to the Rep-PCR data, it was possible to observe different phenotypic characteristics among them. Terzic-Vidojevic et al. (2014) also found that LAB strains isolated from artisanal cheeses with similar genetic profiles formed by Rep-PCR showed certain differences in the phenotypic characteristics.

Additionally, the antimicrobial potential shown for the different tested strains can contribute to the product safety. Numerous researches have demonstrated that the autochthonous microbiota present in artisanal dairy products are associated with antimicrobial activity (Ayad et al., 2000; Cocolin et al., 2007; Dal Bello et al., 2012, 2010; Moraes et al., 2012; Perin and Nero, 2014; Perin et al., 2012; Topisirovic et al., 2006; Tormo et al., 2015).

The artisanal cheeses are considered as the fundamental economic element for the viability of families that depend mainly on this activity. Additionally, gathering information on LAB microbiota in these cheeses can help preventing the loss of microbial biodiversity. It is thus important to know the autochthonous microbiota from raw material, curd and utensils involved in Marajó cheese making.

5. Conclusion

The present study demonstrated the genetic diversity of LAB species obtained from buffalo milk, curd and utensils used in the Marajó cheese making as well as interesting phenotypic characteristics by species of *W. confusa* and *S. infantarius*.

This study has contributed to the identification of a part of the LAB associated with Marajó Cheese making in the Amazon region. The genetic and phenotypic variability showed by strains allows of directing the definition of properties and characteristics of the Marajó cheese in order to facilitate its standardization and commercialization and to meet consumer demands for a good quality product.

Additionally, this study opens new prospects to preservation and development of new cultures for industrial application, especially cultures of *W. confusa* or *S. infantarius*. These species that are not normally associated with artisanal cheeses presented interesting

phenotypic characteristics for future investigation in the manufacturing of traditional products on industrial scale or in quality improvement of existing dairy products as Marajó cheese.

CHAPTER 3

Identification of lactic acid bacteria cocci isolated from Marajó cheese produced in the Amazon region, Brazil during dry and rainy seasons

Abstract

Marajó cheese is a typical artisanal product from Marajó, an island that is part of the state of Pará, located in the Amazon region, Brazil. It is defined as a fresh cheese obtained through natural coagulation of milk by means of the autochthonous microbiota and subsequent curd fusion. The aim of this study was to isolate autochthonous *Lactococcus* spp. from Marajó Cheese during dry and rainy seasons and to evaluate the phenotypic characteristics for potential industrial application. Preliminary characterization as Gram positive cocci and as negative catalase and identification using 16S rDNA sequence analysis and Rep-PCR were undertaken for 97 LAB cocci obtained from all the samples evaluated to identify and characterize the LAB species from Marajó cheese. Five species have been identified as follows: *Lactococcus lactis, Pediococcus acidilactici, Pediococcus pentosaceus, Weissella paramesenteroides, Streptococcus infantarius* and *Enterococcus* genus. Different species of LAB cocci were identified in the two types of Marajó cheese and in the two seasons evaluated. The Rep-PCR was able to identify different genetic profiles showing a high diversity among the evaluated isolates. However, the distinct genetic profiles shown by the strains in different types of Marajó cheese have not determined phenotypic differences in the conditions studied. The phenotypic characteristics showed by LAB cocci in this study allow of opening new prospects for further investigation of the role of each species in the definition of sensory properties of the Marajó cheese.

Keywords: artisanal cheese, buffalo milk, Rep-PCR, 16S rDNA sequencing, *Lactococcus*

1. Introduction

Marajó cheese is a typical artisanal product from Marajó, an island that is part of the state of Pará, located in the Amazon region, Brazil. It is defined as a fresh cheese obtained by the natural coagulation of milk by the autochthonous microbiota and subsequent curd fusion. The autochthonous starter culture is obtained from the whey drained from cheeses made on previous day, which is collected and used for cheese making on the next day (Adepará, 2013).

This cheese shows soft, compact and closed texture, with small and few pores, pleasant aroma with a cylindrical or regular shape (Simões et al., 2014), and it is not subject to any further processing before consumption. According to cheese making process, the Marajó cheese is classified as: (1) Marajó cream type cheese (MC-cheese), which the raw buffalo skim milk is used and the cream obtained from skim milk is added during the curd fusion; (2) Marajó butter type cheese (MB-cheese), which the raw buffalo whole milk is used and butter is added during the curd fusion. Raw cow milk can be mixed with buffalo in a maximum proportion of 40% of the total volume of milk (Adepará, 2013).

The Marajó cheese making is considered a traditional technique that has been passed on from generation to generation for many years, being the main source of income for some cheesemakers. This cheese is known and appreciated throughout the state of Pará as a high quality artisanal product (Seixas et al., 2014a). However, there are few studies on its technological aspects, physico-chemical aspects and on the autochthonous microbiota responsible for natural coagulation of milk that can be associated with the definition of the characteristics of this cheese (Bittencourt et al., 2013; Seixas et al., 2015, 2014b).

Thus, the knowledge of the microbiota of Marajó cheese is fundamental to better understand the species involved in the determination of the quality parameters and to preserve the microorganisms that are important in the characterization of this product that has economic and social representativity. In general, the autochthonous lactic acid bacteria (LAB)

has been widely identified and studied in several types of artisanal cheeses (Dal Bello et al., 2010; Perin et al., 2012; Pogačić et al., 2013; Santos et al., 2015, 2014; Terzic-Vidojevic et al., 2014; Terzić-Vidojević et al., 2015). LAB present in artisanal cheeses are responsible for the sensory attributes and the technological characteristics of each cheese (Dal Bello et al., 2010; Franciosi et al., 2009; Terzic-Vidojevic et al., 2014). Additionally, they can contribute to food preservation by producing organic acids that lower the pH or by producing antimicrobial substances like hydrogen peroxide and bacteriocins (Dal Bello et al., 2012; Ennahar et al., 2000; Moraes et al., 2013; Perin and Nero, 2014).

Among LAB group stands out the *Lactococcus* genus widely used as starter culture in cheese making (Hansen, 2002). Studies in fresh artisanal cheeses have identified the *Lactococcus* as an important genus in the characterization of this type of product (Castro et al., 2016; Pogačić et al., 2010; Terzic-Vidojevic et al., 2014), showing several properties such as the ability to rapidly produce acid, salt tolerance, proteolytic and peptidase activity, production of biogenic amines and nisin, autolytic activity, diacetyl production (Cogan et al., 1997; Dal Bello et al., 2012; Perin et al., 2015; Terzic-Vidojevic et al., 2014); characteristics considered to be fundamental in the selection of potential strains for industrial application (Dal Bello et al., 2012).

Different methods have been used to study of the biodiversity of LAB from artisanal cheeses. The using of genetic fingerprinting techniques contributes to significantly increase the knowledge of the diversity of the microbial ecosystem of the LAB from artisanal cheeses and can facilitate subsequent studies of characterization for the use of the strains in the food industry (Dal Bello et al., 2012, 2010; Franciosi et al., 2009; Terzic-Vidojevic et al., 2014, 2013).

In this context, the aim of this study was to isolate autochthonous *Lactococcus* spp. from Marajó Cheese during dry and rainy seasons and to evaluate the phenotypic characteristics for potential industrial application.

2. Material and methods

2.1. LAB isolation

Forty-four samples of Marajó cheese produced in the Marajó Island, Pará, Brazil from 13 cheesemakers (seven cheesemakers of MC-cheese and six cheesemakers of MB-cheese) were evaluated. The cheeses were collected in local market of Pará state, Brazil, in March and April 2013 (rainy season) and September and October 2012 (dry season) and kept at 4 ºC until microbiological analysis. The samples were categorized based on the industrial process that defines two types of Marajó Cheese as follows: (1) adding cream (cream type cheese) and (2) adding butter (butter type cheese). Portions of 25 g of each cheese were aseptically transferred to individual sterile bags containing 225 mL of 0.85% NaCl (w/v), homogenized, using a Stomacher for 1 min and diluted in the same solutions. From each sample, the decimal dilutions were pour-plated in duplicate on M17 agar (Difco, France), incubated at 30 °C for 48 h under aerobic conditions.

After incubation, 10% of colonies of each sample were randomly picked from the highest dilutions and were classified according to Gram staining and catalase production. Isolates were then purified by streaking, in duplicate on MRS agar plates incubated at 30 °C for 48 h under aerobic conditions. Subsequently, purified isolates were stored at -20 °C in MRS broth media supplemented with 30% (v/v) glycerol. Prior to molecular analysis, the stored colonies were transferred to MRS broth and incubated at 30 °C for 24 h.

2.2. Molecular identification

2.2.1. 16S rDNA sequencing

The isolates characterized as Gram positive cocci and as negative catalase were identified by 16S rDNA gene at species level. The genomic DNA of the isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). For the amplification reaction the primers P027 (GAGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGCTT), used specifically for V9 and V6 regions of the 16S rDNA gene, were used. The PCR amplification products were subject to sequencing of the 16S rDNA gene for identification of the bacterial species (Macrogen, Seoul, Korea). Subsequently, the resulting nucleotide sequences were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) to verify the similarity with strain references.

2.2.2. Rep-PCR analysis

From the 16S rDNA sequencing results, the diversity at the strain level for the isolated LAB was assessed by $(GTG)_5$ Rep-PCR. Rep-PCR fingerprinting that was performed with a single oligonucleotide primer $(GTG)_5$(5'-GTGGTGGTGGTGGTG-3') (Gevers et al., 2001). PCR reactions were performed according to Dal Bello et al. (2010); Perin and Nero, (2014) containing 12.5 µL of Go Taq Green Master Mix 2x (Promega), 100 pMol of the primer, 2 µL of DNA (50 $ng \cdot \mu L^{-1}$) and ultra-pure PCR water (Promega) to a final volume of 25 µL. PCR amplification was carried through the following conditions: 5 min at 95 °C as initial step, 30 cycles of 30 s at 95 °C; 30 s at 40 °C and 8 min at 65 °C for annealing, and final extension of 16 min at 65 °C.

Rep-PCR products were electrophoresed in a 2% agarose gel for 3h at a constant voltage of 90 V in 0,5X TBE buffer (10 $mmol \cdot L^{-1}$ Trisborate, 1 $mmol \cdot L^{-1}$ EDTA, pH 8.0). A 1

Kb DNA ladder (Invitrogen) was used as a molecular size marker. Gels were stained using GelRed (Biotium Inc., Hayward, CA, USA), and the Rep-PCR profiles were visualized and recorded under ultraviolet light using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil). The resulting fingerprints were analyzed by the BioNumerics 6.0 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation with 1% optimization and average linkage dendrograms (UPGMA) were obtained from the isolates.

2.3. Phenotypic characteristics

Thirty strains of LAB that represent the distinct profiles defined by Rep-PCR were selected for evaluation of technological potential. The objective was to carry out a prior identification of strains with phenotypic characteristics for further studies aimed at the development of new products for application in dairy industry.

2.3.1. Acidifying activity

The selected strains were activated in MRS broth and grown at 30 °C for 20h. For the acidifying activity test, the strains (1% v/v) were inoculated in 10 mL of sterile skim milk (RSM 10% w/v) and incubated at 30 °C. The pH was measured after 6 h and after 24 h with a pH meter. The data are expressed on the average of duplicate analysis.

