UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Analysis of bacterial diversity in Brazilian dairy samples by culture-based methods and metataxonomics

Análise da diversidade bacteriana em amostras de laticínios brasileiros através de técnica de cultivo e metataxonomia

DIEGO DE ARAÚJO FRAZILIO

Ribeirão Preto
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Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

Concentration Area: Bioagentes e Biotecnologia Aplicados à Farmácia

Supervisor: Prof. Elaine Cristina Pereira De Martinis, Ph.D.

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Approved on:

Examiners

Prof. Dr. _____

Institution: _____Signature: _____

Prof. Dr. _____

Institution: _____Signature: _____

Prof. Dr. _____

Institution: _____Signature: _____

Prof. Dr. _____

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Prof. Dr. _____

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Prof. Dr. _____

Institution: _____Signature: _____

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“A força reside em conhecer a si mesmo. Aprendi que, quando alguém não conhece a si mesmo, está perdido. Eles se tornam uma ferramenta para os outros”.

Rob Thomas

“Eu não sei sobre os anjos, mas é o medo que dá asas ao homem!”

Max Payne

“O fluxo do tempo é sempre cruel, sua velocidade parece diferente para cada pessoa, mas ninguém pode mudá-la. Uma coisa que não muda com o tempo é a memória dos dias mais jovens”.

Shigeru Miyamoto

” O que é melhor - nascer bom, ou superar sua natureza maligna através de grande esforço? ”.

Elders Scrools

“Todos nós fazemos escolhas na vida, mas no final nossas escolhas nos definem”.

Bioshock

Preface

This thesis is divided in five chapters. In the first part (“Introduction”), there is a brief presentation of the importance of dairy food for health and economy, with emphasis on microbiological standards to guarantee food safety and quality.

The objectives are stated in the second chapter.

Chapter 3 presents a culture-dependent study carried out with samples from selected Brazilian dairy plants (from São Paulo and Goiás states), focusing on isolation of *Listeria monocytogenes*, *Staphylococcus aureus* and the respective accompayining microbiota. Next, are presented the preliminary phenotypic characterization and the molecular identification of the isolates by DNA Sanger sequencing.

In Chapter 4, community 16S rRNA gene sequencing was used to quantify the proportion of taxa present in selected Brazilian dairy plants (São Paulo state), including samples of raw material, food-contact, non-food contact surfaces and final products. The output data was evaluated with bioinformatics tools and discussed from the point of view of food quality and safety.

The Chapter 5 describes a polyphasic approach to determine the dominant microbiota present in dairy samples from two selected dairies from São Paulo state. To accomplish this goal, isolates were cultured from dairy samples and identified by DNA Sanger sequencing. Besides, for community 16S rRNA gene sequencing was performed by High Throughput Sequencing and the results obtained were analyzed by bioinformatics to define a core microbiota.

Finally, general closing remarks are presented in Chapter 6.

RESUMO

FRAZILIO, D. A. **Análise da diversidade bacteriana em amostras de laticínios por meio de técnicas de cultivo e metataxonômica**. 2019. 95p. Tese. Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2019.

A segurança alimentar é uma questão de importância mundial e o conhecimento de microorganismos cultiváveis e não cultiváveis é de aspecto fundamental para compreender a ecologia microbiana, a fim de propor estratégias para a conservação de alimentos e prevenção de surtos de origem alimentar. O leite e os produtos lácteos são altamente perecíveis e suscetíveis à contaminação por bactérias patogênicas. Assim, é importante determinar quais microorganismos estão presentes nas diferentes etapas do processamento de laticínios e nos produtos prontos para consumo. No capítulo 3 desta tese, *Staphylococcus aureus*, *Listeria monocytogenes* e a microbiota acompanhante foram investigadas de 97 amostras de cinco fábricas de laticínios (localizadas nos estados de São Paulo e Goiás - Brasil) e os isolados foram identificados por sequenciamento do DNA utilizando o método de Sanger (gene 16S rRNA). Nenhuma amostra foi positiva para *Listeria sp*, porem a presença de *S. aureus* foi altamente prevalente. A microbiota acompanhante foi composta principalmente por bactérias produtoras de ácido lático (LAB), mas outras bactérias também estavam presentes. Esta parte da tese indicou a necessidade de um melhor controle de *S. aureus* nas fabricadas de laticínios avaliadas. No capítulo 4, amostras de fábricas de laticínios brasileiras (localizada no estado de São Paulo) previamente positivas para *S. aureus* foram analisadas pelo sequenciamento do gene 16S rRNA, dividido em quatro grupos (matéria-prima, produto final, superfícies de contato e sem-contato com o alimento). Os resultados demonstraram altos índices de diversidade alfa (produto final e superfície sem contato com o alimento), mas os índices de diversidade beta foram baixos, as amostras foram separadas em dois grupos principais onde a comunidade bacteriana era dominada por *Macrococcus*, *Alkaliphilus*, *Vagococcus*, *Lactobacillus*, *Marinilactibacillus*, *Streptococcus*, *Lisinibacillus*, *Staphylococcus*, *Clostridium*, *Halomonas*, *Lactococcus*, *Enterococcus*, *Bacillus* e *Psychrobacter*. No capítulo 5, foram analisadas 27 amostras de duas fábricas de laticínios do Estado de São Paulo para cultura da microbiota autóctone e os isolados foram identificados por meio de sequenciamento de DNA. Além disso, o DNA metagenômico foi diretamente extraído das amostras e a microbiota não cultivável foi avaliada através do sequenciamento massivo do gene 16S rRNA. Os resultados de obtidos com as culturas bacterianas, indicaram que a maioria dos isolados eram dos grupos das bactérias láticas, mas não somente essas, foram detectadas também da ordem *Enterobacterales* e das famílias *Staphylococcacceae*, *Bacillaceae*, *Pseudomonadaceae* e *Moraxellaceae*. A partir da metataxonomia, construiu-se um heatmap onde foram determinadas as 20 unidades taxonômicas operacionais (OTUs) mais abundantes, revelando uma significativa dissimilaridade da microbiota de ambos os laticínios. Foram encontradas 12 taxa bacteriana mais prevalentes na microbiota dos laticínios avaliados, com a maior abundância de OTUs de *Tolumonas auensis* e *Lactococcus fujiensis*. No geral, os resultados desta tese revelam a ecologia microbiana altamente complexa de alimentos lácteos e revelaram novas combinações de espécies mistas a partir de abordagens de bactérias cultiváveis e não culturais. A partir desses resultados, é interessante desenvolver novos estudos para avaliar

possíveis correlações positivas ou negativas entre os membros microbianos e suas possíveis implicações para a segurança alimentar.

Palavras-chave: microbiota do leite, metataxonômica, laticinios, microbiota de queijos.

ABSTRACT

FRAZILIO, D. A. **Analysis of bacterial diversity in dairy environment samples using culture dependent and metataxonomic techniques.** 2019. 95p. Thesis. Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2019.

Food safety is a matter of worldwide importance and the knowledge of cultivable and non-cultivable microorganisms is a key aspect to understand the microbial ecology, in order to propose strategies for food preservation and prevention of foodborne outbreaks. Milk and dairy products are highly perishable and susceptible to contamination by spoilage and pathogenic bacteria. Thus, it is important to determine the microorganisms present in different steps of dairy processing and in the readyu-to-eat products. In chapter 3 of this thesis, *Staphylococcus aureus*, *Listeria monocytogenes* and accompanying microbiota were screened from 97 samples of five dairies (located in São Paulo and Goiás states (Brazil) and the isolates were identified by Sanger DNA sequencing (16S rRNA gene). No sample was positive for *Listeria* sp. but *S. aureus* was highly prevalent. The background microbiota was composed mainly by LAB but other bacteria were also present. This part of the thesis indicated a need for a better the control of *S. aureus* in the dairies evaluated. In chapter 4, Brazilian dairy samples (from São Paulo state) that were previously known to be positive for *S. aureus* were analyzed by 16S rRNA gene sequencing, divided in four groups (raw material, final product, food-contact and non-food contact surfaces). The results showed high alpha-diversity indexes (final product and non-food contact surfaces) but, beta-diversity indexes were low. The samples were separated in two main clusters and the bacterial community was dominated by *Macrococcus*, *Alkaliphilus*, *Vagococcus*, *Lactobacillus*, *Marinilactibacillus*, *Streptococcus*, *Lysinibacillus*, Staphylococcus, Clostridium, Halomonas, Lactococcus, Enterococcus, Bacillus and Psychrobacter. In chapter 5, a total of 27 samples from two dairies of São Paulo state were sampled for culture of autochthonous microbiota and the isolates were identified by DNA sequencing. Moreover, metagenomic DNA was directly extracted from samples and the unculturable microbiota was evaluated with massive 16S rRNA gene sequencing. Culture results indicated most isolates were lactic acid bacteria, but *Enterobacterales* and families *Staphylococcacceae*, *Bacillaceae*, *Pseudomonadaceae* and *Moraxellaceae* were also detected. From metataxonomics, a heatmap was constructed and the top20 OTUs (operation taxonomics units) were determined, revealing a significant dissimilarity of the microbiota from both dairies. There were 12 most prevalent bacterial taxa in the core microbiota of the dairies evaluated, with the highest abundance of OTUs from *Tolumonas auensis* and *Lactococcus fujiensis*. Overall, the results of this thesis reveal the highly complex microbial ecology of dairy foods and revealed novel mixed species combinations from culture and non-culture based approaches. From these results, it is interesting to develop novel studies to evaluated possible positive or negative correlations among the microbial members and their possible implications for food safety.

Keywords: milk microbiota, metataxonomics, dairies, cheese microbiota.

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SUMMARY

1 Introduction

1.1 INTRODUCTION

Foodborne diseases are a global public health problem and their prevention has always been a challenge, which depends on the foods consumed, food processing, handling, storage and sensitivity of consumers. To reduce illnesses cases, it is important to consider besides the scientific evidences, the culture and eating habits of different populations. For example, banning unpasteurized milk may be acceptable to some countries, but not to others (ICMSF, 2006). In fact, in Brazil there is a specific legislation on artisanal Minas cheeses made from raw milk (MINAS GERAIS, 2012) and that cheesemaking process is considered a cultural Brazilian heritage (BRASIL, 2008). YOON et al. (2016) affirmed the autochthonous microbiota of raw milk cheeses contribute for products with more intense and varied flavor, preferred by many consumers. There is a large debate on the abolition of raw milk for cheese making because some advocate that even pasteurization do not necessarily ensures that the cheese will be free of pathogens, since post-pasteurization contamination of cheeses may also occur. However, the use of raw milk is of special concern for those cheeses with low acidity and high moisture, which emphasizes the need for implementation of food safety plans to protect consumer's health, and the ideal situation is always to move from responding to microbial contamination to preventing it (JOHNSON, 2017; YOON et al., 2016). According to YOON et al. (2016) the risk associated with raw milk cheeses may be minimized by appropriate aging and adoption of adequate hygienic practices, emphasizing the need for constant monitoring of hygiene in milking farm, in dairy processing facilities and at post-manufacturing stages.

1.2 CHEESE PROCESSING AND DAIRY MICROBIOTA

There are evidences of milk products being a major component of diets since prehistoric times, as shown by the studies on milk residues in ancient pottery vessels, and this suggests that production of cheese, would have been a critical development due to the preservation of milk products, providing a more digestible commodity for early prehistoric farmers (SALQUE et al., 2013).

The process of cheese production starts with the milk, which is often standardized before cheese making to optimize the protein and fat proportion, to

guaranty high quality and yield. The use of heat treated milk for cheese making aims to avoid pathogenic microorganisms, to reduce the number of spoilage microorganisms and to improve conditions for the efficiency of starter cultures (CARAFA et al., 2019; SALES et al., 2018).

There are ca. 1,000 cheese varieties worldwide and more than 80% of the production is represented by cheese of the familes Dutch, Swiss, Pasta-filata, Cheddar and Parmesan. Although no classification scheme is entirely satisfactory, many attempts have been made to classify cheese varieties, and the main characteristics considered are texture and composition, methods of milk coagulation and ripening indices. Considering the milk coagulation methods, all cheese varieties can be classified in three superfamilies: enzymatic coagulation (75% of total production); acid or lactic coagulation; and coagulation by combination of heating and addition of acid or salt (PAULA; CARVALHO; FURTADO, 2009; MCMAHON; OBERG, 2017; MCSWEENEY; OTTOGALLI; FOX, 2017). These processes allow the concentration of milk by separating solid components (mainly protein and fat) from the whey - composed of water, soluble proteins, lactose and other soluble solids (PAULA; CARVALHO; FURTADO, 2009).

Depending on the variety, cheeses can be aged from a few to several months, or even years. The maturation times of several cheeses can be seen in table 1.

Table 1 - Maturation time of some cheeses

Cheese Type	Maturation chesse – month
Parmesan (Reggiano)	14
Cheddar	3-6
Swiss cheese	2-6
Prato cheese	1
Minas Cured cheese	1
Gouda	1
Camembert	3 weeks

Source: Tronco (1996).

The dairy industry in Brazil has been highly influenced by macroeconomic changes, but milk production in Brazil has experienced a constant increase, reaching ca. 30.7 billion liters in 2010, with the largest production by Southeast region. The dairy sector has high potential to grow and there are many factors driving this scenario,

such as increase in family income, population growth, and changes in consumption habits (SPERS; WRIGHT; AMEDOMAR, 2013). Dairy products are of greater economic importance especially for some local communities, for which the production of artisanal cheeses may provide an additional family profit and to promote a better quality of life, with social inclusion (MARTINS et al., 2018).

The most popular type of cheese in Brazil is the “Minas”, which is prepared by enzymatic coagulation of milk, with no ripening. Other popular cheese varieties are mozzarella, *prato* and *requeijão*, among others. There is a very important system of sanitary inspection in place in Brazil (Ministry of Health, through the National Health Surveillance Agency - ANVISA and Ministry of Agriculture, Livestock and Food Supply - MAPA), but there are still informal sales of homemade cheeses in some regions due to cultural and economic factors (GOMES et al., 2013; USDA, 2017). Recently, a new legislation was approved (BRASIL, 2018) to allow the trade of artisanal state inspected food products of animal origin over the country (CASTRO, c2018). With regard to cheeses, there is even a specific state legislation on artisanal “Minas” cheeses, which is made by enzymatic coagulation from raw milk (MINAS GERAIS, 2012), and that cheesemaking process is considered a cultural Brazilian heritage (BRASIL, 2008).

The main steps to produce cheeses prepared with enzymatic coagulation are: 1) milk preparation (selection, filtration, clarification, standardization and pasteurization); 2) cooling (32-35ºC); 3) addition of coagulating ingredients (lactic ferment, calcium chloride and/or enzymes), addition of other ingredients (optional); 4) coagulation (32-35ºC); 5) curd cutting; 6) agitation and sineresis, with acidification and slow heating; 7) removal of whey; 8) pressing and molding; 9) brining (PAULA; CARVALHO; FURTADO, 2009).

