UNIVERSIDADE DE SÃO PAULO

FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

SARAH HWA IN LEE

Staphylococcus aureus and *Listeria monocytogenes* Isolated from Dairy Plants: Occurrence, Evaluation of Biofilm Formation Ability and Inactivation by Peracetic Acid and Cold Plasma

(*Staphylococcus aureus* e *Listeria monocytogenes* Isolados de Laticínios: Ocorrência, Avaliação da Capacidade de Formação de Biofilmes e Inativação por Ácido Peracético e Plasma a Frio)

Pirassununga

2015

SARAH HWA IN LEE

Staphylococcus aureus and *Listeria monocytogenes* Isolated from Dairy Plants: Occurrence, Evaluation of Biofilm Formation Ability and Inactivation by Peracetic Acid and Cold Plasma

(*Staphylococcus aureus* e *Listeria monocytogenes* Isolados de Laticínios: Ocorrência, Avaliação da Capacidade de Formação de Biofilmes e Inativação por Ácido Peracético e Plasma a Frio)

Versão corrigida

Tese apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para o para a obtenção do título de Doutor em Ciências.

Área de Concentração: Ciências da Engenharia de Alimentos.

Orientador: Prof. Dr. Carlos Augusto Fernandes de Oliveira

Pirassununga

2015

Dados Internacionais de Catalogação na Publicação

Serviço de Biblioteca e Informação da Faculdade de Zootecnia e Engenharia de Alimentos
da Universidade de São Paulo

L481s

Lee, Sarah Hwa In

Staphylococcus aureus and *Listeria monocytogenes* isolated from dairy plants: occurence, evaluation of biofilm formation ability and inactivation by peracetic acid and cold plasma /Sarah Hwa In Lee. -- Pirassununga, 2015.

97 f.

Tese (Doutorado) -- Faculdade de Zootecnia e Engenharia de Alimentos – Universidade de São Paulo.

Departamento de Engenharia de Alimentos.

Área de Concentração: Ciências da Engenharia de Alimentos.

Orientador: Prof. Dr. Carlos Augusto Fernandes de Oliveira.

1. Biofilm 2. Cold plasma 3. Dairy farms 4. Dairy plant 5. *Listeria monocytogenes* 6. PAA 7. *S. aureus*. I. Título.

ABSTRACT

LEE, S. H. I. ***Staphylococcus aureus* and *Listeria monocytogenes* Isolated from Dairy Plants: Occurrence, Evaluation of Biofilm Formation Ability and Inactivation by Peracetic Acid and Cold Plasma.** 2015. 97p. Thesis (PhD) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2015.

In the present study, a set of three experiments were conducted aiming to evaluate the occurrence of *Staphylococcus aureus* and *Listeria monocytogenes* in three dairy plants (A, B and C) from Southeast region of Brazil from December 2013 to April 2015 (Experiment 1), the efficiency of peracetic acid (PAA) and cold plasma (CP) jet treatment to inactivate the isolates at different times (Experiment 2) and the ability of the isolates to produce biofilms on polystyrene and stainless steel surface, along with inactivation and removal of biofilms by PAA (Experiment 3). In Experiment1, samples of milk and cheese, food contact surfaces and non-food contact were analyzed. *L. monocytogenes* was isolated in only one sample (0.3%, N=349) of drain sponge swab in dairy plant B, while 6 (1.7%, N=349) *S. aureus* strains were isolated from handlers' glove in dairy plant A, brine in dairy plant B and cheese surface, cheese utensil, worker's boot and worker's left hand in dairy plant C. Although the incidences of those two food-borne pathogens in the dairy plants evaluated were low, their presence also indicates the need for control strategies to prevent their persistence and cross-contamination. In Experiment 2, PAA (0.5%) and CP jet treatment were applied directly on suspensions of *S. aureus* and *L. monocytogenes* strains. Reduction of bacterial load (nearly 7 log cycles) was achieved with 15 sec. of PAA treatment of all strains, whereas CP treatment reduced approximately 2 log cycles after 2 min. Hence, plasma treatment has a potential for reducing the bacterial load on surfaces, although further studies using longer CP treatment times are necessary to fully describe the kinetics of this technology for inactivation of important food pathogens. In Experiment 3, PAA (0.5%) treatment at different times (0-control, 15, 30, 60 and 120 sec.) was evaluated for removing of adherent cells of 4 strains of *S. aureus* and one strain of *L. monocytogenes* on polystyrene plates, as well as for inactivation of biofilms of those strains on stainless steel. PAA treatment removed ($p<0.05$) all the *S. aureus* cells from the surface, with no difference ($p>0.05$) in the reduction of the biofilm-forming index at the treatment times. However, no effect ($p>0.05$) was observed on *L. monocytogenes* adhered cells. Epifluorescence microscopy showed that all bacterial strains tested were partially and completely inactivated after 15 sec. and 30 sec., respectively. Results indicate a potential use of PAA against biofilms formed by *S. aureus* and *L. monocytogenes*, and the need of further studies with CP to determinate the ideal parameters for inactivation of food-borne pathogens.

Key Words: plasma jet, farms, dairy plant, PAA, pathogenic bacteria.