2.3.2. Proteolytic activity

Extracellular proteolytic activity was determined following the modified method of Franciosi et al. (2009). Briefly, ten microliters of activated strains were placed on the surface of a Plate Count Agar (PCA, Difco, France) medium supplemented of 10% (w/v) skim milk

powder and incubated at 30 °C for 4 days. Proteolytic activity was indicated by a clear zone forming around the colonies.

2.3.3. Diacetyl production

Diacetyl production was determined according to the adapted method of (Dal Bello et al., 2012). Revitalized strains (1% v/v) were inoculated in 10 mL of skim milk and incubated at 30 °C for 24 h. One mL of each cell suspension was combined with 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) and incubated at 30 °C for 30 min. Diacetyl production was indicated by the formation of a red ring on the top of the tubes.

2.3.4. Potential of antimicrobial activity

The antimicrobial activity was tested by an agar diffusion assay according to the adapted method of Hungaro et al. (2013). A top agar layer was prepared containing 1 mL of pathogenic bacteria in 5 mL of soft-agar (0.7% w/v) and applied to the surface of BHI (Himedia, India) agar plates. After solidification of the top agar layer on the plates, micro droplets of 20 µL of the LAB selected were placed on and incubated at 37 °C for 24 h. A clear zone of inhibition around the droplets was taken as a positive signal for possible antimicrobial activity. The top agar layer was prepared containing *Escherichia coli* O157:H7, *Salmonella* Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 7644 or *Staphylococcus aureus* ATCC 25923 separately.

3. Results and discussion

Among different isolates from 44 samples of Marajó cheese evaluated, 120 isolates have been obtained which 97 showed compatible characteristics with *Lactococcus* genus

(Gram-positive cocci and negative-catalase), being 68 from MC-cheese and 29 from MB-cheese. The other isolates that do not correspond to the initial characteristics considered for *Lactococcus* were excluded of this work. The M17 medium was selected for selectivity in isolating and enumeration of LAB cocci in dairy products (Dal Bello et al., 2012; Luiz et al., 2016; Perin et al., 2015; Pogačić et al., 2013, 2010) in comparison with MRS, a medium classically used for isolating of LAB in general. Some studies have used different conditions for isolating and enumeration of LAB cocci (Castro et al., 2016; Perin and Nero, 2014; Perin et al., 2015; Pogačić et al., 2010). However, even in different conditions other studies have also identified the isolation of other bacterial genera in M17 (Dasen et al., 2003; Gonçalves et al., 2009; Van Hoorde et al., 2008). Perin and Nero (2014) used different conditions of incubation for isolating of LAB cocci (35 ° C and 42 ° C for 48 h) in raw goat milk and identified LAB bacilli in this culture media. While Terzic-Vidojevic et al. (2014) used the same conditions of incubation (30 ° C for 48 h) of this study and reported the predominance of *Lactococcus* genera among the isolates from artisanal cheese. Thus, the selectivity of M17 medium is not related with the incubation conditions used in this study.

All the isolates that showed compatible characteristics with *Lactococcus* were submitted to 16S rDNA sequencing and were identified as LAB. Among them were identified the genera *Enterococcus* (n = 59), *Pediococcus* (n = 31), *Weissella* (n = 4), *Streptococcus* (n = 2) and *Lactococcus* (n = 1). From MC-cheese samples were identified the *Enterococcus* genus (n = 35) and the species *Pediococcus acidilactici* (n = 17), *Pediococcus pentosaceus* (n = 14) and *Weissella paramesenteroides* (n = 2). The strains of *P. acidilactici* (n = 17), *P. pentosaceus* (n = 14) and *W. paramesenteroides* (n = 2) were distributed among the samples as coming from rainy season, while that *Enterococcus* genus (n = 35) and only a strain of *P. acidilactici* were identified as coming from dry season.

From MB-cheese samples were identified the *Enterococcus* genus (n = 24) and the species *W. paramesenteroides* (n = 2), *Streptococcus infantarius* (n = 2) and *Lactococcus lactis* (n = 1). *Enterococcus* (n = 20) and *S. infantarius* (n = 2) that were distributed among the cheeses coming from dry season, whereas *W. paramesenteroides* (n = 2), *L. lactis* (n = 1) and *Enterococcus* (n = 4) were identified as coming from rainy season production.

Different species of LAB were identified in the two types of Marajó cheese and in the two seasons evaluated. According to Terzic-Vidojevic et al. (2014), this difference can be influenced by various factors including the seasonally variable chemical composition of the milk and the specificities of the production process as well as the geographical location and particular environmental conditions of the household. And according to Feutry et al. (2012); Franciosi et al. (2009) the composition of microbiota of artisanal cheeses is influenced by microbiota present in the raw milk used and practices conducted during the cheese making.

All the 97 isolates were submitted to Rep-PCR to determine the genetic diversity. The molecular characterization of isolates by Rep-PCR (Fig. 3.1 and Fig. 3.2) was able to identify different genetic profile among the evaluated isolates, especially regarding the evaluated seasons. The strains with different genetic profiles were grouped according to the season, which shows a possible influence of the season to determine the genetic profile of the strains. Similar results have been found by Terzic-Vidojevic et al. (2014) using Rep-PCR to characterize the genetic diversity of LAB isolated from artisanal Travnik cheeses during four seasons. These authors determined genetic differences among the isolates from different seasons. Among the 68 isolates from MC-cheese, the Rep-PCR has defined 17 distinct genetic profiles and 13 distinct genetic profiles among the 29 isolates from MB-cheese with 90% of similarity. Among the isolates from MC-cheese samples 6 distinct profiles have been identified for *Enterococcus*, 6 profiles for *P. acidilactici*, 5 profiles for *P. pentosaceus*, and 1 profile for *W. paramesenteroides* (Fig. 3.1). Among the isolates from MB-cheese samples

have been identified 10 distinct profiles for *Enterococcus*, 1 profile for *W. paramesenteroides*, 1 profile for *S. infantarius* and 1 profile for *L. lactis* (Fig. 3.2).

Strains of *P. acidilactici* (n = 8) and *P. pentosaceus* (n = 4) were considered of the same genetic profile of the strains of *Enterococcus* (profile 4 and profile 6) with 90% of similarity (Fig. 3.2). In this case, the Rep-PCR was not able to differentiate distinct genetic profiles among the different species. This result can be explained by the sensitivity of the Rep-PCR method in identifying the genetic differences by amplification of repetitive bacterial DNA elements by a single primer (Gevers et al., 2001). However, the Rep-PCR is a rapid technique , easy-to-perform, reproducible and at a low cost that allows of understanding the dynamics and diversity of bacteria (Cocolin et al., 2011; Gevers et al., 2001) and studies have used the Rep-PCR to evaluated the genetic diversity in different species of LAB (Pogačić et al., 2013; Terzic-Vidojevic et al., 2014, 2013).

Strains with the same genetic profile could be identified as coming from different cheesemakers in the both types of evaluated Marajó cheese (Fig. 3.1 and Fig. 3.2). However, strains from different types of Marajó cheese showed distinct genetic profiles by Rep-PCR, being therefore considered as distinct strains (Fig. 3.3). Only the strains of *Enterococcus* (Ettm 40) and *P. acidilactici* (Patc 2) showed the same genetic profile among the samples of the different types of Marajó cheese. Similar results were showed by Freitas et al. (2015) upon evaluating the biodiversity of *Propionibacterium* by pulsed field gel electrophoresis (PFGE) from different dairy farms in Minas Gerais, Brazil. These authors suggest that each location (dairy farm) represents a specific niche.

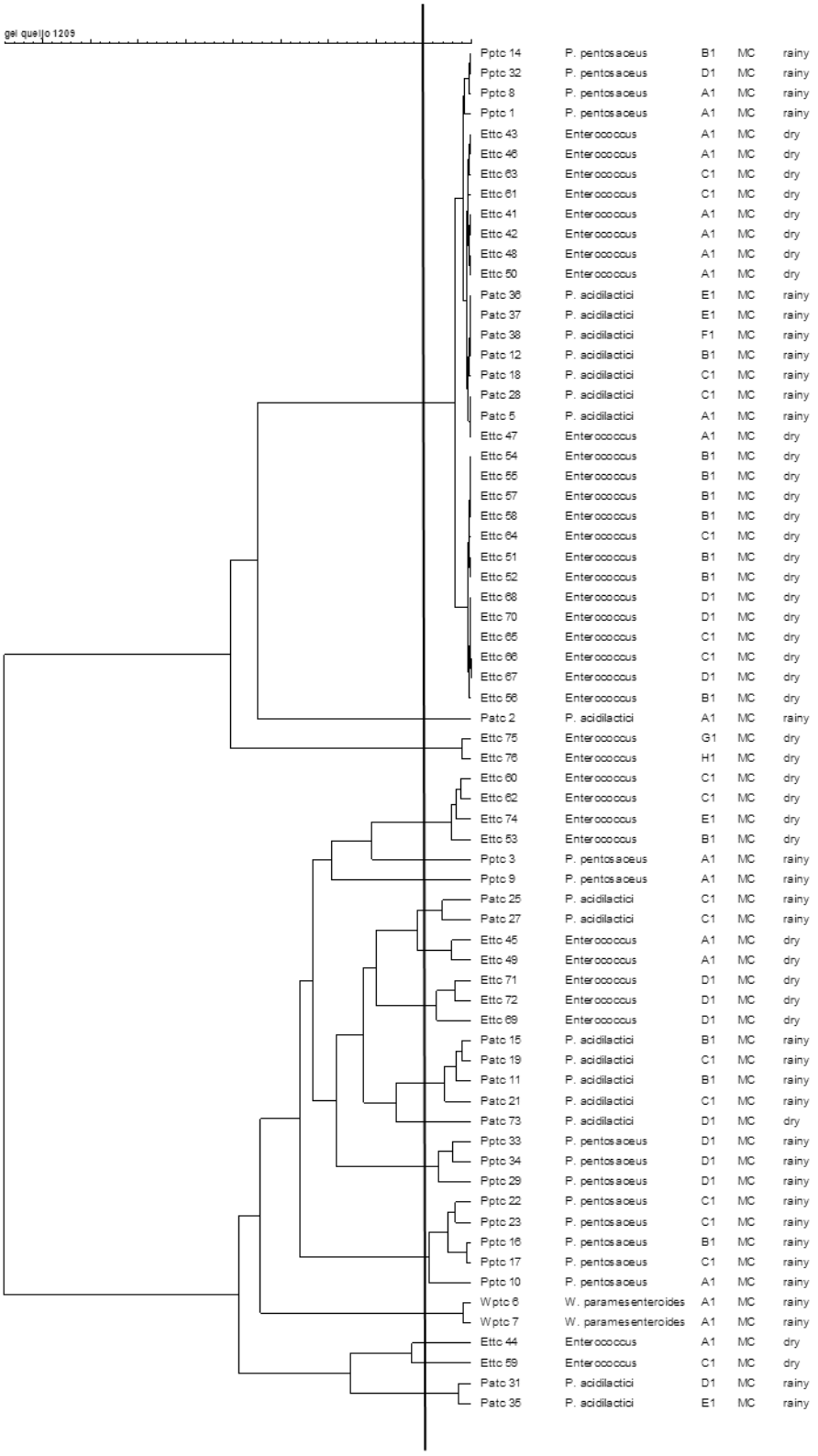

Figure 3.1 - Dendrogram generated after cluster analysis of the digitized Rep-PCR fingerprints of strains from Marajó cream type cheese (MC). A1: cheesemaker A1; B1: cheesemaker B1; C1: cheesemaker C1; D1: cheesemaker D1; E1: cheesemaker E1; F1: cheesemaker F1; G1: cheesemaker G1; H1: cheesemaker H1.