Milk and dairy products are nutrient rich and thus provide ideal growth conditions for many microorganisms, and the large amount of cheese variety results in great part from a diverse microbiota, which can come from raw materials or can be introduced in the product from other sources, such as processing, packaging, maturation, handling and storage, depending on the type of cheese (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2019; SKEIE et al., 2019). Microorganisms can contribute positively for cheese quality, for example, when used as starter cultures or probiotics. However, the cheese microbiota may include spoilage organisms that can be tolerant to heat or can survive at refrigerated storage. Besides, foodborne pathogens can also be

associated with dairies which includes Gram-positive (i.e., *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*) and Gram negative bacteria (i.e., *Salmonella* spp., *Cronobacter* spp. and *Campylobacter jejuni*), among others (KENNY et al., 2013).

More recently, with the advent of “omics” technologies, there is a great potential to advance the knowledge of cheeses as complexes biological ecosystems, and this will certainly impact the understanding of cheese flavour and safity (AFSHARI et al., 2018).

1.2. MICROBIOTA OF CHEESES

Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, microaerophilic or anaerobic bacteria that produce lactic acid as the major end product of sugar fermentation. LAB generally have a low GC content (<50 mol%) and are typically catalase and cytochrome negative, fastidious, aerotolerant, and acid tolerant. The most common food-related genera of LAB are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Carnobacterium*, and *Weissella* (GEMECHU, 2015, PAPADIMITRIOU et al., 2016).

LAB play a prominent role in dairy food fermentation, performing a key role in bioconversions. They can metabolize hexoses by a homofermentative pathway (lactic acid is the primary product) or by a heterofermentative pathway (producing lactic acid, CO_2, acetic acid, and/or ethanol) (MAKAROVA et al., 2006).

Given that dairy food usually represent complexes biological ecosystems, the advances in culture-independent based techniques have been essential to understand

Metagenomics is a very useful molecular tool to study mixed microbial communities. According to Almeida and De Martinis (2018), metagenomics utilizes the total DNA previously extract from microbial communities of a sample of interest and analyses it without the need of culturing. Metagenomics joints concepts of statistics and of meta-analysis, and it quantitatively correlates large genomic datasets with another sequences already studied, integrating all data (SCHLOSS; HANDELSMAN, 2003, ALMEIDA; DE MARTINIS, 2018).

Meta-analysis is fundamental to understand the results obtained from high-throughput DNA-sequencing and makes possible to discuss the dynamic of

microorganisms and their interactions in a microbial community (ALMEIDA; DE MARTINIS, 2019).

Metagenomics is the collection of genes and genomes from the members of a microbiota, which is obtained through shotgun DNA sequencing of a sample, followed by the assembly or mapping to a reference database and annotation. In the other hand, metataxonomics is based on the sequencing of phylogenetic markers (usually 16S *rRNA* for bacteria and ITS for fungi) providing taxonomic profiles identification of a sample (MARCHESI; RAVEL, 2015; QUINCE et al., 2017).

Metataxonomics is crucial to reveal the members of a microbial community and to monitorshifts in microbial profiles (ALMEIDA; DE MARTINIS, 2019).

Recently, metagenomics has been applied to elucidate the microbial communites of Brazilian cheeses. Kamikura et al. (2019) analyzed natural Brazilian cheese starter cultures known as “pingo”. They evaluated 11 different types of artisanal cheeses from five geographical areas of Brazil and found that LAB dominated in all cheeses although *Enterobacteriaceae* and *Staphylococcus* were also present. Sant’Anna et al. (2019) evaluated raw milk and “pingo”, from Minas artisanal cheeses. They observed *Planococcaceae* and *Streptococcaceae* dominated during ripening time, and the former family, seemed to develop strong interactions with the *Leuconostocaceae* on cheese surface. Those authors also reported environmental aspects of the region, are likely to contribute for the microbial signature of the products analyzed

With regard to the most concerning foodborne disease agents related to dairy food, *L. monocytogenes* and *S. aureus* are of great importance (KENNY et al., 2013).

L. monocytogenes was first described in 1926 but only in the 80’s it was identified as a foodborne pathogen. Since them, despite the moderately low number of infections per year reported, the lethality rate of this infection is the very high, ranging from 20 to 30% (RADOSHEVICH; COSSART, 2018).

Listeriosis is the name for a general group of disorders caused by *L. monocytogenes* and it may manifest by fever and muscle aches, which can be sometimes be preceded by diarrhea and other gastrointestinal problems. Mild to severe gastroenteritis is the symptom experienced by most healthy adults after ingestion of highly contaminated food (up to 10^9 bacteria). However, in the case of children, the elderly, immunocompromised individuals and pregnant women, severe

listeriosis may manifest even after ingestion of food with low level of contamination (ca. 10^2–10^4 bacteria). The invasive disease is characterized by bacterial sepsis, Central Nervous System (CNS) infections and/or transplacental contamination, resulting in abortion or perinatal infections (RADOSHEVICH; COSSART, 2018; CHURCHILL et al., 2019). The majority of infections caused by *L. monocytogenes* involve the serotypes 1/2a, 1/2b and 4b (ALMEIDA et al., 2017).

In the invasive disease, *L. monocytogenes* is internalized by cells into a vacuole, leading to cytoskeletal rearrangement. The bacterium disrupts the vacuolar membrane by the action of potent virulence factors (LLO and two phospholipases, PlcA and PlcB). Next, it survives and divides in the cytosol and can spread from one cell to another by co-opting actin-based motility. More recently, factors that hijack cellular processes and, in some cases, induce epigenetic changes that influence host gene expression have been reported for *L. monocytogenes* (RADOSHEVICH; COSSART, 2018).

L. monocytogenes does not form spores but it is considerably tolerant to the effects of freezing, drying and heating (ALMEIDA et al., 2017; BUCHANAN et al., 2017). Outbreaks of listeriosis have been associated mainly with ingestion of contaminated refrigerated ready-to-eat food, but diverse food items have been incriminated in outbreaks, including: meat products, raw vegetables, ice creams, milk and cheeses among others (BUCHANAN et al., 2017).

Raw or inadequately pasteurized milk, as well as soft cheeses, are food items of great concern with regard to contamination by *L. monocytogenes*. The control of this foodborne pathogen demands on-farm control measures such as hygienic husbandry and herd health management. However, there is also need to control contamination in food processing and during food handling (KENNY et al., 2013; BUCHANAN et al., 2017).

Staphylococcal food poisoning was has been know since late 19th century, and still nowadays it is one of the most common foodborne diseases. The symptoms manifest quickly after ingestion of food contaminated with enterotoxins, depending on the quantity. *S. aureus* is capable of growing in a high range of temperatures (7^o to 48.5^o C), in very dry environment and under many adverse conditions (KADARIYA; SMITH; THAPALIYA, 2014). *S. aureus* produces heat stable enterotoxins that are classified basically into serological types, mainly SEA, SEB, SEC, SED, SEE, SEG,

SEH, SEI, and SEJ. All staphylococcal enterotoxins (SEs) cause emesis but their mechanisms of action are not clear. It is lilkely the SEs directly affect intestinal epithelium and vagus nerve, stimulating the emetic center. Staphylococcal toxins also present superantigenic activity, interfere in the immunological system and start an inflammatory process in intestinal cells that results in degranulation and diarrhea (KADARIYA; SMITH; THAPALIYA, 2014).

Besides the foodborne intoxication, *S. aureus* has also been implicated with hospital-acquired infections, raising special attention due to the spread of antibiotic resistant strains, particularly methicillin-resistant *S. aureus* - MRSA. In spite of the this, most of times *S. aureus* is only colonizer in humans, and ca. 20% of adults are persistent carriers (GRUMANN; NÜBEL; BRÖKER, 2014).

In the view of the growing economic and social importance of dairy food in Brazil, considering also the recent advances in the characterization of microbial communities it is crucial to investigate more in depth the composition and possible interactions of authoctonous microbiota and pathogens in Brazilian dairies.

2 Objectives

2.1 GENERAL OBJECTIVE

To characterize the in house the microbiota of selected Brazilian dairies to contribute with the improvement of food safety.

2.1.1 Specific objectives

a) To identifiy the culturable microbiota from samples of raw material, food-contact surfaces, non food-contact surfaces and final products in five Brazilian dairies (states of São Paulo and Goiás), targeting *Listeria monocytogenes*, *Staphylococcus aureus* and accompanying bacterial microbiota. To confirm the identification of isolates by sequencing of*16S rRNA* gene.
b) to determine the bacterial members of the microbiota of raw material, food-contact surfaces, non food-contact surfaces and final products in two Brazilian dairies (state of São Paulo), in metataxonomics approach (*16S rRNA*).
c) to perform experiments to compare the culturable and culture-independent microbiota of selected dairy samples, employing classical bacterial isolation and high throughput *16S rRNA* sequencing.

3 Screening of dairy food and environmental samples for *Listeria monocytogenes*, *Staphylococcus aureus* and the respective accompanying microbiota

Screening of dairy food and environmental samples for *Listeria monocytogenes*, *Staphylococcus aureus* and the respective accompanying microbiota

Abstract

Food safety is of increasing importance in a global economy. To evaluate microbial contamination rates and spots during food processing might help to propose strategies to prevent foodborne diseases. In especial, milk and dairy products are largely consumed food items and they have been incriminated in many outbreaks involving *Staphylococcus aureus* and *Listeria monocytogenes.* In this study, the prevalence of *S. aureus* and *L. monocytogenes* were analyzed and the accompanying microbiota of positive samples was also cultivated. For that, 97 samples from five dairies of São Paulo and Goiás states (Brazil) were screened, using selective and non selective culture media. The final identification of the isolates was confirmed by Sanger DNA sequencing based on the 16S rRNA gene. The results revealed no sample was positive for *Listeria* sp. but *S. aureus* was highly prevalent, with positive samples in all dairies evaluated. The background microbiota was composed mainly by LAB and among those, *Enterococcus casseliflavus* was the most prevalent. Several other Gram-positive bacteria were members of the mixed microbiota but *Serratia nematodiphila* was the only Gram-negative bacteria detected. This study indicates a need for the control of *S. aureus* in the dairies evaluated and provides isolates that might be useful for further research aiming to develop strategies for food safety.

Key words: mixed dairy microbiota, *S. aureus*, *L. monocytogenes*, cheese.

3.1 INTRODUCTION

Cheeses are of great importance for Brazilian economy, and Minas cheese is the most popular type consumed in Brazil, which is prepared by enzymatic coagulation of milk, with no ripening. The other most popular cheese varieties are mozzarella and *prato*, among others. Despite the large availability of dairy products with sanitary inspection, there are informal sales of homemade cheeses in some regions due to cultural and economic factors (GOMES et al., 2013). Ranking among the most concerning microbiological hazards in dairy foods, there is the staphylococcal food poisoning (SFP) (GOMES et al., 2013; KADARIYA et al., 2014).

SFP is an intoxication resulting from the consumption of contaminated food with adequate levels of pre-formed enterotoxins and it manifests usually within 2-8 hours after ingestion, by causing nausea, vomiting and abdominal cramping, with or without diarrhea that typically resolve within 24-48 hours. The number of people affected by SFP is only an estimative due to misdiagnosis and minor outbreaks that are not reported (KADARIYA et al., 2014).

Due to the wide variety of cheeses available, it is difficult to recommend microbiological standards that are applicable to all types of cheese but research of coagulase positive staphylococci or *Staphylococcus aureus* is strongly recommended due to the significant potential of enterotoxin formation (INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS, 2011).

Another significant hazard associated with cheeses is the bacterium *Listeria monocytogenes*, which is ubiquitous and psychrotolerant, with high persistence in food production premises (GOMES et al., 2013; OXARAN et al., 2017; MELERO et al., 2019). *L. monocytogenes* can cause a disease called listeriosis, which is highly invasive and potentially lethal in immunocompromised. This disease is also important as a cause of abortion and perinatal infections. However, a milder infection may manifest in healthy adults, with symptoms of gastroenteritis (RADOSHEVICH; COSSART, 2018)

In this scenario, the objective of this research was to evaluate the prevalence of *S. aureus* and *L. monocytogenes* in Brazilian dairy samples, and to characterize the background microbiota in cheese processing environments, aiming to elucidate

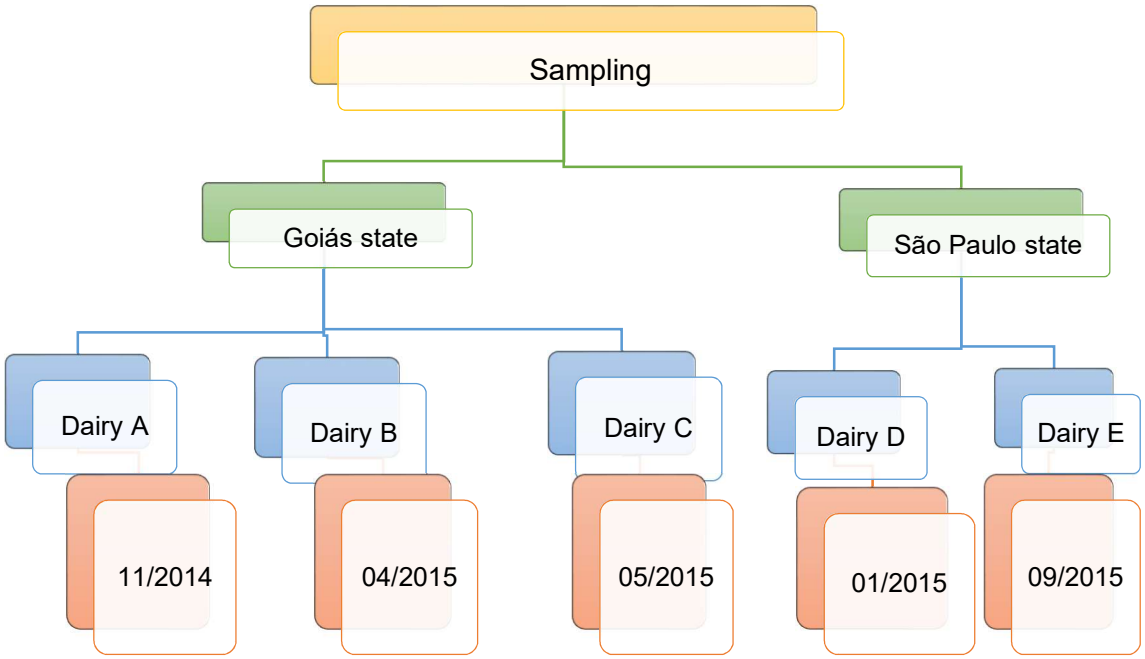
important pathogen and autochthonous microbiota combinations with potential to affect food safety.