RESUMO

LEE, S. H. I. ***Staphylococcus aureus* e *Listeria monocytogenes* Isolados de Laticínios: Ocorrência, Avaliação da Capacidade de Formação de Biofilmes e Inativação por Ácido Peracético e Plasma a Frio.** 2015. 97p. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2015.

No presente estudo, um conjunto de três experimentos foram conduzidos com o objetivo de avaliar a ocorrência de *Staphylococcus aureus* e *Listeria monocytogenes* em três lacticínios (A, B e C) localizados na região sudeste do Brasil de dezembro 2013 a abril de 2015 (Experimento 1), a eficiência do tratamento com ácido peracético (APA) e jato de plasma a frio (PF) para inativar os isolados em diferentes tempos (Experimento 2) e a capacidade dos isolados produzir biofilmes na superfície de poliestireno e de aço inoxidável, juntamente com inativação e remoção de células aderidas pelo APA (Experimento 3). No Experimento 1, foram analisadas amostras de leite e queijo, superfícies com e sem contato com alimentos. *L. monocytogenes* foi isolada em apenas uma amostra (0,3%, N = 349) de ralo no laticínio B, enquanto seis (1,7%, n = 349) *S. aureus* foram isolados de luvas de manipuladores em laticínio A, salmoura no laticínio B e superfície do queijo, utensílio, bota e mão esquerda de trabalhador no lacticínio C. Apesar das incidências desses dois agentes patogênicos de origem alimentar nos lacticínios avaliados foram baixo, sua presença também indica a necessidade de controle estratégias para impedir a sua persistência e contaminação cruzada. No Experimento 2, tratamento com APA (0,5%) e jato de PF foram aplicados diretamente sobre suspensões de isolados de *S. aureus* e *L. monocytogenes*. A inativação bacteriana (aproximadamente de 7 ciclos log) foi alcançada em 120 seg. com o tratamento com APA para todos os isolados, enquanto que o tratamento com plasma a frio reduziu aproximadamente 2 ciclos log nas superficies. Outros estudos usando tratamentos de plasma a frio mais longos são necessários para a total descrição da cinética desta tecnologia para a inativação de importantes patógenos de origem alimentar. No Experimento 3, o tratamento com APA (0,5%) em diferentes tempos (0-controle, 15, 30, 60 e 120 seg.) foi avaliada para a remoção de células aderidas de quatro isolados de *S. aureus* e um isolado de *L. monocytogenes* em microplacas de poliestireno, assim como para a inativação de biofilmes dos isolados em aço inoxidável. O tratamento com APA removeu (p<0,05) células aderidas de todos os isoladoas estudados *S. aureus* da superfície, sem diferenças (p> 0,05) no indice de formação de biofilmes nos tempos de tratamento. No entanto, nenhum efeito (p> 0,05) foi observado em células aderidas de *L. monocytogenes*. A microscopia de epifluorescência mostrou que todas as bactérias testadas foram parcialmente e completamente inativadas após 15 seg e 30 seg.–respectivamente. Os resultados indicam um potencial para a utilização de APA contra biofilmes formados por *S. aureus* e *L. monocytogenes*, e da necessidade de novos estudos com a PF para determinar os parâmetros ideais para a inativação dos patógenos de origem alimentar.

Palavras chave: APA, biofilme, laticínios, *Listeria monocytogenes*, plasma a frio, propriedades leiteiras, *S. aureus*

LIST OF ABREVIATION AND ACRONYMS

ANVISA	Agência Nacional de Vigilância Sanitária
APA	ácido peracético
APP	atmospheric pressure plasma
ATB	Leibniz Institute of Agricultural Engineering Potsdam-Bornim
BFI	biofilm-forming index
BHI	brain heart infusion broth medium
BHIYE	brain heart infusion with yeast extract
CDC	Centers for Disease Control and Prevention
cFDA	5(6)-carboxyfluorescein diacetate mixed isomers
CFU	colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
CP	cold plasma
CV	crystal violet
DiOC	3,3'-diethyloxacarbocyanine iodide
ECDC	European Centre for Disease Prevention and Control
GMP	Good Manufacturing Practices
IMI	intramammary infection
LM	*Listeria monocytogenes*
L. monocytogenes	*Listeria monocytogenes*
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
MIC	bacterial minimum inhibitory concentration
MTP	microtiter plate
PAA	peracetic acid
PBS	sterile phosphate-buffered saline
PEF	pulsed electric fields
PF	plasma a frio
PI	propidium iodide
PSW	peptone saline water
RDC	Resolução da Diretoria Colegiada
SA	*Staphylococcus aureus*
S. aureus	*Staphylococcus aureus*
SCP	*Staphylococcus* coagulase positive
SE	staphylococcal enterotoxin
sec.	second
SFP	staphylococcal food poisoning
TO	thiazole orange
TSA	tryptone soya agar
TSAYE	tryptone soya agar with yeast extract
TSB	tryptone soya broth
TSBYE	tryptone soya broth with yeast extract
UVM	University of Vermont medium
VBNC	viable but nonculturable bacteria

SUMMARY

1. INTRODUCTION

Staphylococcus aureus is a Gram- positive cocci, non-sporulating and non-motile. This aerobic, catalase positive bacterium belongs to the commensal microbiota of several animal species. This bacterium is not only a nosocomial pathogen but also is one of the greatest common causes of food illness outbreaks. *S. aureus* is also the most common agent causative of bovine mastitis;

Listeria monocytogenes is also an important food-borne pathogen. The presence of *L. monocytogenes* in the environment of the dairy plants can be a potential source of contamination of the final product, especially when it is protected by milk solids and spread from the processing environment to the final product through the ventilation system, dripping and splashing, or by workers.