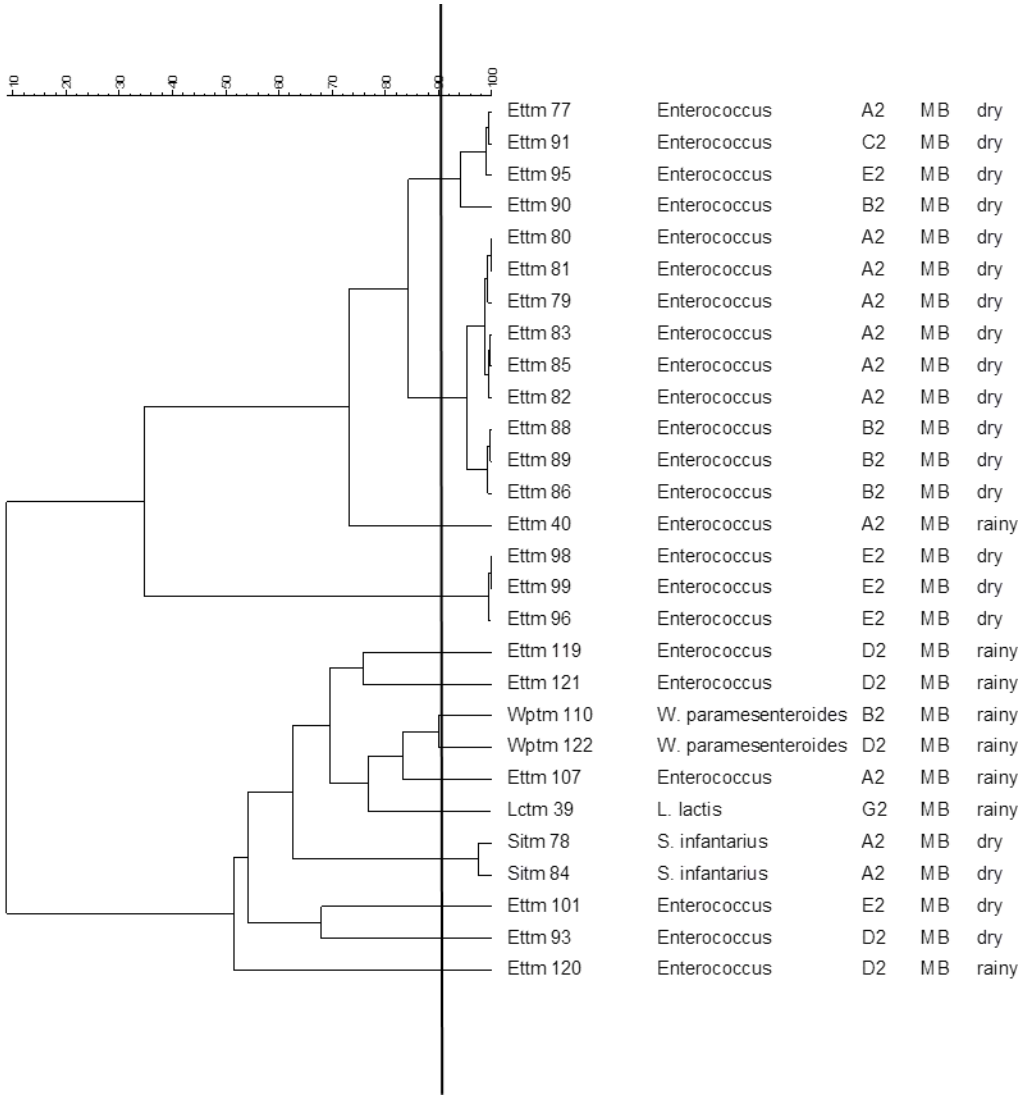

Figure 3.2 - Dendrogram generated after cluster analysis of the digitized Rep-PCR fingerprints of strains from Marajó butter type cheese (MB). A1: cheesemaker A1; B1: cheesemaker B1; C1: cheesemaker C1; D1: cheesemaker D1; E1: cheesemaker E1; F1: cheesemaker F1; G1: cheesemaker G1; H1: cheesemaker H1.

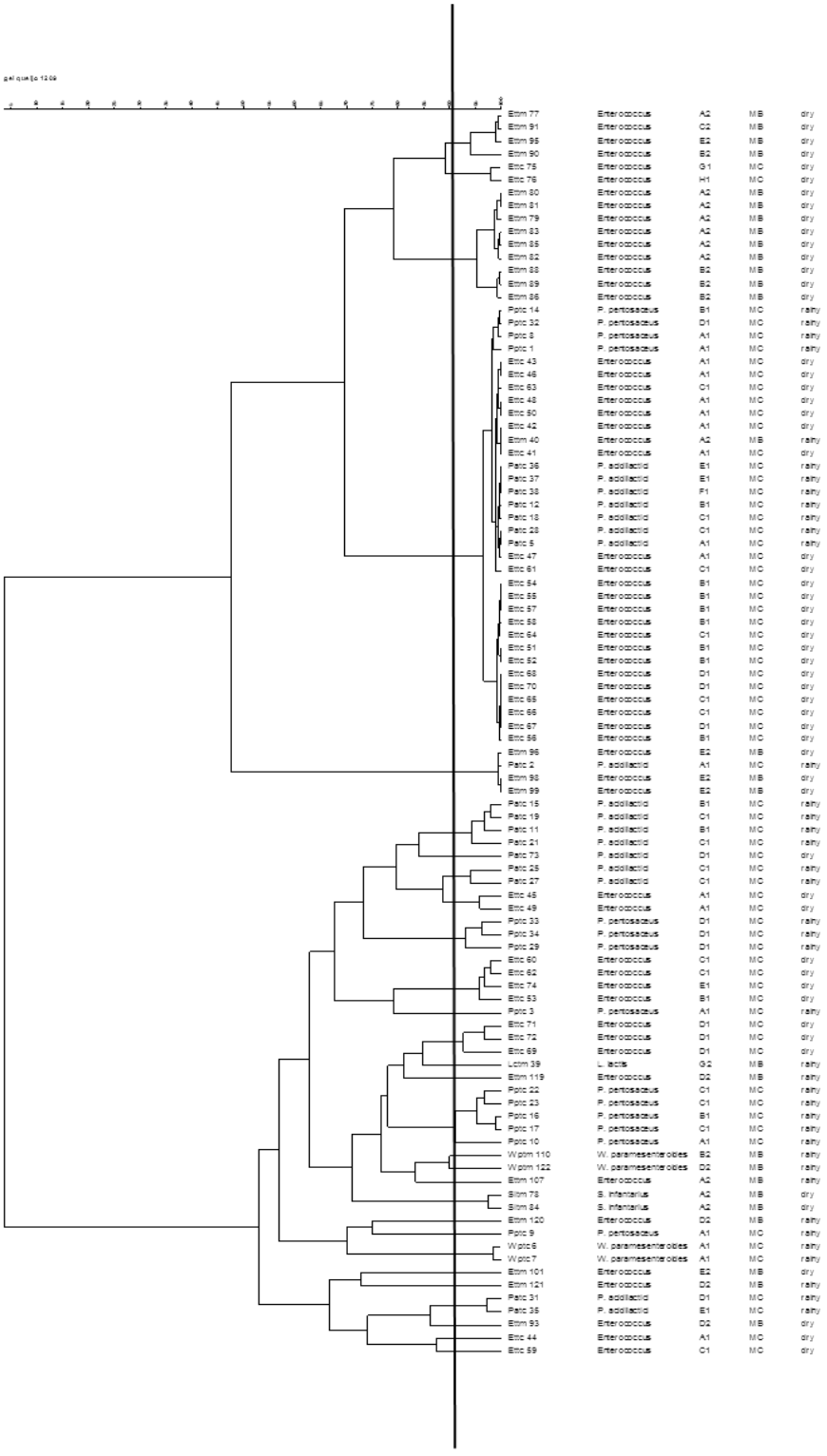

Figure 3.3 - Dendrogram generated after cluster analysis of the digitized Rep-PCR fingerprints of strains from Marajó cream type cheese (MC) and Marajó butter type cheese (MB). A1: cheesemaker A1; B1: cheesemaker B1; C1: cheesemaker C1; D1: cheesemaker D1; E1: cheesemaker E1; F1: cheesemaker F1; G1: cheesemaker G1; H1: cheesemaker H1.

The distinct genetic profiles shown by the strains in different types of Marajó cheese have not determined distinct phenotypic characteristics in the studied conditions. Although *Enterococcus* was the genera most frequently isolated in the both types of Marajó cheese, in general, the phenotypic characteristics showed for this genus were common to both types of cheese. All the strains were considered proteolytic and only three strains produced diacetyl. However, the strains from MC-cheese had a best potential of antimicrobial activity showing zone of inhibition around *E. coli*, *S. aureus*, *L. monocytogenes* and *Salmonella* (Table 3.1). Studies have indicated a potential application for food preservation for *Enterococcus* coming from artisanal cheeses (Moraes et al., 2012; Rivas et al., 2012; Santos et al., 2015).

Lactococcus was isolated at a low incidence in Marajó cheese. In contrast, a larger number of *L. lactis* strains was isolated in raw buffalo milk used for Marajó cheese making (Chapter 2). The main role of lactococci in dairy products is its involvement in the process of acidification of milk (Cogan et al., 1997). The strain identified in this work showed a good acidification in skim milk 10% (Table 3.1), confirming the characteristic of this genus as starter culture in cheeses (Hansen, 2002).

On the other hand, Pediococci typically are not considered as starter cultures, not being able to quickly produce acid from lactose (Papagianni and Anastasiadou, 2009). This can be confirmed by the results shown in this study once Pediococci strains have not shown a good acidifying activity in skim milk 10% (Table 3.1). In general, Pediococci are associated with cheese ripening (Beresford et al., 2001; Caldwell et al., 1996; Van Hoorde et al., 2008).