3.2 MATERIALS AND METHODS

3.2.1 Sampling at dairies

Samples were collected in five dairy plants as illustrated Figure 3.1, which also shows the dates of sampling. Samplings were done in São Paulo and Goiás states, in collaboration with the Universidade Federal de Goiás (Prof. Virgínia F. Alves). For the present thesis, the daires were named as A, B and C according to Alves et al. (2018). It is important to point out the plant A had been previously designated as Dairy 2 in the paper by Dittmann et al. 2017. The daires A, B and C were all located in the state of Goiás. They were artisanal processing plants and produced ca. 20-50 pieces of Minas Frescal cheese per day, prepared from 150–250 liters of bovine milk, from single herds raised *in situ*. All cheeses from dairies A, B and C were produced from unpasteurized milk, with commercial rennet. Dairies D and E were both located in the state of São Paulo. Plant D processed ca. 900 l of pasteurized buffalo milk a day, to produce a variety of cheeses. Plant E was also an artisanal dairy, which produced Minas Frescal cheese from raw bovine milk by processing ca. 50 l per day, once a week. Depending on the availability at the processing plant, samples of different categories were acquired, as follows: raw material (e.g., raw milk, curd, whey, brine, n = 22), food contact surfaces (e.g., milk transport gallons, buckets, processing tanks, jar, freezer, cloth mould, n = 36), non-food contact surfaces (floor, sink and freezer, n = 8) and final product (ready-to-eat cheeses, n = 31). The samples were processed for bacterial isolation as described in the next section. Dairies A, B and C were sampled by partners at Universidade Federal de Goiás for isolation of *S. aureus* (Alves et al., 2018) and background microbiota. Samples from dairies D and E were analyzed from the beginning at FCFRP-USP, for presumptive identification of *S. aureus* and isolation of background microbiota. The molecular identification of all isolates was performed by this author at FCFRP-USP. The samples positive for the pathogen tested are described in more detail in the Tables 3.1, 3.2, 3.3 and 3.4.

Figure 3.1. Flowchart indicating the dairies where samples were collected and the dates of sampling.



3.2.2 Bacterial isolation

The samples were analyzed for Presence or Absence of *Listeria monocytogenes* based on Hitchins and Jinneman (2011), with modifications. Primary enrichment was done with *Listeria* Enrichment Broth (UVM1 formulation), and seletive secondary enrichment was done with Fraser broth. Blackening of Fraser broth was considered a presumptive positive result for the presence of *Listeria* sp., and isolation followed by plating on agars Oxford, agar PALCAM and/or agar ALOA (Agar Listeria Ottaviani & Agosti), which is a chromogenic medium for phenotypic differentiation of *L. monocytogenes*.

Isolation of *Staphylococcus aureus* was done based on Dittmann et al. (2017) by plating on Baird Parker agar, and presumptive identification was carried out by Gram staining, catalase test and at least on isolate from each plate was tested for coagulase (Coagu-plasma, Laborclin, Brazil) and/or for clumping test (Staphiclin, Laborclin, Brazil), according to the manufacturer's instructions.

Background microbiota was isolated according to Bagge-Ravn et al. (2003), with modifications. For that, samples were diluted and spread plated on Brain Heart

Infusion (BHI) agar and de Man, Rogosa, Sharpe (MRS) agar. At least three colonies from each selected plate were purified for presumptive identification of isolates, based on Gram staining and catalase test.

All isolates presumptively identified by phenotypic tests were kept frozen at -80°C, in Brain Heart Infusion (BHI) broth (Oxoid, UK) with 20% glycerol.

The molecular identification of the isolates was done by DNA extraction and 16S rRNA gene sequencing, according to Tulini (2014), as described in the next section.

All culture media for bacterial isolation were acquired from Oxoid (Basingstoke, UK), unless otherwise noted.

3.2.3 DNA extraction and 16S RNA gene sequencing by Sanger method

Working cultures were prepared from frozen stocks in Brain Heart Infusion broth (37ºC/24h) and streaked on Brain Heart Infusion agar plates (37ºC/24h). A single colony of each isolate was inoculated in BHI broth (37°C, 24 h) and a 1 ml aliquot was collected for genomic DNA extraction using the Illustra™ bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK), following the instructions of the manufacturer. DNA extract was checked for purity by spectrophotometry (Nanodrop 2000c, Thermo Fisher, USA) and used as template for PCR amplification of the 16S rRNA genes with the universal primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'GCTTACCTTGTTACGACTT3'), based on Lane (1991) and Turner et al. (1999). According to Tulini (2014), the PCR reactions (50 µL) were prepared with 84 µL of the Taq Platinum® Blue PCR SuperMix (Invitrogen) 30 *p*mol of each universal primer (Invitrogen) and 300 ng of genomic DNA. Amplification was performed on thermocycler (model MG96+, LongGene®, China) programmed for 30 cycles, with denaturation at 94°C/1 minute, annealing at 55°C/1 minute and extension at 72°C/2 minutes. To confirm the amplification of target DNA regions, amplicons were analyzed by agarose gel electrophoresis (SANDERSON et al., 2014). Next, the amplicons were purified with the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and sequencing was performed by automated Sanger method with the 3500 Genetic Analyzer (ThermoFisher Scientific, USA) available at the facility for nucleic acid sequencing of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Brazil. Sequences generated were analyzed using

FinchTV software (Geospiza Inc., https://digitalworldbiology.com/FinchTV) and the alignment of the DNA sequences, composed of high-quality regions, selected through the FinchTV software, was carried out using the software Basic Local Alignment Search Tool (BLASTn - https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the NCBI. The isolates were classified at the species level based on the data resulting from the alignment that presented the highest score of similarity, identity and coverage with the taxon output from the database.

3.3 RESULTS AND DISCUSSION

Listeria monocytogenes was not isolated from the samples analyzed in this study, in a total of 97 samples, from five dairy plants from Goiás and São Paulo states, during the years of 2014 and 2015. We also make note that no *Listeria* monocytogenes was isolated either, which could be an indicator for the presence of other listeria species (Liu et al., 2009). *L. monocytogenes* has been highly associated with dairy products, especially soft cheeses (SWAMINATHAN; GERNER-SMIDT, 2007) and the isolation rates from this kind of product vary from 0 to 41.4%, as indicated by Oxaran et al. (2017). In fact, our results are good agreement with Oxaran et al. (2017), who reported a very low isolation rate for *L. monocytogenes* from dairy samples previously collected from the same Brazilian regions (Goiás and São Paulo). Those authors reported that out of 421 samples from dairy plants analyzed, a single one was positive for *L. monocytogenes*, which was isolated from the processing environment (drain). These findings point towards low contamination rates by *L. monocytogenes* in Brazilian dairies in recent years, which might in part be attributed to implementation of regulatory practices to improve sanitation.

In the other hand, all the dairies included in this study presented high contamination rate by *Staphylococcus aureus*. The isolates were presumptively assigned by phenotypic tests and their identities were later confirmed by DNA sequencing (Tables 3.1, 3.2, 3.3. and 3.4). The results for *S. aureus* contamination were partially reported by this author, in co-authorship with Alves et al. (2018). Overall, the present data show per category of sample, the occurrence rates of *S. aureus* were 45% for raw materials (10 out 22 samples), 38.9% for food contact surfaces (14 out of 36 samples), 37.5% for non-food contact surfaces (3 out of 8 samples) and 29% for final products (9 out of 31). Besides *S. aureus*, other closely related bacteria were also

isolated: *Staphylococcus agnetis* and *Staphylococcus chromog'enes* (dairy plant C) and *Macrococcus caseolyticus* (dairy plant A).

Selected *S. aureus* isolates reported in this thesis (designated with the code "SABRC", in Tables 3.1, 3.2. and 3.3 from dairy plants A, B and C with GenBank accession numbers from MF158055, MF158084 and from MF423380 to MF423415 were also analyzed by the present author, in collaboration with Alves et al. (2018), by using Multi Locus Sequence Typing (MLST), was performed trough the analysis of the DNA sequences of seven conserved housekeeping genes including arcC (carbamate kinase), aroE (shikimate dehydrogenase), glpF (glycerol kinase), gmk (guanylate kinase), pta (phosphate acetyltransferase), tpi (triosephosphate isomerase) and yqi (acetyl coenzyme A acetyltransferase) amplified using specific primersand and the test to antimicrobial susceptibility was done with penicillin 10 U (PEN); cefoxitin 30 mg (CFO); gentamicin 10 mg (GEN); erythromycin 15 mg (ERM); tetracycline 30 mg (TET); ciprofloxacin 5 mg (CIP); clindamycin 2 mg (CLIN); trimethoprime sulfamethoxazole 1.25/23.75 mg (SXT) and chloramphenicol 30 mg (CLO), performed by disk diffusion (Sensifar, Brazil). Those strains were assigned to five known sequence types (ST), namely ST1, ST97, ST126, ST3531, in addition to the newly described ST3816. According to alves et al. (2018), the clonal complex 1 (CC1) was dominant and there was one methicillin resistant strain (CC126), which could represent a public health concern. It has also been recently reported MacFadyen et al. (2019) that *Macrococcus* may present genes associated to resistance of methicillin.

For dairy plants A and B, Lactic Acid Bacteria (LAB) were co-isolated with *S. aureus* in 14 out of 19 samples, and most isolates were enterococci, to know: *Enterococcus casseliflavus* (12 isolates), *Enterococcus faecium* (3 isolates), *Enterococcus galinarum* (1 isolate). The other LAB detected were *Lactococcus lactis* and *Lactococcus garviae* (unpasteurized milk, plant A) plus *Carnobacterium maltaromaticum* (refrigerated milk storage tank, plant B). The co-ocurrence of LAB with *S. aureus* was lower in dairy plant C, with only 2 isolates of *Lactococcus garviae* from one sample of Minas cheese (surface), out of 15 positive samples for *S. aureus*. *Leuconostoc mesenteroides* and *Streptococcus salivarius* were co-isolated from a *S. aureus* positive sample of packed mozzarella ball and no LAB was isolated from *S. aureus* background microbiota of dairy E. Among the LAB of food importance, enterococci are the most controversial members, mainly due to their capability of

transferring antibiotic resistance genes and to likely indicate of fecal contamination, depending on the food. In contrast, enterococci may also present beneficial aspects, such as production of antimicrobial peptides and other desirable technological and health-related traits (FRANZ et al., 1999). Enterococci are able to survive in food because of their ability to withstand high temperatures, making them hard to be eliminated by mild heat processing (CHAJECKA-WIERZCHOWSKA et al., 2016). In particular, *Enterococcus casseliflavus* and *Enterococcus faecium*, are commonly found in dairy samples (HWANHLEM et al, 2017; PERIN et al., 2017).

Carnobacterium maltromaticum (2,38%) is a lactic acid bacterium that can grow in milk samples and can inhibit the growth of microorganisms such as *Listeria monocytogenes* because its capacity of producing bacteriocins (AFZAL et al., 2010).

Lactococcus garvieae (7,13% found on dairy A and C) is a bacterium associated with severe infections in humans and animals, and can cause several diseases such as endocarditis, liver abscesses and others (CHAO et al., 2013), *Staphylococcus chromogenes* (2,38%) is a bacterium associated with mastitis in cattle but is not pathogenic in humans (SANTOS et al., 2016).

Leuconostoc mesenteroides (4,76%) belonging to lactic bacteria and very common in food factories and fermentative processes (D'ANGELO et al., 2017); *Streptococcus salivarius* (2,38%) is a bacterium that has anti-inflammatory properties and are the first colonizers of the human oral cavity and are very common in fermented processes such as ethanol and dairy (KACI et al., 2014).

In the present research, other less usual Gram-positive bacteria were also isolated: *Bacillus aryabhattai* (dairy plant A, from Minas cheese) and *Kocuria* sp. (dairy plant C, from a freezer). *B. aryabhattai* has been reported to significantly promote the growth of soybean (Park et al., 2017) but, to the best of our knowledge, its association with ready-to-eat food has not been reported. *Kocuria* is a genus of strictly aerobic bacteria that has been reported as a relatively common contaminant in breweries (SUZUKI, 2015) but it has been recently associated with opportunistic human infections (PURTY et al., 2013).

The only Gram-negative bacteria co-isolated with *S. aureus* in this study was *Serratia nematodiphila* (from raw milk, dairy plant A). *Serratia* sp. belongs to the *Enterobacterales* order regarded mainly as a spoilage organism in food but it can also carry antibiotic resistance genes. *S. nematodiphila* has been recently reported to to

have symbiotic-pathogenic life cycle, on the multilateral relationships with an entomopathogenic nematode but its significance is still not fully elucidated (KWAK et al., 2015).

To highlight the importance of this data to plan for food safety strategies, it is very interesting to emphasize the paper recently published by Oxaran et al. (2018) on the influence of the background microbiota in the survival of the pathogens *L. monocytogenes* and *S. aureus*. Those authors reported specific members or associations in mixed-species biofilm can protect *L. monocytogenes* and *S. aureus* from biocide action. These findings indicate the isolates from the present investigation provide a very interesting culture collection for further studies to improve the safety of dairy food.

Table 3.1. Number and/or designation of *Staphylococcus aureus* isolates from Dairy Plant A and the respective accompayining microbiota *

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Sampling site	Number of isolates (n) **	Accompanying microbiota (n)
Minas cheese (surface) freshly prepared	*S. aureus* (1)	*Enterococcus casseliflavus* (2)
Minas cheese 1 (surface)	*S. aureus* (3)	*Enterococcus casseliflavus* (1)
Minas cheese 2 (surface)	*S. aureus* (3)	*Bacillus aryabhattai* (1) *Enterococcus casseliflavus* (1)
Minas cheese 3 (surface)	*S. aureus* (2)	*Macrococcus caseolyticus* (1)
Minas cheese 1	*S. aureus* (3)	*Enterococcus casseliflavus* (1)
Minas cheese 2	*S. aureus* SABRC1	*Enterococcus faecium* (1) *Enterococcus casseliflavus* (1)
Minas cheese 3	*S. aureus* (3)	*Enterococcus faecium* (1)
Minas cheese 4	*S. aureus* SABRC11 *S. aureus* SABRC12	*Enterococcus casseliflavus* (1)
Raw milk	*S. aureus* SABRC2 *S. aureus* (2)	*Serratia nematodiphila* (1) *Enterococcus casseliflavus* (1)

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Unpasteurized milk	*S. aureus* (1)	*Lactococcus lactis* (1) *Lactococcus garvieae* (1)
Cloth mold	*S. aureus* SABRC3 *S. aureus* SABRC4	*Enterococcus casseliflavus* (1)
Sink near to area of cheese production	*S. aureus* SABRC5 *S. aureus* SABRC6 *S. aureus* SABRC7	No growth
Processing tank	*S. aureus* SABRC8	*Enterococcus casseliflavus* (2) *Enterococcus gallinarum* (1)
Curd basket	*S. aureus* SABRC9 *S. aureus* SABRC10	*Enterococcus casseliflavus* (1)
Minas cheese 4 (surface)	*S. aureus* (1)	*Macrococcus caseolyticus* (1)

* (n) = number of isolates
** The isolates designated with the code “SABRC” were analyzed by MLST (Multi locus Sequence Typing), as presented by Alves et al. (2018).