Table 3.1 Phenotypic characteristics of strains isolated from Marajó cheese

Strain code	Strain	Acidification			Diacetyl production	Proteolytic activity	Antimicrobial activity
		pH t_0	pH t_{6h}	pH t_{24h}			
Marajó cream type cheese							
Pptc 3	*P. pentosaceus*	6.67	6.31±0.01	5.90±0.02	-	+	SE
Pptc 16	*P. pentosaceus*	6.67	6.37±0.01	5.86±0.01	-	+	EC
Pptc 29	*P. pentosaceus*	6.67	6.36±0.06	6.20±0.01	-	++	-
Pptc 10	*P. pentosaceus*	6.67	6.46±0.03	6.11±0.05	-	+	-
Pptc 14	*P. pentosaceus*	6.67	6.35±0.06	6.19±0.01	-	+	SA
Pptc 1	*P. pentosaceus*	6.67	6.49±0.01	6.00±0.01	-	+	-
Patc 19	*P. acidilactici*	6.67	6.48±0.08	6.35±0.01	-	-	-
Patc 73	*P. acidilactici*	6.67	6.40±0.07	6.20±0.01	+	+	EC; SA; SE
Patc 31	*P. acidilactici*	6.67	6.48±0.07	6.35±0.06	-	-	-
Patc 2	*P. acidilactici*	6.67	6.44±0.03	6.00±0.01	-	+	-
Wptc 6	*W.paramesenteroides*	6.67	6.35±0.03	5.15±0.04	-	+	-
Ettc 69	*Enterococcus*	6.67	6.00±0.01	5.00±0.04	+	+	SA; SE
Ettc 53	*Enterococcus*	6.67	6.35±0.01	5.93±0.06	-	+	LM
Ettc 44	*Enterococcus*	6.67	6.24±0.01	5.30±0.02	-	+	EC
Ettc 41	*Enterococcus*	6.67	6.17±0.01	5.55±0.01	-	+	-
Ettc 55	*Enterococcus*	6.67	6.32±0.02	5.93±0.07	-	+	SE
Ettc 76	*Enterococcus*	6.67	6.21±0.03	5.66±0.01	-	+	-
Marajó butter type cheese							
Ettm 107	*Enterococcus*	6.67	6.41±0.08	5.96±0.05	-	+	-
Ettm 121	*Enterococcus*	6.67	6.34±0.04	5.41±0.09	+	+	-
Ettm 119	*Enterococcus*	6.67	6.32±0.02	5.05±0.01	+	+	-
Ettm 96	*Enterococcus*	6.67	6.28±0.04	5.75±0.09	-	+	-
Ettm 99	*Enterococcus*	6.67	6.28±0.02	5.72±0.09	-	+	-
Ettm 85	*Enterococcus*	6.67	6.24±0.01	5.74±0.01	-	+	-
Ettm 120	*Enterococcus*	6.67	6.24±0.04	5.52±0.01	-	+	-
Ettm 88	*Enterococcus*	6.67	6.38±0.01	5.94±0.04	-	+	SE; LM
Ettm 40	*Enterococcus*	6.67	6.16±0.03	5.50±0.01	-	+	-
Sttm 78	*S.infantarius*	6.67	6.35±0.03	5.94±0.01	+	+	LM

Sttm 84	*S.infantarius*	6.67	5.73±0.04	4.92±0.01	+	+	-
Wptm 122	*W.paramesenteroides*	6.67	6.27±0.01	5.58±0.01	-	-	EC
Lctm 39	*L.lactis*	6.67	6.00±0.03	4.60±0.03	-	+	-

(+) positive; (-) negative. EC = *E. coli*; SA = *S. aureus*; LM = *Listeria monocytogenes*; SE = *Salmonella* Enteritidis

The strains that showed the best phenotypic characteristics of diacetyl production and proteolytic activity were *Enterococcus* (Ettc 69), *Enterococcus* (Ettm 121), *Enterococcus* (Ettm 119), *P. pentosaceus* (Pptc 29), *P. acidilactici* (Patc 73), *S. infantarius* (Sttm 78) and *S. infantarius* (Sttm 84). Among them, only *S. infantarius* (Sttm 84) had a good acidifying activity. While *P. acidilactici* (Patc 73) showed the best potential of antimicrobial activity against *E. coli, S. aureus* and *Salmonella*. In general, from all the 30 selected strains, 12 showed potential of antimicrobial activity for at least one of the pathogens (Table 3.1).

The abilities of LAB wild strains from artisanal cheeses to produce attractive compounds associated to flavor and antimicrobial activity may contribute to development of strategies to food preservation and to the increasing demand for cheeses with different or improved sensorial properties (Dal Bello et al., 2012; Moraes et al., 2012; Perin and Nero, 2014; Van Hoorde et al., 2010). The phenotypic characteristics showed by LAB cocci in this study allow of opening new prospects for further investigations of the role of each species in the definition of sensory properties of the Marajó cheese.

4. Conclusion

The identification of autochthonous LAB cocci microbiota present in Marajó cheese is important because it allows a better association between the occurrence of certain species on sensorial and technological properties of these cheeses. From this study, it is possible to

contribute to information that may in the future be included in legislation related to the characteristics that define this cheese. And to start a better characterization and selection of LAB cocci that may be exploited in Marajó cheese making in order to help defining a better standardization of this cheese and to preserve the isolated strains. Consequently, this could be applied to the production of Marajó cheese with a better quality, control and standardization. And it will be possible to sell it in other regions of Brazil without losing its historic and cultural identity.

CHAPTER 4

Tracking amazonian cheese microbial diversity: Development of an original, sustainable and robust starter by spray drying/freeze drying

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Abstract

The Marajó cheese produced with raw buffalo milk in the Amazon region can be considered as a good source of wild lactic acid bacteria (LAB) strains with yet unexplored promising characteristics. The aim of this work was to evaluate the effects of different carrier media on the survival rate, acidifying activity and probiotic properties of *Lactobacillus plantarum* isolated from Marajó cheese and subjected to either freeze drying or spray drying. There was a significant ($p<0.05$) decrease in the survival rates of freeze dried samples, in comparison with spray dried samples. The spray dried cultures remained approximately $10^9 CFU \cdot g^{-1}$, while the freeze dried samples showed $10^7 CFU \cdot g^{-1}$ after 60 days of storage at 4 °C. All the spray dried samples showed a greater ability to decrease the pH in 10% skim milk during 24 hours. The spray dried samples showed a greater resistance towards acid conditions and towards the presence of bile salts. In addition, under heat stress conditions the reduction was less than 2 log cycles in all the samples. This study can direct further

investigations to preserve LAB probiotics in order to produce starter and adjunct cultures by spray drying with a high number of viable cells to be used for industrial application through a cost-effective method.

Keywords: *Lactobacillus plantarum*, probiotic, sweet whey, skim milk, Amazonian artisanal cheese

1. Introduction

In the food industry, the starter/adjunct cultures can be obtained by two methods of concentration/preservation: culture in frozen conditions and culture in a dried form, such as freeze drying. The major disadvantage of using frozen cultures is the cost of transport, storage and manipulation (Carvalho et al., 2004b; Santivarangkna et al., 2007). Dried preparations have the double advantage of long-term preservation and convenience in handling and storage. Freeze drying is a widespread technique and a large number of freeze dried cultures are commercially available (Santivarangkna et al., 2007), being widely used in the implementation of LAB cultures (Carvalho et al., 2003; Kandil and Soda, 2015; Madhu et al., 2011; To and Etzel, 1997).

Nevertheless, the process of freeze drying itself is expensive and complex, and can take days to complete for large product loads due to the slow energy and water transfer needed to dry the material. This, as well as the large commercial interest in microbial culture starters, explains the continuing research to develop alternative drying techniques (Carvalho et al., 2004b; Morgan et al., 2006; Santivarangkna et al., 2007; Silva et al., 2011).

Spray drying is a good example of a most used technique in industrial drying foods today and can be used for drying bacteria. The cost of spray drying is approximately 10 times lower than the freeze drying. The versatility of this process and the considerable progress made through technical innovation have led to a greater flexibility to meet biotechnological

requirements, especially low heat treatments to avoid loss of activity (Schuck et al., 2013). For beneficial dairy bacteria, spray drying has to be adapted in order not to affect viability of bacteria, by a fine tuning of drying air temperature. This can be achieved by modulating the concentration of the drying medium (Schuck et al., 2013) or of the culture medium when directly spraying fresh cultures (Huang et al., 2016).

Maximizing the survival of LAB cultures during drying and ensuring a subsequent storage for long periods is essential to maintaining technological characteristics and economic viability. The biological activity of a lactic culture that includes cell viability and physiological state is necessary to evaluate the quality as a starter culture (Peighambardoust et al., 2011). Microbial cell survival throughout drying and storage is dependent on many factors, including initial concentration of microorganisms, growth conditions, growth medium, drying medium, storage conditions and rehydration conditions (Carvalho et al., 2003, 2004a, 2004b).

Both freeze drying and spray drying are currently used to dry probiotic cultures (Maciel et al., 2014; Shokri et al., 2015; Soukoulis et al., 2014; Utami et al., 2016). Probiotics are defined as ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2001). However, these health benefits may depend on the viability of the probiotic cells and the maintenance of their probiotic properties in commercial cultures and food products during the storage (Rathnayaka, 2013).

Drying processes expose the bacteria to heat stress, cold stress and osmotic stress, which potentially could lead to cell death (Castro et al., 1997, 1996; Corcoran et al., 2006; Dijkstra et al., 2014). The challenge of preserving probiotic cultures by drying is to maintain the viability and the functional properties after the drying process (Madhu et al., 2011; Reddy et al., 2009).

The composition of the growth and drying media also can influence the protection during storage of dried cells (Carvalho et al., 2003, 2004a). Skim milk powder has been selected as drying medium for most LAB cultures. The skim milk prevents cellular injury by stabilizing the cell membrane constituents (Carvalho et al., 2004a; Maciel et al., 2014; Zamora et al., 2006) and it is one of the media used for bacterial protection during drying (Fu and Chen, 2011).

Whey is a food co-product from cheese manufacturing. The large amount of lactose and whey proteins in sweet whey can make it an ideal medium for growing LAB (Huang et al., 2016). Moreover, several researches have demonstrated that milk components could have protective effects on probiotics against adverse stresses conditions (Ananta et al., 2005; Huang and Chen, 2013; Huang et al., 2016, 2014; Maciel et al., 2014).

Therefore, the use of a nutritional and low-cost drying medium that maintains the viability and functional properties of LAB cultures during storage in association with a low-cost drying technology can promote the development of an optimal probiotic/starter culture for industrial application.

The use of wild strains is promising for starter/adjunct culture production with particular characteristics desirable in the food industry. Artisanal cheeses are considered as good sources of new LAB biodiversity. The biodiversity found in these cheeses allows the identification of microbial strains with a wide range of technological and probiotic characteristics (Castro et al., 2016; Freitas et al., 2015; Randazzo et al., 2009, 2007; Terzic-Vidojevic et al., 2014a; Van Hoorde et al., 2008).

Brazil stands out in the production of an artisanal cheese produced with buffalo milk on the island of Marajó, state of Pará, located in the Amazon region. This cheese is known as Marajó cheese. The curd coagulation is caused only by the autochthonous microbiota in raw milk and in the processing environment, which makes it a product characteristic of the region.

Consequently, this cheese can be considered as a good source of isolation of the wild LAB strains of industrial application.

The aim of this work was to evaluate the effects of different carrier media on the survival rate, the acidifying activity and probiotic properties of *Lactobacillus plantarum* isolated from Marajó cheese, subjected to freeze drying and spray drying.

2. Material and methods

2.1. Bacterial Strain and Growth Conditions

The strain *L. plantarum* (UFV-Lb26) isolated from Marajó cheese and present in the culture collection of the INOVALEITE Laboratory, Department of Food Technology of the University Federal of Viçosa, Brazil, was selected to study the effect of freeze drying and spray drying. This strain exhibits the ability to produce diacetyl, as well as a proteolitic activity, an acidifying activity and an antimicrobial activity against food-borne pathogens (data not shown), in addition to the potential probiotic abilities like tolerance to low pH and a high bile salt concentration up to 1%. The strain was preserved at -20 °C in Eppendorf tubes containing Man, Rogosa and Sharpe broth (MRS, Difco) added with 30% (v/v) glycerol.

2.2. Preparation of cells for drying experiments

The frozen culture (−20 °C) was grown in 10 mL sterilized Lactobacilli MRS broth (Difco, France) at 30°C for 18 h (stationary phase). An aliquot of 1% of the grown culture was transferred and re-grown under the same conditions. Approximately 10^9 $CFU \cdot mL^{-1}$ cells density was obtained after incubation.

The culture was transferred under aseptic conditions into 1 mL sterile eppendorf's tubes to prepare for the freeze drying process and into 1 L sterile centrifuge tubes to the spray

drying process. After, the cultures were centrifuged at 8,000×*g* for 5 min at 4°C. The supernatant was discarded and the harvested cells in the form of pellets were washed once using phosphate buffer saline pH 7.0 (PBS, Difco, France). The cell suspensions were centrifuged as previously; after discarding the supernatant, the pellets were suspended separately into the drying carrier media with enhanced high dry matter, i.e. 20 % (w/v) of reconstituted skim milk powder (RSM) (Lactalis, Bourgbarré, France), reconstituted sweet whey powder (RSW) (Lactalis) and reconstituted sweet whey permeate powder (RSWP) (Lactalis) 20 % (w/v).

2.2.1. Freeze drying

The cell suspensions resuspended (10^9 CFU.L^{-1}) in the drying carrier media (item 2.1) were frozen for 24 h and then freeze dried (S.G.D. SERAIL, France) for 24 h. For the after-drying experiments, the samples of freeze dried powder were stored at 4 °C. Small amounts of powder were subjected to rehydration and viability enumeration.

2.2.2. Spray drying

Dried samples consisted in one liter samples of fresh cultures, resuspended (10^9 CFU.L^{-1}) in carrier media with enhanced high dry matter, i.e. 20% (w/v) mentioned in item 2.1. Such concentrations are widely used for spray drying and allow limited heating, compared to unconcentrated milk or whey. The concentrated microbial suspensions were spray dried in a pilot-scale Mobile Minor™ spray-dryer (GEA Niro A/S, Denmark) as previously described (Huang et al., 2016). A two-fluid spray nozzle with an orifice diameter of 0.8 mm was used.

The outlet spray drying temperatures were 75 °C with inlet temperatures fixed at 180 °C. The experiments were carried out in triplicate. The feed rate was maintained at around 30 $mL \cdot min^{-1}$ to obtain the outlet air relative humidity at 10±1% for all the samples. For the after drying experiments, spray dried powders were stored in plastic bags under vacuum at 4 °C and 20 °C, respectively. Small amounts of powder (approximately 10 mg) were subjected to rehydration and viability enumeration.

2.3. Water profile in the powder (spray dried)

2.3.1 Water content measurements

The water content of the powders were tested according to the methods described by (Schuck et al., 2012). The water content in the spray dried samples was calculated as follows: approximately 1 g of powder was spread on a petri dish containing sand and put in the water content analyzer, which heated the powder up to 102 °C ± 2 °C until the mass equilibrated to a final value. Afterward, the petri dish was weighed and the moisture was calculated in percentage. This analysis was conducted in duplicate.

2.3.2 Water activity

Water activity (aw) was measured in triplicate with a Novasina water activity meter (RTD-33 TH-2, Pfäffikon, Switzerland) at a constant temperature of 25 °C.

2.4. Enumeration of the bacteria

The freeze dried and spray dried powders were rehydrated in sterile triptone water (sodium chloride 5 $g \cdot L^{-1}$ and tryptic hydrolysate of casein 10 $g \cdot L^{-1}$) to the same solid content as the original suspension. The rehydrated samples were kept at room temperature for 30 min

to allow complete rehydration. The count of colony forming units was carried out in triplicate before the freeze drying, after the freeze drying (time 0), seven, thirty and sixty days of storage at 4 °C; before the spray drying, after the spray drying (time 0), seven, thirty and sixty days of storage at 4 °C and 20 °C.

The resulting samples were subjected to serial dilutions using triptone water. Each dilution was pour plated on an MRS agar (Difco, France) and the plates were incubated at 30 °C for 48 h. Enumeration of the bacteria was performed in triplicate and the total counts of the viable bacteria were expressed in log colony forming units per gram of powder ($CFU \cdot g^{-1}$).

2.5. Acidifying activity

The acidifying activity was performed to evaluate the biological activity of freeze dried and spray dried cells of *L. plantarum* in 10% reconstituted skim milk. Biological activity of a lactic acid starter that includes cell viability and physiological state is needed to evaluate starter quality. Biological activity is defined as the ability of a lactic acid starter to acidify a certain medium (Peighambardoust et al., 2011).

The freeze dried and spray dried samples were reconstituted in triptone water and 1 ml of the culture was added separately to 100 mL of reconstituted skim milk (RSM, Lactalis, France) 10% (w/v). Then, the production of acids and the acidification kinetics were evaluated by checking the pH with CINAC software (Wcinac 32 V 2.1) during 24 hours.

2.6. Stress conditions

The freeze dried and spray dried cells were subjected to stress conditions (acid stress, thermal stress and bile salts stress) to assess their resistance and probiotic potential.

In the acid stress conditions, freeze dried and spray dried cells were resuspended for 30 min as described above in item 2.5 in tryptone water and centrifuged (8000 x *g* / 5 min). Then, the supernatant was discarded and the same volume of solution was added (Sodium lactate 10 g; Tryptone 10 g; Yeast extract 10 g; + K_2HPO_4, $3H_2O$ 2.5 g; $MnSO_4$, H_2O 5 mg) at pH 2.5. The monitoring of colony forming units was performed in triplicate during 1 hour every 15 min.

Alternatively, the same bacterial suspensions were subjected to thermal stress at 60°C in a water bath. Bile salts stress was applied as described previously (Leverrier et al., 2003) by adding $1g \cdot L^{-1}$ bile salts to the suspensions.

2.7. Statistical Analysis

A comparison of the effect of the drying media and of the drying process on survival rate, the acidifying activity and the stress conditions were evaluated by ANOVA and Tukey test ($p < 0.05$)/($p < 0.01$) for comparison between means.

3. Results

3.1. Effect of Freeze drying and Spray drying on the Survival Rate

Figure 4.1 shows the results of the survival rates of *L. plantarum* during the storage period (0, 7, 30, and 60 days) at 4°C for both freeze dried and spray dried cultures. Before both drying processes the viability of *L. plantarum* cells was $10^9 CFU \cdot g^{-1}$. In time 0 (zero), there was no significant ($p>0.05$) loss of viability of spray dried samples. However, there was a reduction of 1 log cycle in the freeze dried samples (RSW and RSWP). There was a significant ($p<0.05$) decrease in the survival rates of freeze dried samples in comparison with spray dried samples during the storage. The spray dried cultures remained approximately

10^9CFU·g^{-1} after 60 days of storage at 4°C, indicating approximately 98% of viability, while the freeze dried cultures had a reduction of 1 log cycle in RSM (10^8CFU·g^{-1}) and 2 log cycles in RSW (10^7CFU·g^{-1}) and RSWP (10^7CFU·g^{-1}) after 60 days of storage at 4°C, indicating 93, 90 and 91% of viability, respectively. There was no significant ($p>0.05$) difference between carrier mediums used for spray dried cultures in the survival rate of *L. plantarum*. The spray dried culture storage at 20 °C also maintained the survival rate at 10^9CFU·g^{-1} during the storage period of 60 days (data not shown).

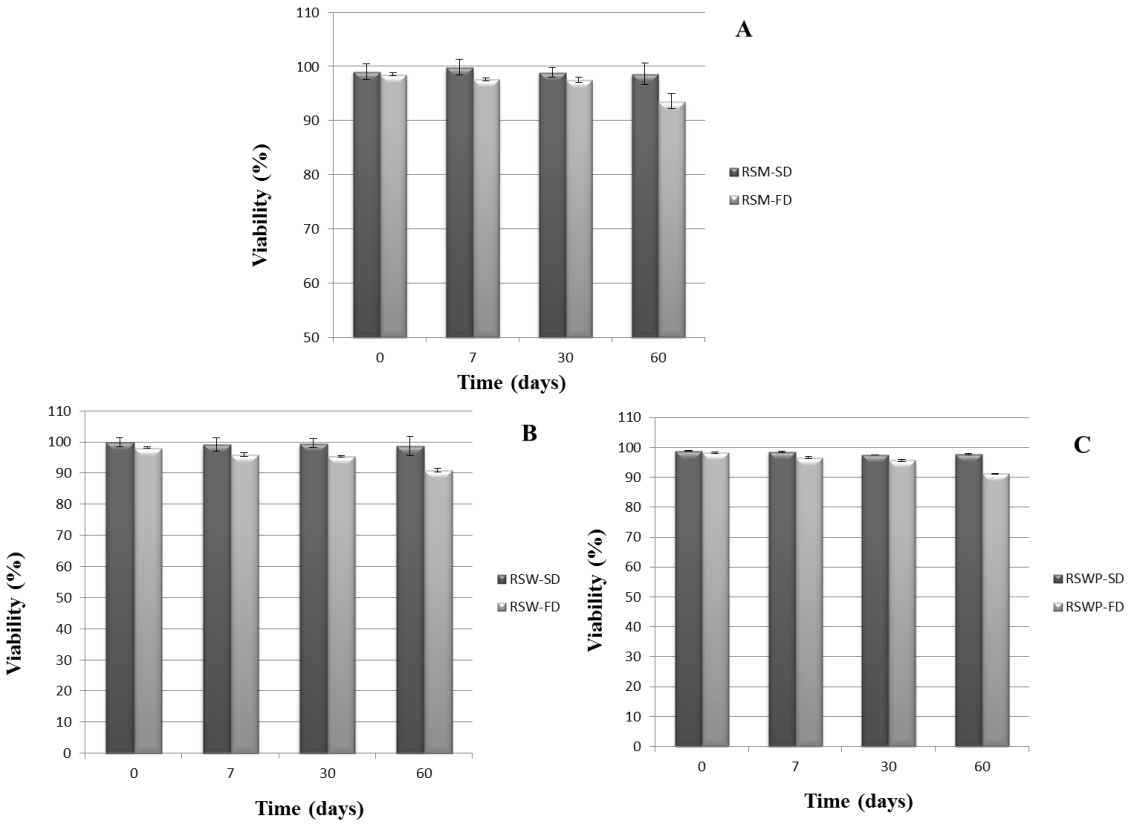

Figure 4.1 - The survival rates of *L. plantarum* at the first day and during the storage period (7, 30, and 60 days) at 4 °C in triplicate. SD (Spray drying); FD (Freeze drying). A – Drying samples in reconstituted skim milk; B - Drying samples in reconstituted sweet whey; C - Drying samples in reconstituted sweet whey permeate.

3.2. Water profile in the powder (spray drying)

Table 4.1 shows the water profile in the powder of spray dried cultures.