Table 3.2 - Number and/or designation of *Staphylococcus aureus* isolates from Dairy Plant B and the respective accompanying microbiota *

Sample	Number of isolates (n) **	Accompanying microbiota (n)
Brine	*S. aureus* SABRC13 *S. aureus* SABRC14 *S. aureus* SABRC15	No growth
Curd	*S. aureus* SABRC17 *S. aureus* SABRC18 *S. aureus* (1)	*S. aureus* (1)
Processing tank	*S.aureus* SABRC19 *S. aureus* (1)	*S. aureus* (1) *Enterococcus faecium* (1)
Milk storage tank 5°C	*S. aureus* SABRC20	*S. aureus* (1) *Carnobacterium maltaromaticum* (1)

*The strains designated with the code "SABRC" were analyzed by MLST (Multi locus Sequence Typing) in co-authorship and the results are presented by Alves et al. (2018).

(n)**Number of isolates found in each sample.

Table 3.3 - Number and/or designation of *Staphylococcus aureus* isolates from Dairy Plant C and the respective accompanying microbiota *

Sample	Number of isolates (n) **	Accompanying microbiota (n)
Freezer	*S. aureus* (1)	*Kocuria* sp (1)
Unpasteurized milk	*S. aureus* (1) *S. agnetis* (1)	No growth
Raw milk 2	*S. aureus* (1)	*Staphylococcus agnetis* (1)
Minas cheese 1 (surface)	*S. aureus* SABRC24	No growth
Minas cheese 1	*S. aureus* SABRC21 *S. aureus* SABRC22	No growth
Minas cheese 2 (surface)	*S. aureus* SABRC30 *S. aureus* (2)	*Lactococcus garvieae* (2) *S. aureus* (1)
Minas cheese 2	*S. aureus* (3)	*Staphylococcus agnetis* (1)
Milk gallon	*S. aureus* (1) *S. aureus* SABRC27	*S. aureus*
Sink 1	*S. aureus* (2)	No growth
Raw milk 2	*S. aureus* (2)	No growth
Whey	*S. aureus* SABRC25 *S. agnetis* (1)	*Staphylococcus chromogenes* (1)
Jar for transferring raw milk	*S. aureus* (1)	*Staphylococcus agnetis* (1)
Whey + curd	*S. aureus* SABRC29	*S. aureus* (1)
Minas cheese 3 (surface)	*S. aureus* (1)	No growth
Minas cheese 3	*S. aureus* SABRC26 *S. aureus* (2)	*Staphylococcus agnetis* (1) *Staphylococcus aureus* (1)

*The strains designated with the code “SABRC” were analyzed by MLST (Multi locus Sequence Typing) in co-authorship and the results are presented by Alves et al. (2018).
(n)**Number of isolates found in each sample.

Table 3.4 - Number of *Staphylococcus aureus* isolates from Dairy Plants D and E, with the respective accompanying microbiota

Sample	Number of isolates (n)	Accompanying microbiota (n)
Whey (tray)	*S. aureus* (4)	*Staphylococcus agnetis* (1)
Packed mozzarella ball	*S. aureus* (1)	*Leuconostoc mesenteroides* (2) *Streptococcus salivarius* (1)

Listeria monocytogenes was absent in the 97 samples from five dairies from São Paulo and Goiás states analyzed in this study.

Staphylococcus aureus was detected in all the five dairies studied, indicating a need for a better sanitary control to avoid staphylococcal food poisoning.

The accompanying microbiota of *S. aureus* was composed mainly by LAB, with emphasis on enterococci, but other less usual bacterial groups were also present.

4 Metataxonomics unravels the accompanying microbiota of in a Brazilian dairy

ABSTRACT

Metataxonomics unravels the accompanying microbiota in a Brazilian dairy

The microbiota of dairy food and processing plants are very important to determine the survival of pathogens and it can also impact directly on food safety. For example, *S. aureus* can be protected from biocides, depending on specific members in association. In this research, the microbiota of Brazilian dairy samples was analysed by community 16S rRNA gene sequencing. For that, samples were combined in four groups (raw material, final product, food-contact and non-food contact surfaces), and the results showed high alpha-diversity indexes (final product and non-food contact surfaces) but, beta-diversity indexes were low. The samples were separated in two main clusters and the bacterial community was dominated by *Macrococcus, Alkaliphilus, Vagococcus, Lactobacillus, Marinilactibacillus, Streptococcus, Lysinibacillus, Staphylococcus, Clostridium, Halomonas, Lactococcus, Enterococcus, Bacillus* and *Psychrobacter*. These results highlight rare taxa co-occur with *S. aureus* in dairies and may further aid in developing strategies for food protection.

Key-words: cheese microbiome, Brazilian dairy microbiota, cheese metataxonomics.

4.1 INTRODUCTION

4.1.1 MILK AND DAIRY

Milk and dairy products are consumed worldwide daily and represent a vital source for human nutrition, with key economic importance in industrialized as well in developing countries. However, the nutrient richness of milk and dairy products also offer an ideal growth environment for many microorganisms, including spoilage and pathogenic bacteria. Dairy contamination by the enterotoxigenic bacterium Staphylococcus aureus is of great concern and it may enter the food chain due to mastitis in dairy herds, poor milking conditions and lack in good manufacturing and hygiene practices (KENNY, 2013).

Considering that Brazil is an important producer and consumer of milk and dairy products (USDA, 2017), there is an increased scientific interest in determining the microbiota and prevalence of pathogens in Brazilian dairies, as reported recently by several authors (ALVES et al., 2018; CAMPAGNOLLO et al., 2018; DITTMANN et al., 2017; GOMES et al., 2013; LUIZ et al., 2017; OXARAN et al., 2017; SILVA et al., 2015). However, it is noteworthy there are scarce publications on the molecular profiling of the autochthonous microbial communities from Brazilian milk, dairy plants and products. Oxaran et al. (2018) highlighted the importance of determining the composition of mixed microbial communities from dairies to guarantee food safety, by demonstrating the survival of S. aureus may be favored by particular members or their associations in biofilms.

To gain more insight into the structure of microbial communities, High-Throughput Sequencing (HTS) of DNA is a very powerful tool to generate data with high resolution, in a metagenomic approach (ERCOLINI, 2013; ESCOBAR-ZEPEDA et al., 2016). Metagenomics refers to the theoretical collection of genes and genomes from the members of a given microbiota obtained by shotgun DNA sequencing of the DNA from a sample. On the other hand, metataxonomics is a HTS culture-independent technique based on the amplification of specific regions of the 16S rRNA gene of a given microbial community (MARCHESI; RAVEL, 2015).

The community 16S rRNA gene sequencing allows to quantify the proportion of taxa present in a given food sample and to monitor changes in the microbial community profile (ERCOLINI, 2013; KABLE et al., 2016). For metataxonomics, the total nucleic

acid content is extracted directly from the food matrix, cleaned up, amplified with 16S rRNA specific primers, tagged and sequenced to compare with reference databases to determine sequences correlation with Operational Taxonomic Units (OTUs). Thus, the number of matches between sequences and OTUs is calculated and a quantification of the occurrence of each OTU per sample is obtained (ERCOLINI, 2013).

Some authors have successfully investigated the microbiota of milk and dairy food using HTS (DE FILLIPIS et al., 2014; DE PASQUALE et al., 2014; RIQUELME et al., 2015; CALASSO et al., 2016; DALMASSO et al., 2016; KABLE et al., 2016; KAMIMURA et al., 2019) but additional investigation is necessary for a more complete comprehension of these intricate microbial interactions. In particular, to guarantee the safety and quality of dairy products, it is important to evaluate the microbiota at several steps, from farm to industry, because it is known that the core microbiota may be remarkable influenced by the processing facility (KABLE et al., 2016).

Based on this scenario, our research hypothesis is that previously overlooked background microbiota members should be considered for a better evaluation of the food safety and pathogen survival in dairy food and environmentsTo reach this goal, samples of raw material, final products, food-contact and non-food contact surfaces from a Brazilian dairy plant (DITTMANN et al., 2017), were submitted to Next-Generation DNA Sequencing followed by metataxonomics analysis to determine the culture-independent microbiota and, the possible implications of the results for food safety and quality are discussed.

4.2 MATERIALS AND METHODS

4.2.1 Sampling

In this study, 90 selected samples that had been previously studied by Dittmann et al (2017) were analyzed. More specifically, the samples were previously collected at dairy plant 5, located in São Paulo state, in 2014, as shown in Table 1. Aliquots of 5mL of the selected samples homogenized in peptone (0.1% w/v) salt (0.85% w/v) water were kept frozen and stored at -80^{o}C to be analyzed by High Throughput Sequencing of metagenomic 16S rRNA.

Table 4.1. Number of samples separated per category belonging to raw material, final product, food-contact surfaces and non-food contact surfaces obtained in a Brazilian Dairy (São Paulo state 5)

Sampling date	Number of samples per category				Total
	Raw material	Final product	Food-contact surfaces	Non-food contact surfaces	
January 2014	3	3	7	6	19
February 2014	3	9	3	4	19
March 2014	3	13	10	4	30
July 2014	3	4	7	8	22
Total	12	29	27	22	90

4.2.2 DNA extraction and HTS of dairy and environmental samples

For HTS, the 90 samples were grouped and pooled into four categories, to achieve at least 5 ng/µl of total DNA for 16S Metagenomic Sequencing Library Preparation, according to Illumina® (San Diego, USA). Table 4.1 shows the number of samples included in each category, comprising 12 raw material (group A - raw milk, pasteurized milk, brine), 29 final products (group B - soft and semi-hard cheeses), 27 food-contact surfaces (group C - shelves and worker gloves) and 22 non-food contact surfaces (group D - sink, drain, floors and staff's boot).

Total genomic DNA was extracted from pooled samples using the PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, USA), following the protocol recommended by the manufacturer.

Sequencing was performed on a MiSeq system (Illumina®, San Diego, USA) at the facility for nucleic acid sequencing of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Brazil.

For that, PCR was performed to amplify the V3-V4 regions of 16S rRNA genes with specific primers (forward=5´CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW

GCG3'; reverse=5´ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGTATCTAATCC 3') with overhang adapters attached. 16S rRNA gene amplicons were prepared according to instructions of the manufacturer by performing a first stage PCR, followed by a purification step using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, USA) to remove free primers and primers dimers. A second stage PCR was performed to attach dual indices and Illumina sequencing adapters, using the Nextera XT Index kit® (Illumina®). The inserts were further purified with Agencourt AMPure XP beads (Beckman Coulter), the libraries were quantified by real-time PCR (Kapa HiFi Hot Start ready Mix, Kapa Biosystems, Wilmington, USA), normalized and pooled, according to instructions of the 16S Metagenomic Sequencing Library Preparation guide from Illumina®. Next, the libraries were denaturated with sodium hydroxide and samples were loaded onto MiSeq flow cell using the MiSeq reagent kit V3 (600 cycles). Each run included a minimum of 5% of PhiX control kit v3 (Illumina®) for internal control of low diversity libraries, preventing the run to fail. For HTS, there were four categories of samples: raw material (group A, which included raw milk, pasteurized milk, brine), final product (group B, that comprised soft and semi-hard cheeses), product-contact surfaces (group C, which included shelf and worker gloves) and non-food contact surfaces (group D, which included sink, drain, floors and staff's boot). These samples were pooled in order to achieve the minimum DNA concentration of 5 ng/µl for running the sequencing, according to the 16S Metagenomic Sequencing Library Preparation from Illumina®.

4.2.3 Bioinformatics and statistical analysis pipeline

The raw reads generated from the sequencing on Illumina® MiSeq platform were quality-assessed, trimmed and taxonomically annotated using the BaseSpace Illumina® cloud bioinformatics pipeline.

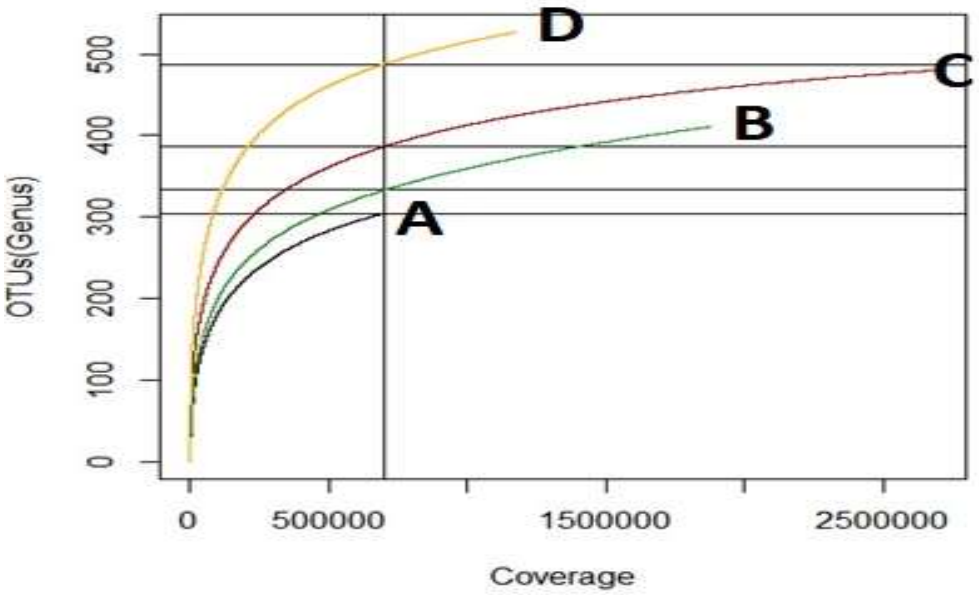
The statistical and bioinformatics downstream analyses were performed in R platform using as input the OTU table with reads counts by sample (see Supplementary Material). To draw a rarefaction curve we employed the Vegan R package (OKSANEN et al., 2018). The heatmap related to the general abundance was plotted using phyloseq R package (MCMURDIE; HOLMES, 2013), an open-source package to analyze microbiome data. The Top10 OTUs heatmap was plotted based on Bray-

Curtis dissimilarity distance. The microbiome R package (LAHTI et al., 2018) was used to determine the core microbiota composition, the Shannon's and the Chao1 indexes using the archive of OTUs counts.