Table 4.1 – Water profile in the powder of spray dried cultures in different media

Medium	Water content (%)	Water activity (-)
RSM	3.97±0.11	0.18±0.011
RSW	3.80±0.20	0.17±0.003
RSWP	3.10±0.44	0.12±0.004

3.3. Effect of Freeze drying and Spray drying on the acidifying activity

The biological activity of dried *L. plantarum* was monitored by testing the ability of the cells to acidify 10% skim milk after the freeze drying and spray drying processes. Figure 4.2 shows the results of the acidification kinetics during 24 hours of the skim milk inoculated with freeze dried and spray dried cultures.

All the spray dried samples showed a greater ability to decrease the pH in skim milk 10% in the same period in comparison with freeze dried samples. The initial pH was approximately 6.5. The spray dried samples in RSM (Figure 4.2-A) reduced the pH down to 4.80 in 24 hours, while RSW (Figure 4.2-B) and RSWP (Figure 4.2-C) samples reduced pH to 4.50. For the freeze dried samples the reduction was not significant ($p>0.01$) in the three samples evaluated, RSM (6.20), RSW (6.15) and RSWP (6.12). Finally, the spray dried samples were significantly different ($p<0.01$) from the freeze dried samples.

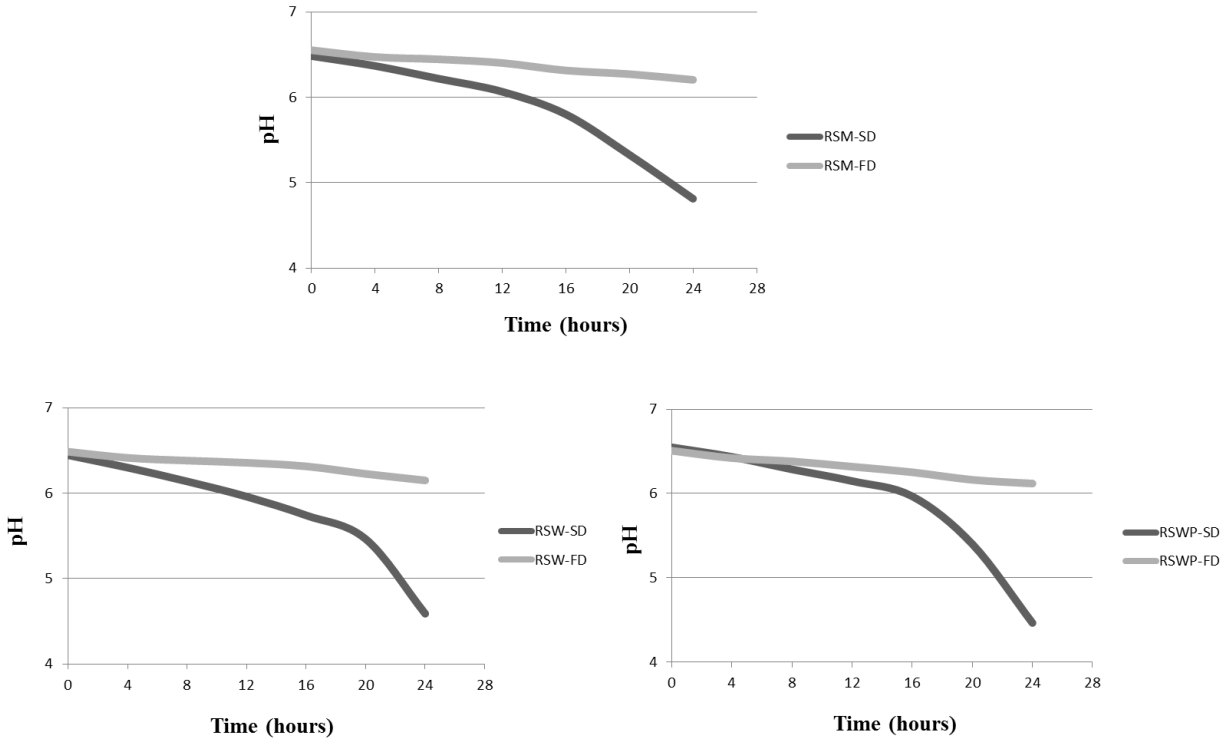

Figure 4.2 - The acidification kinetics during 24 hours of the skim milk inoculation with freeze dried and spray dried cultures. SD (Spray drying); FD (Freeze drying). A – Samples dried in reconstituted skim milk; B - Samples dried in reconstituted sweet whey; C - Samples dried in reconstituted sweet whey permeate.

3.4. Effect of Freeze drying and Spray drying on stress tolerance

Figure 4.3 shows the impact of acid stress on *L. plantarum* viability during 60 minutes of challenge at pH 2.5, for freeze dried and spray dried cultures in different medium. The spray dried samples showed a greater resistance under acid conditions. However, the freeze dried samples showed a low resistance in pH 2.5, except the freeze dried cells in RSW that reduced just 1 log cycle after 60 minutes. The freeze dried cells in RSM reduced 4 log cycles and RSWP reduced 5 log cycles.

The spray dried cells in RSWP showed a limited loss of viability (approximately 1 log cycle) in comparison with other spray dried samples. This reduction was significant ($p<0.01$), confirming the influence of different dairy matrices in the carrier medium on the survival rate

of the cells. The freeze dried samples also showed significant ($p<0.01$) reduction between the carrier media.

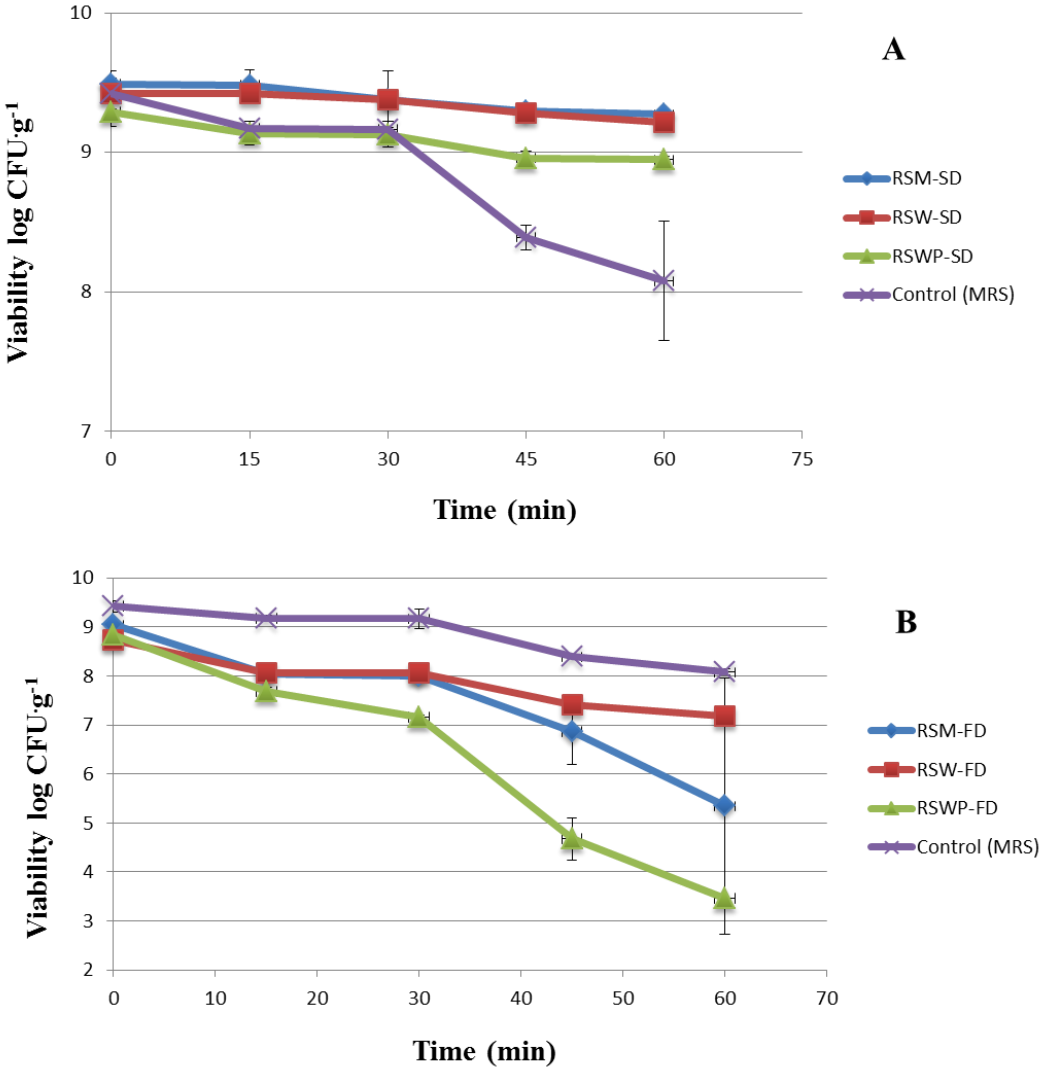

Figure 4.3 – Acid stress during 60 minutes of freeze dried and spray dried cultures in different medium in triplicate. SD (Spray drying); FD (Freeze drying). A – Spray drying samples; B – Freeze drying samples.

None of the samples evaluated showed a significant ($p>0.01$) viability loss in the presence of bile salts (Figure 4.4). In addition, under heat condition stress (Figure 4.5), the reduction was less than 2 log cycles in all the samples, and all the RSWP samples showed lower resistance under heat stress. In this case, the influence of the carrier medium was bigger than the influence of the drying method.