4.3 RESULTS

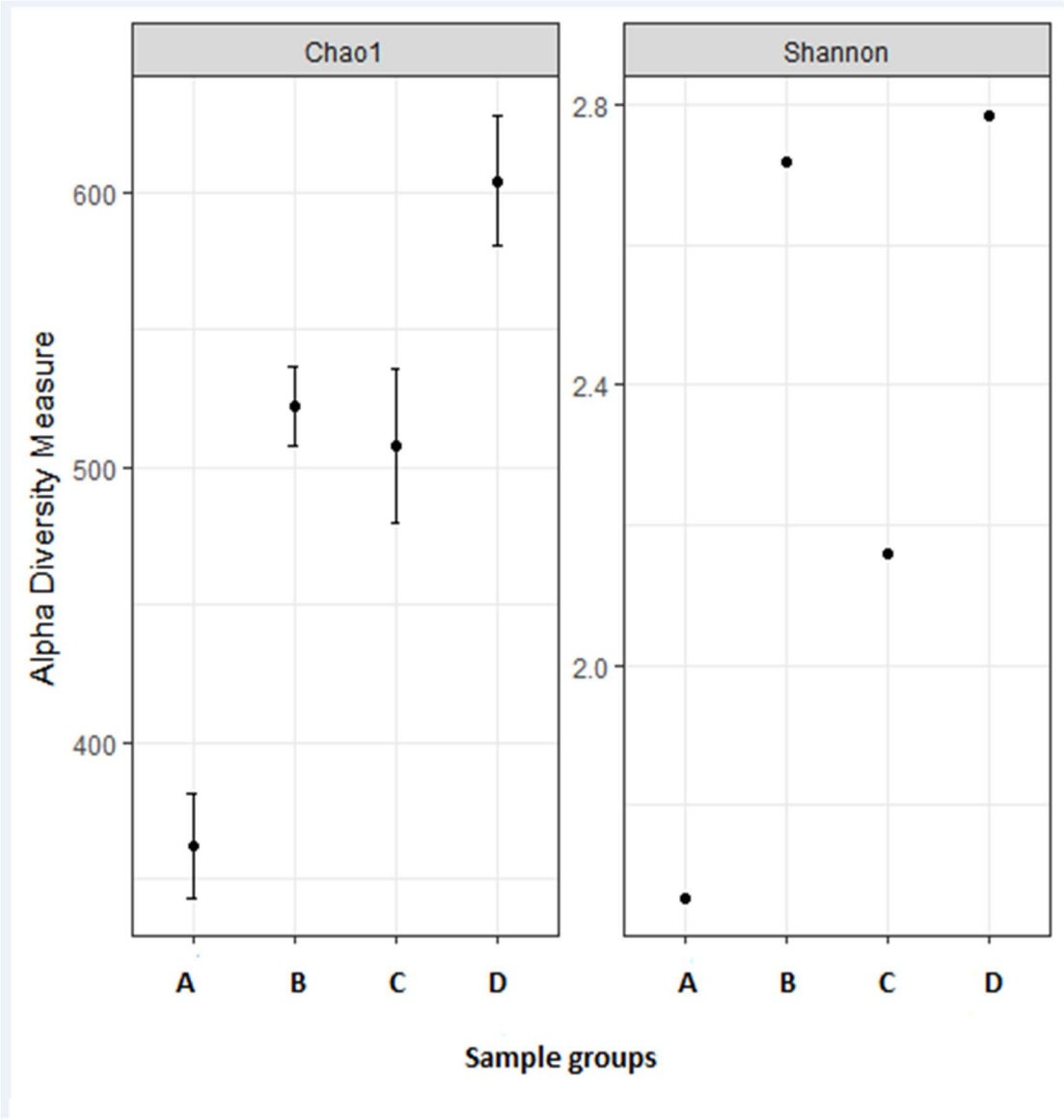
The number of reads generated in HTS was plotted *versus* the sequencing coverage to draw a rarefaction curve, in order to determine that the optimal sequencing depth was reached, as shown in Figure 4.1.

Figure 4.1 - Rarefaction curves showing the sequencing coverage from Illumina® MiSeq 16S rDNA metataxonomics results for samples of raw material (A), final product (B), food-contact surfaces (C) and non-food contact surfaces (D) from a Brazilian Dairy (São Paulo state 5). The mean sequencing coverage was 200,000 and all samples reads were well sequenced, as indicated by the plateaus.



The α-diversity indicates the richness and uniformity within a community and the β-diversity is the variation of the composition of microbial diversity among communities (ALMEIDA; DE MARTINIS, 2018). The alpha-diversity was calculated by using the Shannon and Chao1 indexes (Figure 4.2), which indicate the groups B (final product) and D (non-food contact surface) were the richest and most diverse ones.

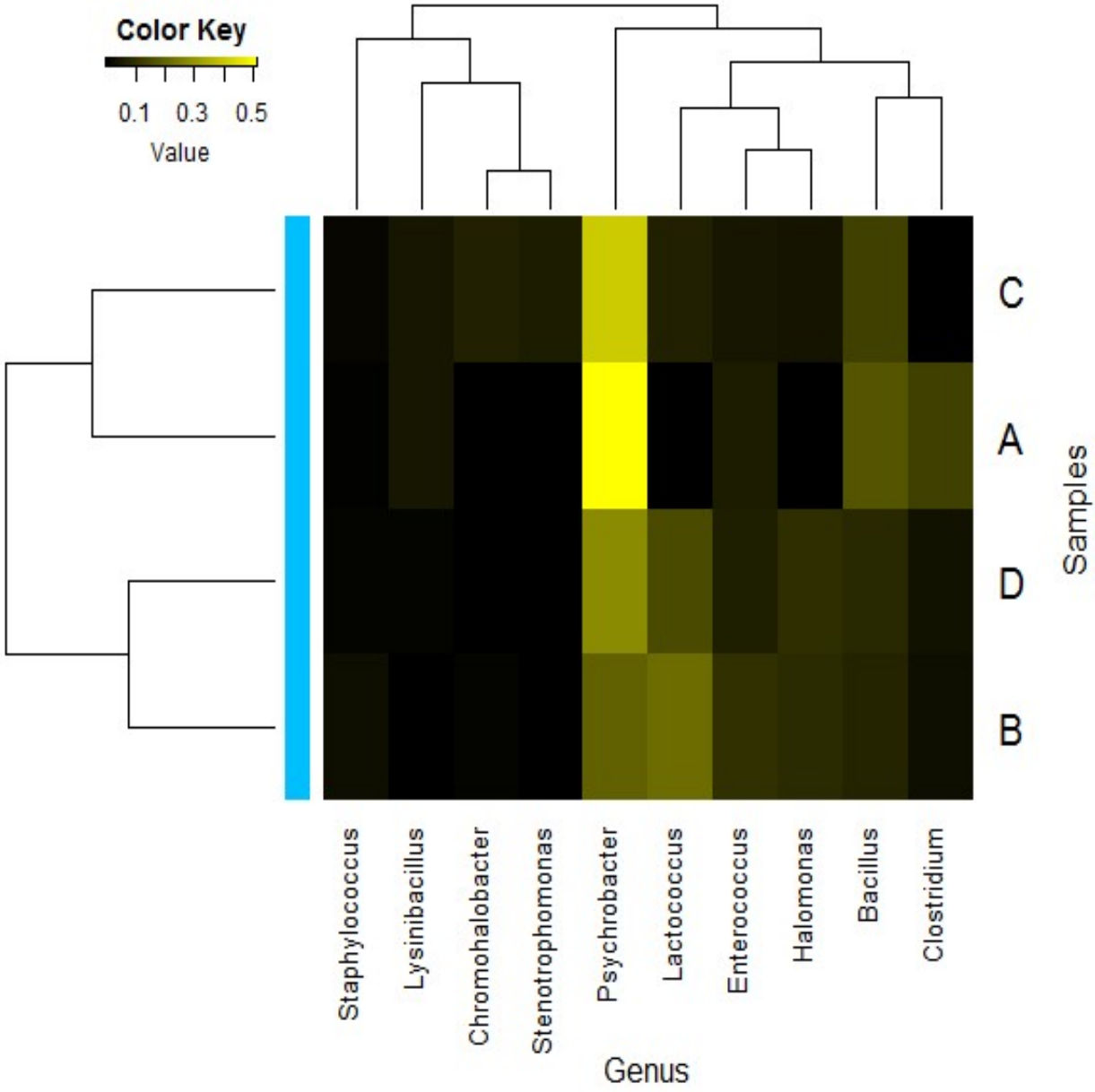
Figure 4.2 - Chao1 and Shannon diversity indexes calculated from Illumina® MiSeq 16S rDNA metataxonomics results for samples of raw material (A), final product (B), food-contact surfaces (C) and non-food contact surfaces (D) from a Brazilian Dairy (São Paulo state 5).



The beta-diversity was compared by clustering studies and the results are shown in a heatmap (Figure 4.3). For this cluster analysis we cut-off the taxa that were lower than 1% in relative abundance and many OTUs were deleted from the heatmap, which indicate that our samples have low diversity. According to Figure 4.3, there are two main clusters were formed by the group of samples A (raw material) – C (food contact surface) and by B (final product) - D (non-food contact surface). The abundance of

reads from the genera *Psychrobacter* and *Bacillus* join the groups A and C, which are separated respectively by *Clostridium* and *Lactococcus*. The groups B and D are held together by the genera *Psychrobacter* and *Lactococcus* but they are split by the high levels of *Enterococcus* reads in the final product.

Figure 4.3 - The Top10 OTUs clustered according to its abundances, obtained with Illumina® MiSeq 16S rDNA sequencing for samples of raw material (A), final product (B), food-contact surfaces (C) and non-food contact surfaces (D) from a Brazilian Dairy (São Paulo state 5).



These results are corroborated by Figure 4.4, which shows the total read counts, irrespective of the abundance, for each sample group. Despite the great number of reads present in the samples, the majority of OTUs reads are not key to represent the

bacterial diversity of the core in-house identity, which is determined based on the prevalence of taxa among samples.

Figure 4.4 - Abundance of 16S rDNA reads by sample groups of raw material, final product, food-contact surfaces and non-food contact surfaces from a Brazilian Dairy (São Paulo state 5), according to results of Illumina® MiSeq system.

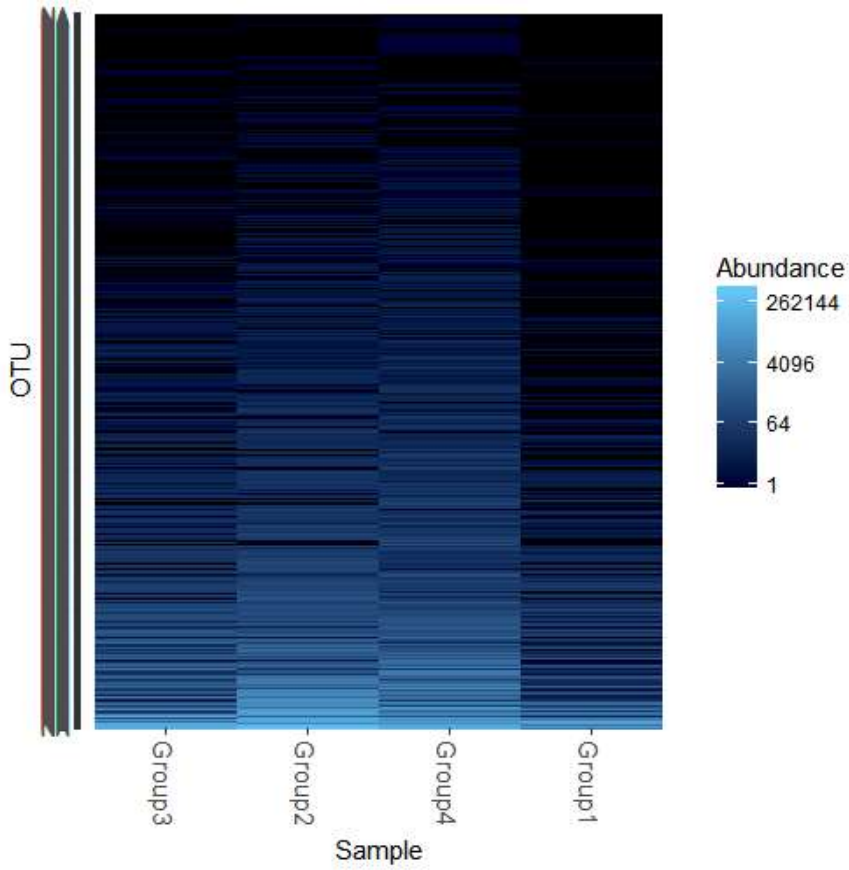
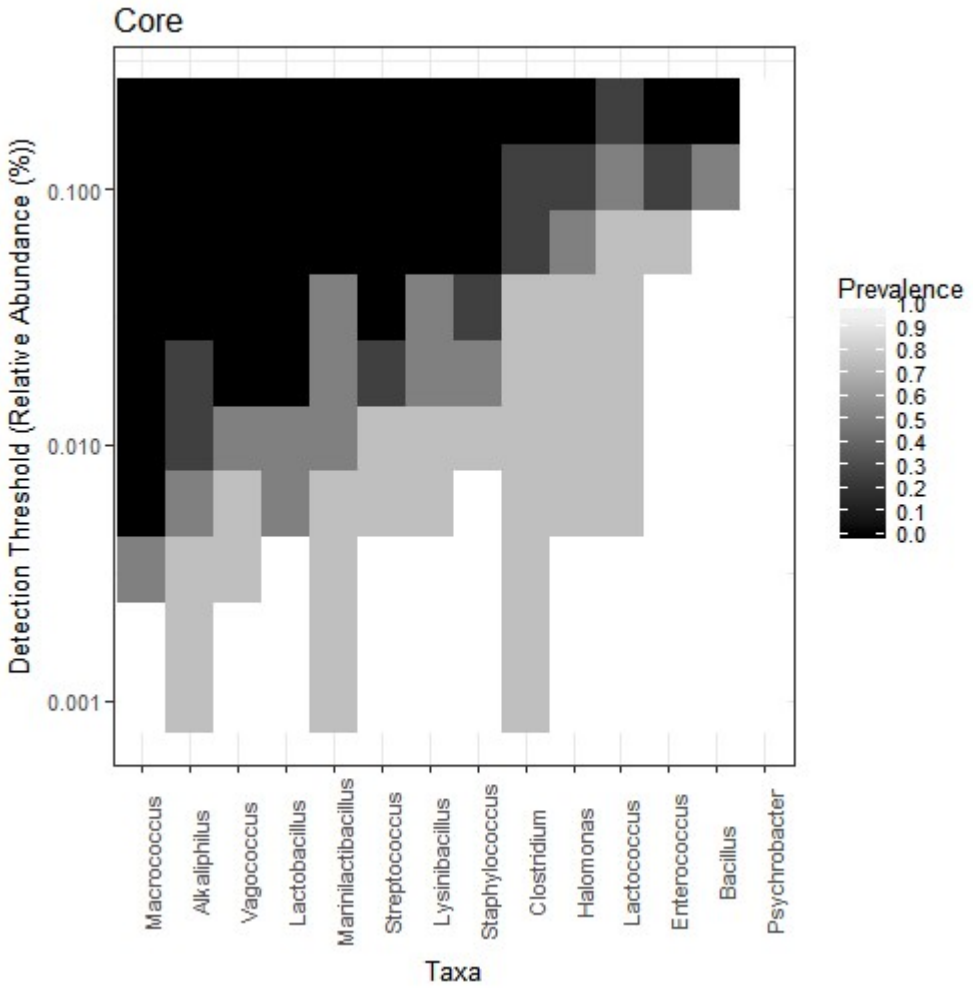


Figure 4.5 shows hierarchically the OTUs that compose the core microbiome. The bacterial community was dominated, in a crescent order, by *Macrococcus*, *Alkaliphilus*, *Vagococcus*, *Lactobacillus*, *Marinilactibacillus*, *Streptococcus*, *Lysinibacillus*, *Staphylococcus*, *Clostridium*, *Halomonas*, *Lactococcus*, *Enterococcus*, *Bacillus* and *Psychrobacter*.

Figure 4.5 - The Core Microbiome for samples analysed with Illumina® MiSeq system for 16S rDNA, according to groups of raw material (A), final product (B), food-contact surfaces (C) and non-food contact surfaces (D) from a Brazilian Dairy (São Paulo state 5)



.

This study contributes to determine the mixed microbial communities from Brazilian dairies. With regard to quality control parameters of DNA sequencing by NGS, the plateaus reached for all the sample groups shown in the rarefaction curves (Figure 4.1) indicate that the whole theoretical bacterial diversity was covered. This data is important because it assures the quality of the metataxonomics downstream analysis, besides being useful to determine the optimal sequencing depth for future studies on dairy microbiome.

To describe the total diversity of microbial communities in different samples, the Shannon's index is very useful because it represents a combined measurement of the richness and abundance (MORRIS et al., 2014; DELGADO-BAQUERIZO et al.,

2016). Shannon's index increases with diversity and uniformity in samples, but it decreases as a result of the presence of rare OTUs in highly disproportional rates, such as singleton reads (HILL et al., 2006). On the other hand, Chao1 calculates the minimal theoretical number of OTUs present in a sample (LEMOS et al., 2011; HAEGEMAN et al., 2013). The estimation of the α-diversity using these two mathematical approaches reinforces the coverage of total diversity and indicates the richest and most diverse samples. In this research, the higher diversity was observed for final product and non-food contact surfaces (Figure 4.2). In food processing environments, it is possible to envisage multiple points of cross contamination due to handling, hygienic conditions and flow from raw to final products. Jonnala et al. (2018) compiled factors influencing the increase or decrease of several bacterial genera in cheeses as determined by HTS, pointing out that the abundance of OTUs of several lactic acid bacteria and enterobacteria vary with the addition of herbs and salt, as well as it may be influenced by a myriad of factors such as temperature, pH, moisture, contact with wooden surfaces, smoking and production practices.

With regard to the bacterial genera found in this study, in Figure 4.3 it is shown there is one cluster formed by the grouping of raw material with food contact surface. Psychrobacter and Bacillus were very abundant in raw material and food contact surfaces. The relevance of *Psychrobacter* sp. is not very well described for cheeses, although Deetae et al. (2007) observed that *Psychrobacter* sp. is important due to its potential for producing cheesy aroma notes, such as volatile sulphur compounds. This hypothesis was later confirmed by Deetae et al. (2008) during ripening in a cheese model medium by demonstrating the production of sulphur and non-sulphur volatile compounds as well as the caseinolytic activity of *Psychrobacter* sp*.* The synthesis of volatile flavour components, aminoacids and fatty acids of importance for cheese aroma is also a result of *Bacillus* metabolic activity, although this genus may be of particular concern for the dairy industry as a spoilage spore-former that is able to survive pasteurization and to form biofilms in pipes and equipment (GOPAL et al., 2015; JONNALA et al., 2018).

Figure 4.3 also revealed higher abundance of *Clostridium* sp. in raw material compared to food contact surfaces, which instead, presented high number of *Lactococcus* sp. reads. According to Quigley et al. (2013), *Clostridium* sp. has been associated with raw milk and it is important as a potential spoilage microorganism.

Diversely, *Lactococcus* sp. is a common member of the microbiota of dairy products associated with technological and health promotion aspects.

The other cluster seen in Figure 4.3 refers to final product and non-food contact surface, which had in common the genera *Psychrobacter* and *Lactococcus*, but they were separated due to the detection of high levels of Enterococcus reads in the final product only. Gomes et al. (2008) conducted an extensive research on enterococci commonly found in Brazilian cheeses and reinforced the dualistic aspects of this genus with regard to food safety: some isolates of enterococci present potential to spread virulence/resistance traits but others can play a role as bioprotective or even probiotic cultures.

Taking into account the total read counts generated by HTS in this research (Figure 4.4), it is possible to affirm that, despite the large number of reads there was a predominance of selected OTUs. In this sense, a core microbiota analysis was very useful to determine how deeply bacterial taxa were shared among samples. A core microbiome is defined as the common members composition in two or more microbial assemblies related to a habitat. It is important because it shows the central composition of the OTUs, which is regarded as critical for the understanding of the functional activity of a microbial community (SHADE; HANDELSMAN, 2012).

The core microbiota for the dairy samples analyzed in this study indicated the bacterial community was dominated, in a crescent order, by *Macrococcus*, *Alkaliphilus*, *Vagococcus*, *Lactobacillus, Marinilactibacillus, Streptococcus*, *Lysinibacillus, Staphylococcus*, *Clostridium*, *Halomonas*, *Lactococcus*, *Enterococcus*, *Bacillus* and *Psychrobacter*.

These results are in accordance with Li et al. (2018) who observed *Bacillus* had a strong positive correlation with the presence of *Alkaliphilus* in raw milk samples collected in China. *Alkaliphilus* belongs to the family *Clostridiaceae* and it has also been reported as a member of the humam gut microbiota (Doré et al., 2013). Moreover, Zakharyuka et al. (2017) affirmed that a number of anaerobic halophilic and *Alkaliphilic* bacteria, including representatives of the genus *Alkaliphilus*, have been isolated from lakes with high salinity and pH varying from 7 to 9.9. Based on this research and on literature data, it seems that the harsh environment from dairy processing plants can selected extremophiles microbes not previously detected by culture-based methods. The *Halomonas* genus present in the core microbiota described in this research is also

halotolerant and it has been recently detected in cheese (DELCENSERIE et al., 2014). One hypothesis for this finding is the high salt contents on the surface of ripened cheeses, which could favor bacteria from marine environment that likely enter the dairy processing plants carried out by the salt used as an ingredient (Monnet et al., 2014). It is also possible that the DNA from these extremophilic microbes have been detected despite the cells were not viable anymore.

Besides, it is interesting to note that spore-formers (*Clostridium*, *Bacillus Lysinibacillus)* were also members of the core microbiota in the present study, which also presented *Staphylococcus* and *Macrococcus*, closely related genera (KLOSS et al., 1998).

Lactic acid bacteria commonly linked to dairy food also composed the core microbiota in this research (*Vagococcus*, *Lactobacillus*, *Streptococcus*, *Lactococcus*) and more intriguing, a lactic acid bacterium named *Marinilactibacillus*, formerly isolated from marine organisms (ISHIKAWA et al., 2003) was also predominant in the Brazilian dairy samples analysed.

The core microbiome of a variety of cheeses have been previously described. De Fillipis et al. (2014) demonstrated for Mozzarella, Grana Padano and Parmigiano Reggiano the genera *Lactobacillus* and *Streptococcus* were the prevalent OTUs. Another cheese of Italian origin, Caciocavallo Pugliese, was evaluated by De Pasquale et al. (2014) and the core microbiome was formed by *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Acinetobacter*, *Yersinia*, *Pseudomonas*, *Chryseobacterium* and *Hafnia*. Diversely, Pico cheese presented seven dominant taxa belonging to the genus *Lactococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, *Lactobacillus*, *Staphylococcus*, Rothia; Pantoea (RIQUELME et al., 2015). Dalmasso et al. (2016) described that the microbiota of Plaisentif cheese in final maturation step was dominated by *Lactococcus*, *Lactobacillus* and *Streptococcus*, which was corroborated with data published by Calasso et al. (2016) about Caciotta and Caciocavallo cheeses and dairy industry environment, with predominance of lactic acid bacteria.

4.4 CONCLUSION

In comparison with the literature data on cheese and dairies, the present results from Brazil are in accordance with regard to the abundance of *Lactococcus*,

Lactobacillus and *Streptococcus* as members of the core microbiota but they diverge due to the predominance of halo and psychrotolerant bacteria. These results show samples from niches of high salinity and low temperature may have contributed for the prevalence of these bacteria.

5 Comparative metataxonomics based on 16S rRNA and culture-dependent analysis of two Brazilian dairies suggests a common core microbiota

ABSTRACT

Comparative metataxonomics based on 16S rRNA and culture-dependent analysis of two Brazilian dairies suggests a common core microbiota

Dairy foods are complex ecosystems composed of microorganisms from different origins (raw materials, food-contact surfaces, and processing environment) that can impact flavor and safety of final products. The objective of this paper was to determine the "in-house" microbiota of two Brazilian dairies and to discuss the possible implications for food protection. In total, 27 samples from dairies were cultured for background microbiota and the isolates were identified by DNA sequencing. Moreover, metagenomic DNA was directly extracted from samples and the unculturable microbiota was evaluated with massive 16S rRNA gene sequencing (Illumina®) and bioinformatic analyses. Overall, the results showed most isolates were lactic acid bacteria, but order *Enterobacterales* and families *Staphylococcacceae*, *Bacillaceae*, *Pseudomonadaceae* and *Moraxellaceae* were also detected. For metataxonomics, a heatmap was constructed and the top20 OTUs were determined, revealing a significant dissimilarity of the microbiota from both dairies. In conclusion, the results from culture method were partially in agreement with metataxonomics and there were 12 most prevalent bacterial taxa in the core microbiota of the dairies evaluated, with the highest abundance of OTUs from *Tolumonas auensis* and *Lactococcus fujiensis*.

Key words: Brazilian cheese, cheese microbiota, dairy metataxonomics, cheese uncuturable microbial diversity

5 INTRODUCTION

5.1 MICROORGANISMS IN DAIRY

Microorganisms in dairy foods can be milk borne or transferred indirectly from food-contact and non-food contact surfaces, which contribute for a complex “in house” microbiota that greatly impacts on flavor development and confers unique characteristics to the final products (BOKULICH; MILLS, 2013; CALASSO et al., 2016, AFSHARI et al., 2018). The study of these intricate microbial communities has greatly advanced with the application of Next-Generation Sequencing (NGS) for the massive and parallel analysis of fractions of the 16S rRNA gene from a pool of DNA, in a metataxonomic approach (ALMEIDA; DE MARTINIS, 2018). Metataxonomics data are useful not only to quantify the proportions of the bacterial taxa in a given sample but also to monitor time-spatial changes in the profiles of microbial communities (ERCOLINI, 2013). In this sense, one application of metataxonomics refers to the determination of the microbiota associated with the geographic origin from different food products.

Brazil represents a huge market for dairy food and many authors have evaluated hygienic-sanitary indicators and bacterial pathogens related to these products (SALOTTI et al., 2006; SANTOS; HOFFMAN, 2010; NASSER et al., 2015; AMORIM et al., 2014). Cheeses rank among the most popular dairy products in Brazil and the most popular types in Brazil include “requeijão” (a spreadable pasteurized processed cheese), mozzarella, “prato” (an intermediate moisture cheese obtained by enzymatic coagulation and addition or not of lactic acid bacteria), and “Minas Frescal” (a typical high moisture Brazilian white cheese, obtained by enzymatic coagulation, added or not of lactic acid bacteria) (BRASIL 1997a, b; USDA, 2017). Moreover, there is a specific Brazilian legislation on artisanal Minas cheeses (MINAS GERAIS, 2012), considered a cultural Brazilian heritage (BRASIL, 2008). Another typical Brazilian cheese is “coalho”, prepared by enzymatic coagulation (complemented or not with lactic cultures) and cooking, yielding a final product with intermediate to high moisture (BRASIL, 2001; SANTOS et al., 2017).

Despite the economic and cultural relevance of Brazilian dairies, there are only a few reports on the use of culture-independent methodologies for studying Brazilian milk and cheese samples. Arcuri et al. (2013) carried out a bacterial community fingerprint analysis (PCR-DGGE) based on 16S rRNA gene marker from “Minas”

cheese autochthonous communities. Those authors reported the band profiles distinguished cheese made from raw or pasteurized milk, and *Streptococcus* sp. was likely to predominate in the artisanal “Minas” cheese. Lacerda et al. (2011) used a culture-independent, clone library-based 16S rRNA gene sequence analysis to identify bacterial communities from “Minas” cheese samples, and reported the most prevalent species were *Lactococcus lactis* and *Streptococcus salivarius*, respectively, after seven and sixty days of ripening. In another paper, Delgado et al. (2013) applied culture, DGGE and pyrosequencing techniques to prospect starter culture candidates from the autochthonous thermophilic bacteria in milk, reporting the predominance of *Lactococcus*, *Leuconostoc* and *Streptococcus*. Moreover, Martins et al. (2018) determined the microbiota of Amazonian artisanal cheeses by TTGE (temporal temperature-gradient gel electrophoresis), and reported the presence of *Lactococcus lactis* subsp. *lactis*, along with non-usual species such as *Macrococcus caseolyticus* and *Corynebacterium variabile*. Only very recently, NGS with 16S gene amplicon sequencing has been used to characterize Brazilian cheeses, as reported by Sant’Anna et al. (2019), who found members of *Streptococcaceae* and *Planococacceae* were prevalent throughout ripening time in “Minas” cheeses. Kamimura et al. (2019) have just published a paper using a metataxonomic approach and reported lactic acid bacteria dominated the microbiota of artisanal Brazilian cheeses, but *Enterobacterales* and *Staphylococcus* were also present. These later authors (KAMIMURA et al., 2019) also pointed out the evaluation of microbial contamination sources are needed to improve cheese quality and safety.

In the context of the recent unfolding microbial characterization of dairy food by NGS, the aim of this work was to apply culture-dependent analysis combined with metataxonomics to characterize the mixed microbiota from food and environmental samples from Brazilian dairies.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

For this research, sampling was done in two cheese processing facilities located in the state of São Paulo, Brazil (Table 5.1). Factory A refers to an artisanal producer which processed ca. 150 liters of raw bovine milk per month, and factory B is a commercial plant uses pasteurized milk for making cheese, that processed ca. 1,500

liters of pasteurized milk per day. The samples were transported on ice in insulated boxes and analyzed on the same day.

Aliquots of 25g of in-process and processed cheeses were analyzed. To increase isolation rates, processed cheeses were also surface sampled with Sani-Sponge (LabPlas, Montreal, Canada). For liquids, 25 mL samples were used for bacterial isolation. To each sample aliquot, 225 ml of saline (0.85% sodium chloride) were added and homogenized, obtaining the 1:10 dilution. From this dilution, 50 mL were saved and frozen (-80°C) for later metataxonomics analyses.