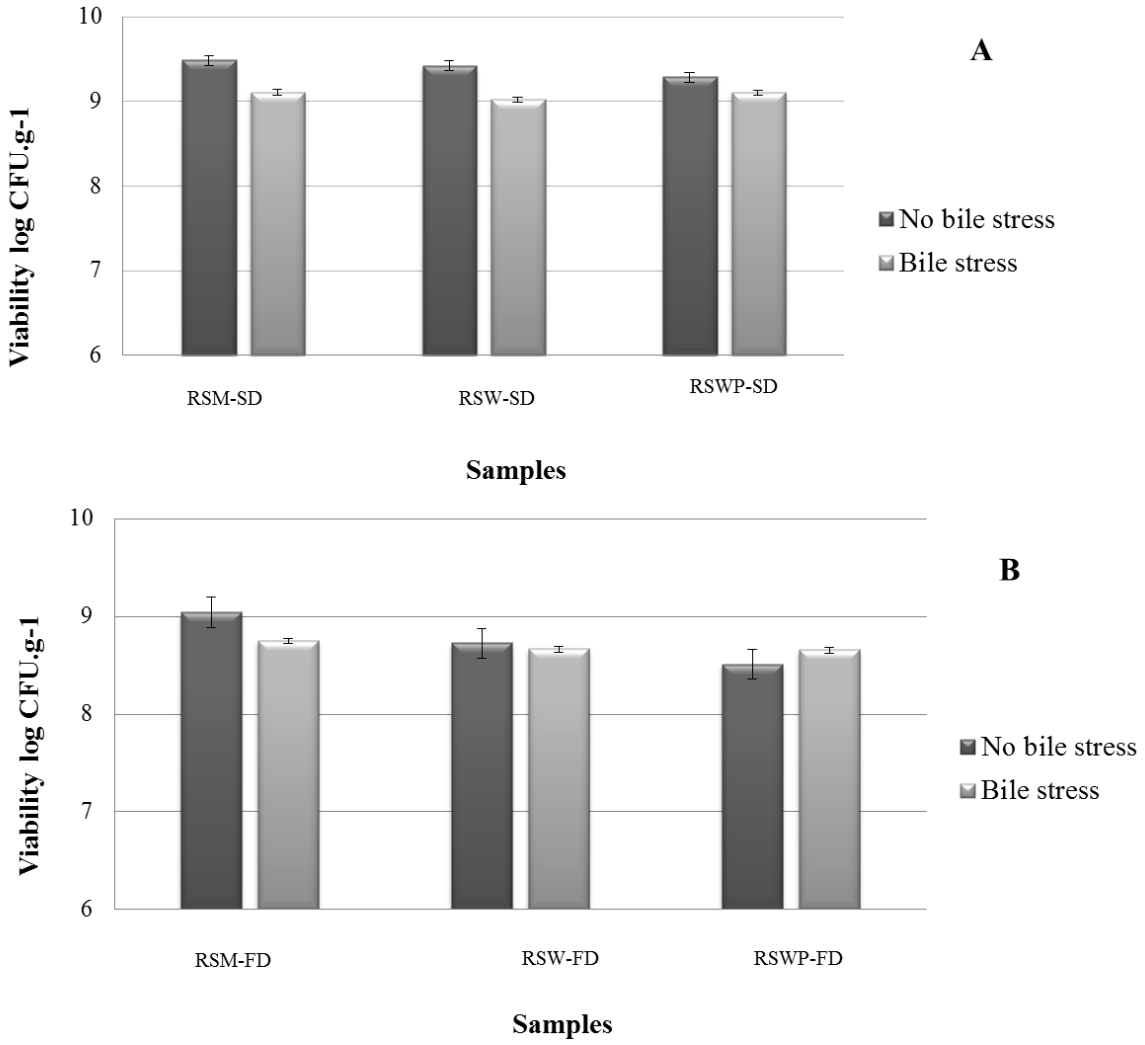

Figure 4.4 – Bile salts tolerance of *Lactobacillus plantarum* in triplicate. A – Spray dried cells; B – Freeze dried cells

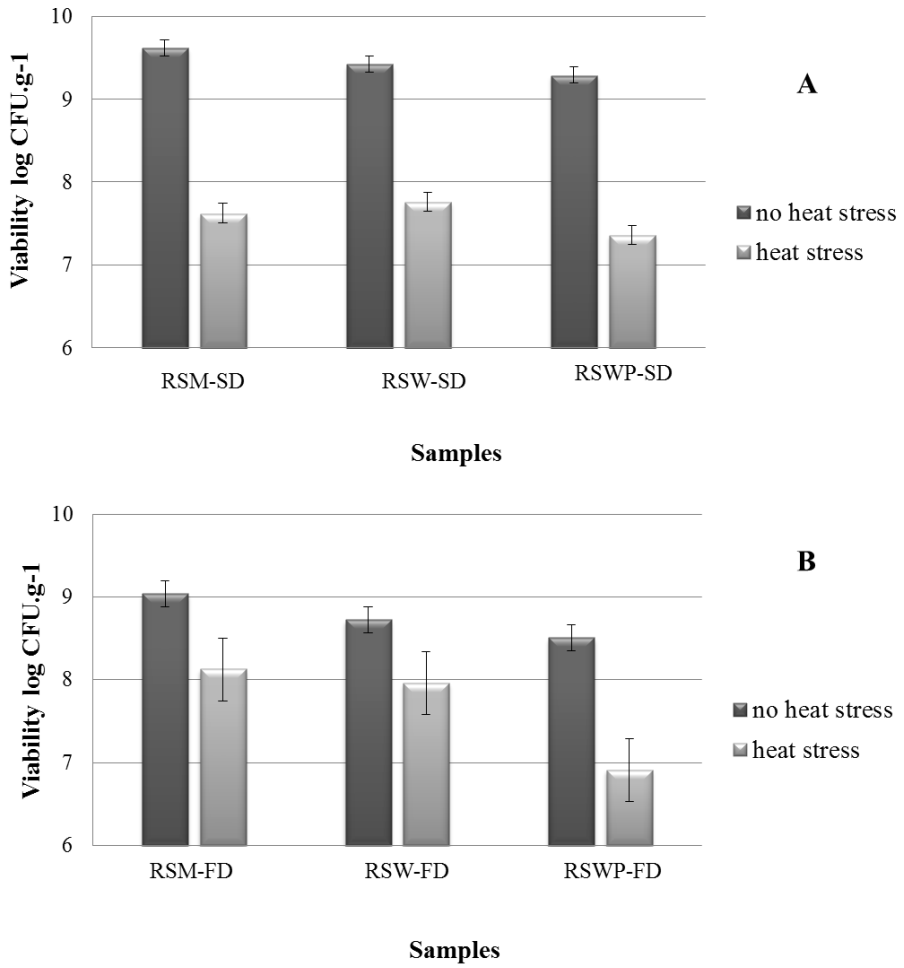

Figure 4.5 – *Lactobacillus plantarum* subjected on heat stress conditions in triplicate. A – Spray dried cells; B – Freeze dried cells.

4. Discussion

4.1. Effect of Freeze drying and Spray drying on the Survival Rate

Freeze drying and spray drying have both been considered for the preservation and storage of bacteria for industrial application (Kandil and Soda, 2015; Reddy et al., 2009; Schuck et al., 2013; Shokri et al., 2015a; Soukoulis et al., 2014; Utami et al., 2016; Zamora et al., 2006). Starter cultures for industrial application require the maintenance of high viabilities and biological activity during both drying and storage processes (Strasser et al., 2009). In this

work all the *L. plantarum* samples evaluated showed high viability over a storage period of 60 days for industrial application, although the cell concentrations in freeze dried samples were lower in comparison with spray dried samples. According to Morgan et al. (2006) the cell concentration ($>1\times10^{8}$ cells.mL^{-1}) has been decided as the higher initial cell concentration that allows the longer viable survival of cells within the freeze dried sample during the storage. The ice crystal formation during the initial freezing step of the freeze drying can lead to damage in the plasmatic membrane, resulting in loss of viability of the cells. Consequently, the DNA can also be affected (Castro et al., 1997). Lievense et al. (1994) considered the plasmatic membrane as the principal site of lethal damage after freeze drying.

Besides, as a result of the low water activity of the medium and the external increase of accumulated solutes due to the composition of the carrier medium, the cells are also subjected to osmotic stress conditions (Kandil and Soda, 2015). To reverse the damage and maintain the osmotic balance, some microorganisms accumulate compatible solutes in high concentration from the extracellular environment (Morgan et al., 2006; Santivarangkna et al., 2008a, 2007). According to Desmond et al. (2001) the accumulation of the compatible solutes also exhibits cross-protection effects with other microbial stresses as heat stress. Therefore, this can explain the better survival rate showed by spray dried cells during storage time.

However, some LAB do not synthesize compatible solutes and need to take up these solutes from the environment (Peighambardoust et al., 2011). These solutes can help the LAB to stabilize proteins and the cell membrane under osmotic stress conditions during the drying process (Morgan et al., 2006). The drying medium's composition influences the survival of bacteria. It is very important to define the better composition and concentration of carrier medium for drying. According to Fu and Chen (2011) the effect of the carrier medium's composition on cell survival during the drying process has an influence on the metabolic flux of microbial cells.

Spray dried cells are also subjected to membrane damage caused by conditions of low water activity and thermal inactivation. In this work we reported that freeze dried and spray dried samples in RSM and RSW showed a better survival rate during the storage period in comparison with freeze dried and spray dried samples in RSWP. According to Maciel et al. (2014), dairy matrices are beneficial for the encapsulation and protection of cell viability of lactic acid and probiotic bacteria in a spray drying process. This protection can probably be related to the presence of milk proteins and lactose in these matrices. Accordingly, the sweet whey permeate does not have significant amounts of protein, and it afforded lower survival rates of bacteria in our work. Ananta et al. (2005) and Zamora et al. (2006) suggested that the use of RSM at a concentration of 20% would be as the optimal solid content to ensure the high residual viability of different strains of LAB. In this study we used the carrier medium with 20% concentration (w/w), which allowed the maintenance of the viability of spray dried samples.

Our results differ from other authors who argue for a lower survival rate of spray dried cells (To and Etzel, 1997). According to Santivarangkna et al. (2008) the outlet air temperature can be the major drying parameter affecting the viability of spray dried starter cultures. However, in this work, the parameters used in spray drying are different in comparison with To and Etzel (1997) who used higher inlet (220 °C) and outlet temperatures (70, 75, 80 and 90 °C). The different temperatures of outlet-air used by To and Etzel (1997) influenced the survival rate of the microorganisms, whose residual viability increased with the decrease of outlet-air temperature.

The use of lower temperatures in our study enabled to maintain the powders in the glassy state, allowing a better conservation of *L. plantarum* in comparison with other studies. Additionally, the control of the spray dryer parameters seeking to obtain a relative humidity constant of outlet-air allowed a low water activity in the final product, justifying the maintain

of survival rate of *L. plantarum* over the storage time. According to Schuck et al. (2005) the optimization of water content and water activity in dairy powders allows the control powder composition and properties that enable obtaining a dehydrated product with lower sticking and clumping, favoring the glassy state.

In these conditions, the powder had a good quality with low water activity (0.12-0.17) and low moisture (3.4-3.9%). According to Shokri et al. (2015) the outlet-air temperature in different parts of a spray dryer should not be above 75 °C in order to avoid serious damage in the cells. In addition, it should not be below 60°C because a lower temperature could result in a high moisture of powder (up to 7%). These authors suggested that the best moisture of powder content is 4-7%. These data are similar to the results obtained in this work. The water activity has been recognized as one of the primary factors influencing the thermal resistance of bacteria in low-moisture foods. Hypothetically, the drying of bacterial cells can reduce the molecular mobility and help stabilize ribosomal units against irreversible damage due to thermal energy in low-moisture environments (Syamaladevi et al., 2016). Besides, the fast feed rate (30 $mL \cdot min^{-1}$) used in this study allowed a short residence time between the hot air and the material, minimizing the damages of thermal inactivation.

4.2. Effect of Freeze drying and Spray drying on the acidifying activity

The capacity to acidify milk is the most important biological property of the lactic acid bacteria that are used as starters in cheese making. According to Beresford et al. (2001) a good strain candidate for inclusion as a starter culture should produce sufficient acid to reduce the pH of the milk lower than 5.3 after 6 h of incubation in milk at 30 ºC.

None of the *L. plantarum* samples tested here was not able to reduce the pH below 6.0 after 6 h of incubation. However, after 24 hours, the spray dried samples reduced the pH more than the freeze dried samples in the same period evaluated. The spray dried cells were able to

adapt better to RSM 10%, probably because these cells had fewer injuries in the drying process, which can be confirmed by the better viability rate observed in the spray dried samples over 60 days of storage. The capacity to acidify milk by the spray dried cells in all the samples was the same, so in this case any of the carrier mediums can be used to preserve *L. plantarum* cells for industrial application as an adjunct culture.