Isolation of autochthonous bacteria (BAGGE-RAVN et al., 2003) was done by surface plating (0.1 mL) on BHI (Brain Heart Infusion), MRS (de Man, Rogosa, Sharpe) and BP (Baird-Parker) agar plates, all purchased from Oxoid, Basingstoke, UK. The seeded plates were incubated at 37°C for up to 48 hours and examined for bacterial growth to select up to 3 colonies with different morphologies (color, shape and size) to be purified and kept frozen (-80°C) for later identification by 16S rRNA gene sequencing

5.2.2 Sanger sequencing of 16S rRNA gene for molecular identification of isolates

Stock bacterial cultures isolated on BHI or BP agars were grown overnight at 37°C in BHI broth; isolates from MRS agar were grown in MRS broth. For genomic DNA extraction, a 1 mL aliquot was processed using the Illustra™ bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK), following the instructions of the manufacturer. DNA extract was checked for purity by spectrophotometry (Nanodrop 2000c, Thermo Fisher, USA) and used as template for PCR amplification of the 16S rRNA genes with the universal primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'GCTTACCTTGTTACGACTT3'), based on Lane. (1991) and Turner et al. (1999). The PCR reactions (50 µL) were prepared with 84 µL of the Taq Platinum® Blue PCR SuperMix (Invitrogen, USA) 30 *p*mol of each universal primer (Invitrogen) and 300 ng of genomic DNA. Amplification was performed on a thermocycler (model MG96+, LongGene®, China) programmed for 30 cycles, with denaturation at 94 °C/1 minute, annealing at 55 °C/1 minute and extension at 72 °C/2 minutes. To confirm the amplification of target DNA regions, amplicons were analyzed by agarose gel electrophoresis (Sanderson et al. 2014). Next, the amplicons were purified with the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare,

Buckinghamshire, UK) and sequencing was performed by automated Sanger method with the 3500 Genetic Analyzer (ThermoFisher Scientific, Waltham, USA) available at the facility for nucleic acid sequencing of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Brazil. Sequences generated were analyzed using FinchTV software (Geospiza Inc., https://digitalworldbiology.com/FinchTV) and the alignment of the DNA sequences, composed of high-quality regions, selected through the FinchTV software, was carried out using the software Basic Local Alignment Search Tool (BLASTn - https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information – NCBI (USA). The isolates were classified at the species level based on the data resulting from the alignment that presented the highest score of similarity, identity and coverage with the taxon output from the database.

5.2.3 Metagenomic DNA extraction from cheese and dairy environment samples

The total volume of each previously frozen sample aliquot was centrifuged for 10 minutes at 10,000g (Sorvall Legend Mach 1.6R, USA), the supernatants were discarded and the pellets were used directly for metagenomic DNA extraction with the PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories, Qiagen, USA), to obtain at least 5ng/µL. Sequencing was performed on a Miseq platform (Illumina®, USA) at the facility for nucleic acid sequencing of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Brazil. V3 and V4 regions of 16S rRNA genes were amplified by PCR with specific primers recommended by Illumina® (forward=5´CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCG3'; reverse=5´ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGTATCTAA TCC3') with overhang adapters attached. The amplicons were purified with Agencourt AMPure XP beads (Beckman Coulter, USA) to remove free primers and primers dimers. A second stage PCR was performed to attach dual indices and Illumina sequencing adapters, using the Nextera XT Index kit ® (Illumina ®). Following, another purification step was performed with Agencourt AMPure XP beads (Beckman Coulter), the libraries were quantified by real-time PCR (Kapa HiFi Hot Start ready Mix, Kapa Biosystems, USA), normalized and the total libraries were pooled, according to instructions provided by Illumina ® for the 16S Metagenomic Sequencing Library

Preparation guide. The libraries were denatured with sodium hydroxide and the pool samples were loaded into MiSeq flow cell using the MiSeq reagent kit V3 (600 cycles). For each run, to provide an internal control of low diversity libraries and to prevent the run to fail, a minimum of 5% of PhiX control kit v3 (Illumina ®, USA) were used.

5.2.4 Bioinformatics and statistical analysis workflow

The output data provided by the Illumina® BaseSpace commercial pipeline in a .csv extension file (available in the supplementary material) was processed in the R environment using the Rstudio v.3.5 software (https://www.rstudio.com/). For the analysis of diversity within the samples, represented by the Chao1 and Shannon indexes, was used the phyloseq package (MCMURDIE; HOLMES, 2013), in which the OTUs count file .csv was converted to a phyloseq object to determine the bacterial diversity in the samples. By means of the “estimate richness” function, a table was generated and it presents the diversity indexes and the relation with the observed number of OTUs. The sequencing coverage (%) was defined in terms of the ratio of the number of observed OTUs and the number of theoretical OTUs calculated by the Chao1 index.

The evaluation of the bacterial composition in the samples from plants A and B was performed from the .csv extension file without any conversion to the phyloseq object. From this file a Heatmap with the Top20 OTUs was plotted, representing the OTUs with the proportion of hits for a given taxon with a value greater than or equal to 1% of relative abundance. To analyse the β-diversity (diversity among the samples), the samples were clustered using the Bray-Curtis dissimilarity distance method available in the Vegan package (OKSANEN et al., 2018).

The core microbiota was determined using the microbiome package (LAHTI et al., 2018) using the "prevalence" function, which calculates the number of OTUs that exceeds the established limit of detection. In this work, this limit was "zero", which means that was not excluded counts with a value greater than or equal to 1. Thus, it was possible to detect all counts in the dataset. Then, was applied the “core” function to filter in the data set only the most prevalent taxa according to the limit of detection established, which accepted OTUs with 0.2% of relative abundance but with a minimum prevalence of 50% by each sample. The plotting of core microbiota Heatmap was performed using the “plot_core” function.

5.3 RESULTS

5.3.1 Culturable microbiota

To assess the bacterial diversity in Brazilian dairies, 27 samples were analyzed from one artisanal and one commercial processing plant. Out of these samples, 59 isolates were identified in total (Table 5.1), with predominance of species belonging to the lactic acid group and to the order *Enterobacterales* (ADEOULU et al., 2016) and families *Staphylococcacceae*, *Bacillaceae*, *Pseudomonadaceae* and *Moraxellaceae*.

Regarding to the artisanal dairy plant (A), LAB members were isolated from all samples analyzed and belonged to the genera *Enterococcus*, *Pediococcus* and *Weissella.* Enterobacterales (*Escherichia coli*) were detected in several samples of dairy plant A, including raw material, food contact surfaces, intermediate and final products (Table 5.1). *Staphylococcacceae* family members detected in dairy plant A Included *Staphylococcus aureus* (raw milk bucket, whey, “Minas” frescal cheese and cheese storage shelf) and *Macrococcus caseolyticus* (raw bovine milk). Sporulated bacteria were detected at dairy plant A in samples of raw bovine milk and shelf of cheese storage (*Bacillus* sp.), and in final product (*Lysinibacillus* sp.).

According to Table 5.1, it is observed in the commercial dairy (plant B) LAB were isolated from 2 out of 13 samples evaluated, and they were identified as *Lactococcus lactis* (raw bovine milk) and *Enterococcus hirae* (cheese mold). The same isolation rate was obtained for *Enterobacterales*, with the detection of *Klebsiella pneumonia* in two samples (cheese curd and cheese fermentation tank). *Staphylococcacceae* were detected in dairy B in several processing steps and the isolates comprised *Macrococcus caseolyticus* (one sample) and *Staphylococcus* sp. (four samples), as also shown in Table 1. *Bacillus* sp. was isolated at dairy B from five samples, which included raw material and food contact surfaces. The non-fermenting bacterium *Pseudomonas* sp. was also detected in raw material and food contact surfaces (5 samples). Besides, *Acinetobacter baumannii* was present in one food contact sample (cheese fermentation tank).

Table 5.1 - Bacterial isolates from dairy plants A (artisanal) and B (commercial) identified by sequencing of 16S rRNA genes and sampling code for metagenomics analyzes.

Samples	Dairy plant A (n)*	Dairy plant B (n)*	Sample code
Raw bovine milk (in-process)	*Bacillus subtilis* *Enterococcus faecium* *Macrococcus caseolyticus*	*Lactococcus lactis* *Pseudomonas* sp. *Pseudomonas putida*	A1, B1
Raw bovine milk (stored)	**ND** ***	No isolates	B2
Raw milk bucket	*Enterococcus faecium* *Escherichia coli* *Pediococcus pentosaceus* *Staphylococcus aureus*	**ND** ***	A3
Heat treated milk**	No isolates	*Bacillus* sp. *Pseudomonas* sp.	A4, B4
Cheese curd	No isolates	*Klebsiella pneumoniae* *Macrococcus caseolyticus* *Pseudomonas* sp.	A5, B5
Cheese fermentation tank	**ND** ***	*Acinetobacter baumannii* *Klebsiella pneumoniae* *Pseudomonas alcaligenes* *Pseudomonas putida*	B6
Curd cheese cutter	No isolates	*Bacillus cereus*	A7, B7
Cheese mold	*Enterococcus faecalis* (2) *Enterococcus faecium* *Escherichia coli*	*Bacillus licheniformis* *Bacillus megaterium* *Enterococcus hirae* *Pseudomonas* sp.	A8, B8

Samples	Dairy plant A (n)*	Dairy plant B (n)*	Sample code
Cheese ripening tank	**ND*** **	*Bacillus megaterium* *Staphylococcus chromogenes*	B9
Whey	*Escherichia coli* *Macrococcus caseolyticus* *Staphylococcus aureus* *Weissella cibaria*	No isolates	A10, B10
Brine	No isolates	*Pseudomonas putida* *Staphylococcus chromogenes*	A11, B11
"Minas Frescal" cheese	*Enterococcus faecium* *Lysinibacillus xylanilyticus* *Macrococcus caseolyticus* *Staphylococcus aureus*	No isolates	A12, B12
"Minas Frescal" cheese (surface)	*Enterococcus faecalis* (4)	No isolates	A13, B13
Semi-hard cheese	*Enterococcus casseliflavus* *Escherichia coli*	**ND*** **	A14
Semi-hard cheese (surface)	*Enterococcus faecalis* (2) *Enterococcus faecium* *Escherichia coli* (2)	**ND*** **	A15
"Coalho" goat cheese (surface)	**ND*** **	*Staphylococcus aureus*	A16, B16
Shelf for cheese storage	*Bacilus cereus* *Enterococcus faecalis* *Macrococcus caseolyticus* *Staphylococcus aureus*	*Bacillus* sp. *Staphylococcus aureus* *Staphylococcus chromogenes*	A17, B17

* **(n)** refers to the number of isolates obtained.
** **Dairy Plant A:** boiling; **Dairy Plant B:** pasteurization.
*** **ND**: not determined – Product or operational step not available at this dairy plant.

5.3.2 Structure of culture-independent bacterial dairy communities

For metataxonomics, the samples were obtained at the processing steps listed in Table 1. DNA was extracted, libraries were successfully constructed and DNA sequencing was adequate for the samples shown in Table 5.2.

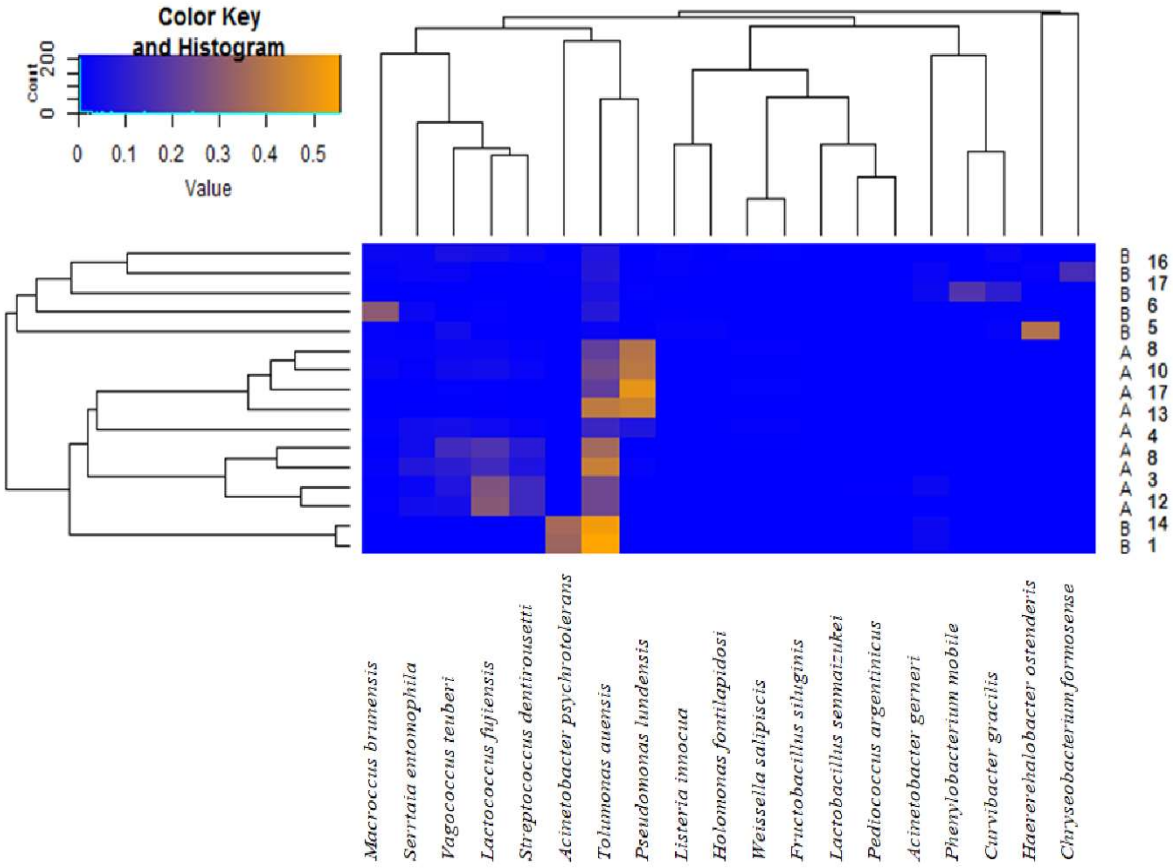
Table 5.2. Number of OTUs observed and calculated diversity indexes and coverages for metataxonomics analyzes of samples from dairy plants A and B.

Samples	Number of observed OTUs	Chao1 index	Shannon index	Coverage (%)
A1	447	598.44	2.52	74.69
A3	494	666.08	2.39	74.16
A4	423	580.68	1.60	72.84
A8	568	756.90	3.14	75.04
A10	474	648.72	2.60	73.06
A13	446	638.45	2.20	69.85
A17	668	843.01	2.50	79.23
B1	546	725.75	1.33	75.23
B2	514	677.37	1.27	75.88
B5	480	696.56	3.30	68.90
B6	1350	1,668.90	4.02	80.89
B8	598	777.11	2.80	76.95
B16	877	1,105.30	4.70	79.34
B17	1180	1387	4.67	85.07

The coverage (%) of sequencing, ranged from 68% (for sample B5) to 85% (for sample B17). Coverage (%) is defined as the ratio between the observed number of OTUs and the Chao1 diversity index, which refers to the theoretical minimum number of OTUs expected in a given sample (LEMOS et al., 2011; HAEGEMAN et al., 2013; ALMEIDA; DE MARTINIS, 2018). Based on these results, it is possible to affirm that no less than 68% of the total theoretical diversity was accessed for all the samples. On the other hand, the Shannon diversity index combines the measurement of sample richness and uniformity (MORRIS et al., 2014; DELGADO-BAQUERIZO et al., 2016), and it is sensitive to the presence of rare OTUs, such as singletons, which can lead to a decrease in its numerical value (HILL et al., 2006). These metrics allow estimating the α-diversity and are crucial to understand the taxa composition within the samples and to assure greater confidence regarding the coverage reached in the sequencing. In our study, the richest and most uniform samples were B17 (shelf of storage cheese), B6 (cheese fermentation tank) and B8 (“coalho” goat cheese surface), according to the highest values of indexes Chao1 and Shannon (Table 5.2).

The Figure 1 shows the structure of the bacterial communities studied, with the Top20 OTUs present with more than 1% relative abundance. The parameter of β-diversity, determined by the Bray-Curtis dissimilarity method, supports the definition of two large clusters, with samples derived from plants A and B clustering independently, which suggests the occurrence of unique in house microbiota.

Figure 5.1 - Heatmap showing the Top20 OTUs that represent the structure of the bacterial communities from processing plants **A** (artisanal) and **B** (commercial).

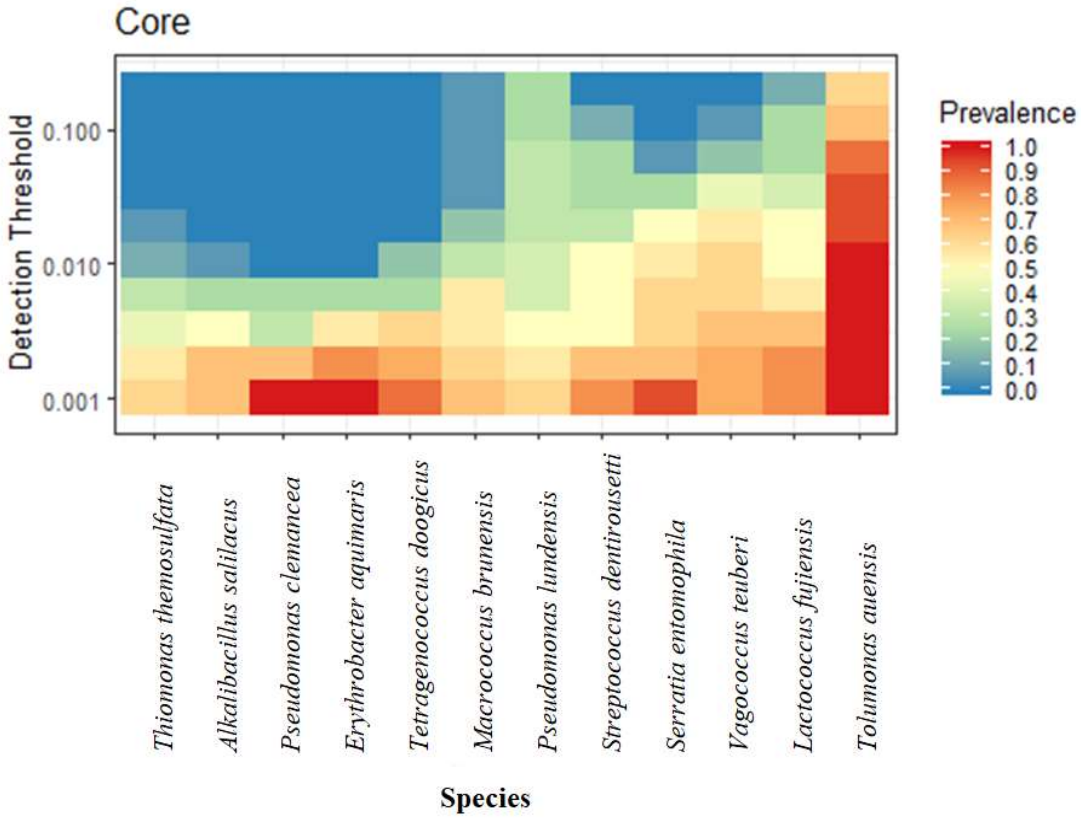


Overall, based on the results shown in Figure 5.1, it is possible to affirm there was a significant dissimilarity of the microbiota from plants A and B. In plant A, it was evidenced the dominance of *Serratia entomophila, Vagococcus teuberi, Lactococcus fujiensis, Streptococcus dentirousetti, Acinetobacter psychrotolerans* and *Pseudomonas ludensis*. On the other hand, the microbiota of plant B was dominated by *Phenylobacterium mobile*, *Curvibacter gracilis*, *Haererehalobacter ostenderis* and *Chryseobacterium formosense*. It is also noteworthy the commercial processing plant (B) presented a less diverse microbial structure in comparison with the artisanal plant (A).

The core microbiota of the two Brazilian dairies evaluated (Figure 5.2) presented 12 most prevalent bacterial taxa, and the majority of OTUs were derived from

Tolumonas auensis, followed by *Lactococcus fujiensis*, with *Thiomonas thermosulfata* determined as the least prevalent species in the core microbiome.

Figure 5.2. The general core microbiota determined for Brazilian dairy plants **A** (artisanal) and **B** (commercial).



5.4 DISCUSSION

Cheeses are complex microbial ecosystem, and the sources for different microorganisms can be the raw materials, added starter or adjuvant cultures, autochthonous microorganisms attached to processing equipment and/or resident in the production environment (AFSHARI et al., 2018). The aim of this research was to characterize the culturable and culture-independent microbiota from selected Brazilian dairies, to get a better understanding on the safety and quality of cheeses.

The analysis of the bacterial isolates (Table 5.1) revealed the prevalence of taxa belonging to the LAB group. The LAB can be functionally divided into two groups: (i) LAB starter (SLAB), which are associated with fermentation processes, with lactose metabolism and production of high concentrations of lactic acid, and (ii) non-starter

LAB (NSLAB), which designates groups of bacteria related to cheese maturation process (SETTANNI; MOSCHETTI, 2010). *Lactococcus lactis*, a SLAB species, was present in the samples from commercial plant (B) but for artisanal plant (A), mostly NSLAB species were detected, including *Pediococcus pentosaceus* and *Enterococcus faecium*, *E. faecalis* and *E. casseliflavus*. This is in accordance with literature reports showing usually higher prevalence of SLAB in commercial dairies (BOKULICH; MILLS, 2013; HANSEN, 2002), which might be related to the widespread use of predefined bacterial cocktails to achieve more standardized products.

Raw bovine milk may present over 100 bacterial genera, which can directly contribute to the increase of the in house microbiota, by contaminating milk pipes and silos for milk storage. The main microbiota from raw milk includes LAB, enterobacteria, *Staphylococcus* sp., and *Pseudomonas* sp. It has been shown that refrigerated storage of milk causes a shift of its microbial profile by favouring the dominance of Gram-negative bacteria (GOBBETTI et al., 2018). This corroborates the observation of the present study with regard to the prevalence of *Pseudomonas* sp., a Gram-negative psychrotrophic bacterium, in samples of raw milk from the commercial dairy (B), which had a refrigerated storage step. According to Marchand et al. (2012) *Pseudomonas* sp. is the most predominant spoilage bacterium in dairies, which usually originates from raw milk and produces several heat-stable enzymes (lipases, proteases and lecithinases). It is remarkable the ability of pseudomonads to form biofilms in the food industry. Those authors also reported *Acinetobacter* sp. may be listed among the psychrotrophic population in refrigerated raw milk, which is also in agreement with the findings of the present study (Table 5.1).

Among the enterobacteria, the fecal coliform *E. coli* was found in several samples in the artisanal plant (A), indicating unsanitary manufacturing conditions (TRMČIĆ et al., 2016). On the other hand, only environmental coliforms were detected in plant B (*Klebsiella* sp.).

Another frequent contaminant in the dairy food, the *Bacillus* genus, was also cultured from samples of dairies A and B. These bacteria may present strong adhesion properties due to hydrophobic characters of their spores, which tolerate heat treatment as well as cleaning processes. *Bacillus cereus* was detected in this research in samples of dairy plant A, which is of special concern due to its potential to cause emetic and diarrheic syndromes (KUMARI; SARKAR, 2016).

The microbial diversity from dairies A and B were also evaluated more in depth with culture independent studies. The results presented in the heatmap (Figure 5.1) indicated the microbiota of the cheese processing plants A and B could be differentiated, and there was a higher bacterial diversity in the artisanal dairy (A) in comparison with the commercial cheese processor (B). This observation is in agreement with a previous study based on PCR-DGGE, which also showed artisanal cheeses presented more diverse microbiota than industrialized ones (COPPOLA et al., 2001). From Figure 5.1, it is interesting to highlight that among prevalent members of the community, the genera *Pediococcus*, *Lactococcus*, *Weissella*, *Macrococcus* and *Pseudomonas* were also detected by plate culture (Table 5.1), despite different species were determined by metataxonomics approach. One hypothesis for this finding is that culture has favored low abundant taxa that overgrew the competitors revealed by culture-independent studies.

To determine the taxonomic fingerprint shared between the plants A and B, a core microbiota was estimated (Figure 5.2), which showed the prevalent bacterial species in both production sites were *Thiomonas thermosulfata*, *Alkalibacillus salilacus*, *Pseudomonas clemancea*, *Erythrobacter aquimans*, *Tetragenococcus doogicus*, *Macrococcus brunensis*, *Pseudomonas ludensis*, *Streptococcus dentinousetti*, *Serratia entomophila*, *Vagococcus teuberi*, *Lactococcus fujiensis* and *Tolumonas auensis*.

To the best of our knowledge, out of the species found in the core microbiome (Figure 5.2), *Thiomonas thermosulfata*, *Erythrobacter aquimans* and *Tolumonas auensis* have not been previously described in dairy related samples. *Thiomonas* sp. are ubiquitous in extreme environments, such as acid mine drainage, which presents high toxic element concentrations, low levels of organic matter and low pH (ARSÈNE-PLOETZE et al., 2010). *Erythrobacter* sp. encompasses aerobic anoxygenic photothophic bacteria from the oceans, with recent taxonomic studies (ZHENG et al., 2016). With regard to *Tolumonas* sp., it is a toluene-producing bacterium firstly isolated from anoxic freshwater lake sediments (FISCHER-ROMERO et al., 1996). Taken together, these results indicated the presence of unusual microorganisms in food processing environments, which can present harsh conditions due processing operations, such as cleaning, sanitizing, heating and cooling. However, the significance of these bacteria for food industry remains to be elucidated.

Halotolerant organisms were also detecting as members of the core microbiome of the dairies evaluated in this research, including species from the genera *Alkalibacillus* and *Tetragenococcus*, which have been previously reported by MARINO et al., (2017) as brine-associated bacteria from Italian cheese processors. The marine microorganism *Alkalibacillus* sp. has not a defined role in dairy processing and it is likely to be carried to the dairy environment by the salt used in cheesemaking. On the other hand, *Tetragenococcus* genus might have beneficial association with dairy products due to its proteolytic and lipolytic activities (MARINO et al., 2017).

The genera of Gram-negative bacteria, *Pseudomonas* and *Serratia* were determined as members of the core microbiota in this study (Figure 5.2), in partial agreement with results from the culture-dependent analyses (Table 5.1). These bacteria are commonly reported as part of the psychrotrophic population found in refrigerated raw milk (MARCHAND et al., 2012), which indicated the need to design cleaning and sanitation strategies targeting these organisms with outstanding ability to act as a pioneering colonizers in biofilms (MARCHAND et al., 2012; STELLATO et al., 2015).

Another core microbiome member detected in this study is *Vagococcus teuberi* (Figure 5.2). It belongs to the lactic acid bacteria group and it has just been isolated from Malian artisanal sour milk by Wullschleger et al. (2018). Those authors reported *Vagococcus teuberi* presented a set of unique genes related to branched chain amino acids metabolism, which are regarded as flavour precursors in fermented food and beverages.

The other genera of lactic acid bacteria detect in this study (Figure 5.2) were *Lactococcus* sp. and *Streptococcus* sp., which is in accordance with literature reports on the core microbiota of Italian cheeses (DE FILLIPIS et al., 2014; DE PASQUALE et al., 2014; DALMASSO et al., 2016; CALASSO et al., 2016). A recent report on the microbial diversity of Brazilian artisanal cheeses also indicated the prevalence of lactic acid bacteria, mainly *Lactobacillus* (KAMIMURA et al., 2019). Diversely, a study on the microbial shifts in Brazilian raw milk cheeses indicated *Planococcaceae* and *Streptococcaceae* were prevalent throughout ripening time, with strong association of the former with the *Leuconostocaceae* family (SANT'ANNA et al., 2019).

In the present study, Macrococci were also determined as a member of the core microbiota. These are dairy-related bacteria, although they are also regarded as widespread commensals of animals. In spite of this, macrococci are being increasingly

recognized as veterinary pathogens, with ability to encode methicillin resistance, similar to the genus *Staphylococcus*, that presents close phylogenetic relationship but is more pathogenic (MACFADYEN et al., 2018).

5.5 CONCLUSION

Taken together, the results of this investigation revealed it is possible to determine a core bacterial microbiota for Brazilian Minas cheese processors, despite indicating also the presence of some unique microorganisms, depending on the processing characteristics, either in artisanal or industrial scales. Based on these novel findings, it seems interesting to continue the studies in this area, with functional analyses and metatranscriptomics, to clarify the complex metabolic networks that make the flavour of Brazilian cheeses unique.

6 Closing remarks

- In this study, the culturable and culture-independent microbiota of samples from dairies of two Brazilian states (Goiás and São Paulo) were evaluated.

- Selected samples presumptive-positives for *Listeria* sp. and *Staphylococcus aureus* and their respective accompayning microbiota were confirmed with 16S rRNA gene sequencing by Sanger method (chapter 3). No sample was confirmed for *Listeria* sp. but *S. aureus* was highly prevalent. LAB was predominant in the background microbiota, mainly *Enterococcus casseliflavus*.

- The microbiota of dairy samples from São Paulo state were analysed by community 16S rRNA gene sequencing (chapter 4). The results revealed the samples could be grouped in two main clusters and the bacterial community was dominated by *Macrococcus, Alkaliphilus, Vagococcus, Lactobacillus, Marinilactibacillus, Streptococcus, Lysinibacillus, Staphylococcus, Clostridium, Halomonas, Lactococcus, Enterococcus, Bacillus* and *Psychrobacter*.

- The “in-house” microbiota of two Brazilian dairies were determined simultaneously by culture-dependent and culture-independent methods (chapter 5). The isolates were mainly LAB, but the order *Enterobacterales* and families *Staphylococcacceae*, *Bacillaceae*, *Pseudomonadaceae* and *Moraxellaceae* were also detected. According to metataxonomics (*16S rRNA*), there were 12 most prevalent bacterial taxa in the core microbiota of the dairies evaluated, with the highest abundance of OTUs from *Tolumonas auensis* and *Lactococcus fujiensis*.

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