4.3. Effect of Freeze drying and Spray drying on the stress tolerance

Acid and bile tolerance are important characteristics for probiotics and constitute a limit to their efficacy. Acid tolerance is required for bacteria to survive the passage through the stomach and bile tolerance is required for survival in the small intestine (Madhu et al., 2011; Shokri et al., 2015a; Utami et al., 2016). The great acid and bile tolerance showed by the spray dried samples can be explained by the possibility of thermal stress generated during the spray dryer to allow an induction of resistance to acid conditions and bile salts as a result of cross-protection. These adaptive responses can protect the cell in other adverse conditions, such as inactivation by dehydration and inactivation for exposition in high or low temperatures during the process (Morgan et al., 2006). Similar results were found by Reddy et al. (2009) where *L. plantarum* CFR 2158 maintained 100% viability when microencapsulated by spray drying using skim milk and exposed to pH 2.0 for 4 h.

Besides, there was a decrease of less than 2 log cycles of the samples subjected to heat stress conditions after 10 minutes. It can be confirmed by the thermal resistance of the *L. plantarum* strain studied, which also explains the better survival rate after spray drying. The *L. plantarum* cells that did not were subjected to spray and freeze drying showed lower thermal resistance (data not shown). It has been reported that the reduction in the survival rate and stress response of some starter cultures is dependent on the species, the method of preservation and the carrier medium (Dijkstra et al., 2014; To and Etzel, 1997).

5. Conclusion

Spray drying and freeze drying of *L. plantarum* were investigated with different dairy fractions as carrier. Surprisingly, spray drying was less damaging than freeze drying. However, it is still necessary to evaluate the parameters of the two processes to optimize the conditions of cell viability and biological activity in relation to the growth and protection media, conduction of proceedings, storage and rehydration for a better comparison between techniques. Spray drying being far more cost-effective than freeze drying, this work opens new prospects for the sustainable production of robust starters adapted to cheese making.

This study can direct further investigations to preserve lactic acid bacteria in order to produce starter and adjunct cultures by spray drying with a high number of viable cells to be used for the industrial application because it is a low-cost technique that allows the maintenance of the probiotic potential and acidifying activity properties.

The retention of probiotic properties after spray drying is very important to confirm *L. plantarum* isolated from Marajó cheese as a potent probiotic candidate for industrial applications. In addition, it is suggested that the development of starter/adjunct cultures from Marajó cheese produced by spray drying using the sweet whey as drying media can be applied on experimental models of cheese to evaluate the efficiency of yeast, generating better prospects for the dairy industry for the production of a product with sustainable characteristics.

The microencapsulation technique can also be studied to as an alternative to its application on experimental models of cheese. This technique has been proposed to improve the survival of probiotic strains to the drastic conditions and it allows a good viability and functionality when subjected to gastrointestinal conditions.

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CHAPTER 5

FINAL CONSIDERATIONS

GENERAL DISCUSSION

Normally, LAB are used as starter or adjunct cultures in several dairy produtcs. The importance of LAB in the development of these products is mainly associated with the production of many antimicrobial compounds, including organic acids (mainly lactic acid), hydrogen peroxide, and bacteriocins, which may inhibit spoiling and the growth of pathogenic bacteria (Castro et al., 2016). They also contribute to the flavor, texture and aroma of cheese and may play a probiotic role, adding value to functional products that have become increasingly widespread in recent years (Kołożyn-Krajewska and Dolatowski, 2012; Tripathi and Giri, 2014).

The use of LAB cultures for food fermentations is a multidisciplinary endeavour, requiring not only an understanding of the food ecosystem (Vogel et al., 2002), but also the characterization of useful technological and physiological features of the predominant strains (Dal Bello et al., 2012).

The study of LAB used as endogenous starter culture from artisanal cheese allows of identifying the species involved in cheese making, mainly related to the ripening and to the safety of artisanal cheeses and to select new strains for development of starter or adjunct cultures with distinct characteristics (González et al., 2015; Terzic-Vidojevic et al., 2013).

However, the processing of some cheeses can eliminate some bacteria with interesting properties in cheese making. Thus, the study of LAB from raw milk and utensils used in fabrication of artisanal cheeses may also have the advantage of the identification of endogenous LAB with interesting properties that could otherwise be accidentally lost during cheese manufacturing (Franciosi et al., 2009).

Our results have shown a diference among the species isolated from Marajó cheese in relation to the isolated species from milk and utensils used in Marajó cheese making. This demonstrates the possible influence of cheese making in the determination of the species in the final product. Therefore, it is important to identify the species present in the raw material to evaluate the influences in the fermentation process, since some species present in the cheese can be related to cross-contamination after cheese making and not contribute to the definition of the sensory characteristics of Marajó cheese that is considered a cheese not matured.

Additionally, the different phenotypic characteristics and the different genetic profiles shown by the strains in this study allows of a better knowledge and understanding of the contribution of each species involved in the Marajó cheese making and could eventually have commercial applications in the industry.

However, one of the main challenges in LAB cultures production is to maintain the biological activity of culture after the preservation processes (Peighambardoust et al., 2011). Freeze drying is a widely used technique in the preservation of lactic cultures for industrial application, though this technique is complex and more expensive than other drying processes (Santivarangkna et al., 2007). Thus, studies have been made to develop alternative drying processes at lower cost, as spray drying (Corcoran et al., 2004; Huang et al., 2016; Maciel et al., 2014; Madhu et al., 2011; Schuck et al., 2013; Silva et al., 2011). The process can be carried out in large-scale production which makes it an ideal technique for producing large amounts of dried cultures, optimizing the processing time (Santivarangkna et al., 2007; Schuck et al., 2013). And the product can be marketed and stored at room temperature (Schuck et al., 2013), decreasing the cost of refrigeration, and facilitating the transport to warmer regions, considering the fact that Brazil is a warm country.

Our results showed that spray drying is less damaging than freeze drying for *L. plantarum* from Marajó cheese. However, several factors of the two techniques can influence the viability and physiological properties of bacteria. These processes expose the bacteria to heat stress, cold stress and osmotic stress, which could potentially lead to cell death (Teixeira et al., 1995; To and Etzel, 1997; Zamora et al., 2006). These factors can be minimized by using different carrier media and optimization of drying technology (Santivarangkna et al., 2007).

Additionally, the appropriate selection of the strains that exhibit thermostability is an alternative for production of viable cultures over storage time. There are no commercial LAB cultures available that are stable at high temperatures (Peighambardoust et al., 2011) and, in general, the studies involving drying LAB by spray dryer do not use wild strains (Huang et al., 2016a; Maciel et al., 2014; Shokri et al., 2015). Therefore, discovering naturally thermo-stable LAB or those which have been genetically modified is important (Peighambardoust et al., 2011). Additionally, to promote adaptations to the different stress conditions that may influence the viability of the cultures after spray drying is a promising alternative.

CONCLUSIONS AND PERSPECTIVES

The methodologies applied in this PhD thesis allowed the study of the biodiversity of wild LAB from Marajó Cheese, milk, curd and utensils used in Marajó Cheese making, produced on Marajó Island, Amazonian region, Brazil. The Rep-PCR was able to discriminate a high genetic variability among the isolates. This microbial diversity can be explained by isolation of LAB from different sources, cheesemakers and types of cheeses. The phenotypic characteristics shown by some strains can be explored to better understand the role that each species may have in the characterization of Marajó Cheese.

The preservation of these wild LAB strains can ensure the maintenance of cultures of industrial interest for long storage time in culture banks in order to allow further studies for development of commercial lactic cultures or to assist in standardizing Marajó Cheese.

The impact of drying conditions by spray drying in this work in the presence of dairy-based carrier media of different compositions was minimal on the viability and biological activity of *L. plantarum. S*pray drying is a simple and cost-effective process to production and preservation of LAB.

The use of sweet whey and sweet whey permeate for drying of bacteria in spray dryer represents a great opportunity for the dairy industry. These dairy co-products are low-cost and show relevant nutritional aspects, interesting for development of new products, including functional products.

The aim of this thesis was to isolate and identify autochtonous LAB from Marajó Cheese and to show their potential for industrial application as well as the possibility of preservation of these cultures by an alternative method by means of dairy co-products. The main results of this thesis are summarized in Table 5.1.

Table 5.1 - The main results of this thesis

Experimental Chapters	Main Results
CHAPTER 2 Biodiversity of autochthonous LAB in raw buffalo milk, curd and utensils	- Identification of 149 LAB cocci and bacilli obtained from all the evaluated samples ; - Higher frequency of isolation of *Weissella* sp. and *S. infantarius*, mainly in milk and curd; - The Rep-PCR was able to identify a high genetic diversity among the isolates coming from different samples and cheesemakers; - Strains considered clones by Rep-PCR showed different phenotypic characteristics.
CHAPTER 3 Identification of LAB cocci in Marajó Cheese	- Identification of 97 LAB cocci obtained from Marajó cream and butter type cheese; - Different species of LAB cocci were identified in two types of Marajó cheese and in two seasons evaluated; - The Rep-PCR was able to identify different genetic profiles, showing a high diversity among the isolates; - The different genetic profiles showed by strains in different types of Marajó Cheese do not determine phenotypic differences in the conditions studied; - The LAB cocci showed good phenotypic characteristics for potential industrial application.
CHAPTER 4 Development of an original, sustainable and robust starter by spray drying/freeze drying	- The spray drying was less damaging than freeze drying on the cells of *L. plantarum*; - The effects of spray drying on *L. plantarum* using three different dairy-based carrier media were minimal;

CHAPTER 4 Development of an original, sustainable and robust starter by spray drying/freeze drying	- The spray dried cells maintain the physiological properties of acidifying activity and bile salts, acid and heat tolerance conditions; - The spray dried cells remained approximately 10^9 CFU·g^{-1} after 60 days of storage at 4 °C and 20 °C.

From the main results and conclusions shown in this work, we propose the continuity of the characterization studies of autochthonous lactic microbiota involved in the Marajó cheese making by molecular techniques that allow a global understanding of all LAB species present in this cheese. Among these molecular techniques, it is suggested the application of Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). This technique is a culture-independent method and has been used in studies of microbial ecology from artisanal cheese (Arcuri et al., 2013; Ercolini et al., 2008; Perin et al., 2015), allowing the knowledge of the interactions and possible influences caused by bacteria in food systems (Perin et al., 2015), and it possibly relates to the geographical area of production of cheese (Ercolini et al., 2008). Thus, PCR-DGGE may assist in a better understanding of influence of Marajó Cheese processing in the isolation of different species coming from raw buffalo milk, curd and utensils in comparison with cheese itself.

We also propose more studies to better characterize the strains that have shown the best phenotypic characteristics. Other properties such as salt tolerance, production of biogenic amines, autolytic activity, antibiotic resistance, phage-resistance, and production of bacteriocins that are considered common technological features to determine the selection of potential strains for industrial applications (Dal Bello et al., 2012).

However, these new strains for industrial application can be exposed to different stress conditions involved in their preservation by spray drying such as thermal, osmotic and

oxidative stress. Thus, it is suggested the research of the different genes related to stress responses of LAB and the application of different strategies to adapt these strains to different stress conditions that can affect their survival rate during spray drying.

The survival rate of bacteria subject to spray drying may differ among different species of LAB. Further studies are necessary to determine the influence of the drying conditions tested in this work.

These studies will allow the preservation of LAB that may be used for the development of new products or to give a better technical support to Marajó cheese making. The utilization of these cultures can improve the quality and standardization of the product as well as to preserve the biodiversity of the product`s LAB that may some day be eventually modified by law requirement to meet the standards of identity and quality which in many cases does not reflect the reality of production of this cheese on farms.

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