The persistence of biofilm on food contact surfaces, and equipment, may constitute a continuous source of contamination. Moreover, several reports have already shown the ability of *S. aureus* and *L. monocytogenes* to produce biofilms on materials commonly encountered in food industry, such as the stainless steel, glass, rubber, polystyrene, titanium, aluminum, and ceramic. Polystyrene and stainless steel are the most widely used materials in the food sector. Biofilm formation in dairy equipment can lead to economic loss due to the deterioration of food and equipment.

Food-borne disease can be transmitted by surface contamination of equipment and utensils; this risk can be effectively reduced by washing and sterilization with a disinfection agent. Sanitizer's effectiveness on bacterial reduction has many factors depending on the product type, target microorganisms, time interval between contamination and washing, and treatment time. Peracetic acid is a strong disinfectant with a wide spectrum of antimicrobial activity that was introduced to wastewater

treatment several years ago, and currently is one of the most common sanitizers used in food processing plants.

Cold plasma (CP) is an emerging nonthermal technology that could potentially decontaminate the surfaces of fresh produce. CP can efficiently kill or inactivate bacteria, yeasts and molds and other hazardous microorganisms, as well as spores and biofilms that are generally very difficult to inactivate. There are only theories about the mechanism action of the CP, probably the decontamination happens due to the formation of free radical components.

With regard to these aspects, this study evaluated the occurrence of *S .aureus* and *L. monocytogenes* in dairy plants, the ability of strains to produce biofilms on polystyrene and stainless steel surface, and the efficiency of peracetic acid and cold plasma jet treatment to inactivate the isolates at different times.

2. LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram- positive cocci, non-sporulating and non-motile. This facultative anaerobic, catalase positive bacterium (HENNEKINNE; DE BUYSER; DRAGACCI, 2011) belongs to the commensal microbiota of humans and various animal species (VANDERHAEGHEN et al., 2010b). This bacterium is not only an established as a nosocomial pathogen but also one of the great common causes of food illness outbreaks (ABDALLAH et al., 2014b) and is the most common etiological agent of bovine mastitis (PERILLO et al., 2012).

This bacterium is responsible for a great number of human infections all around the world (ĆIRKOVIĆ et al., 2015). A typical series of events leading to a Staphylococcal food poisoning (SFP) are: a) The organism is turned out from a host into a food source; b) When temperature abuse and/or inappropriate storage occurs contamination happens; c) The organism multiply and reach a high cell density, which triggers staphyloccoal enterotoxin, SE(s), production; and d) Poisoned food is ingested and the symptoms occur hours later (GUSTAFON et al., 2015). Typical symptoms are nausea, abdominal pain, vomiting, myalgia, diarrhea, dizziness, fever, headache and prostration (HENNEKINNE; DE BUYSER; DRAGACCI, 2012).

S. aureus has an ability to survive defending itself against exogenous stress (BUI et al., 2015) or to adapt to different environments and it represents a primary cause of infection both in humans and in several animal species (ZECCONI, SCALI, 2013). In dairy cows, a high prevalence of *S. aureus* udder infections is observed worldwide. Intramammary mastitis infections caused by *S. aureus* are reported in all the countries with prevalence from 5% up to 70% of cows and 90% of herds (ZECCONI, SCALI, 2013). About one third of cases of clinical mastitis or subclinical

is caused by the pathogen (BOTREL et al., 2010; BRADLEY et al., 2007). Moreover, livestock can also act as a reservoir for the emergence of new human bacterial clones with potential for pandemic spread, highlighting the necessity for preventing the emergence of new human pathogens (SPOOR et al., 2013).

In the food industry *S. aureus* can spread from food handlers and food-contact surfaces to food products (SOSPEDRA, MAÑES, SORIANO, 2012) it may occur when people colonized with *S. aureus* asymptomatically, who handle food, introduce the bacteria into the food chain (ARGUDIN et al., 2010). Since 30–50 % of individuals carry these organisms in their nasal passages and throat or on their hands, so any time a food is exposed to human handling, asymptomatic food handlers can contaminate food during preparation (TANG et al., 2014). Many other sites are also colonized by *S. aureus*, such as the axillae, groin, and gastrointestinal tract.

Stainless steel, a common material in food processing facilities, can serve as an important reservoir for *S. aureus* in the food industry (VÁZQUEZ-SÁNCHEZ et al., 2014). In Brazil, sale of raw milk is not allowed, and restrictive rules and guidelines were published to provide proper monitoring of its quality (BRASIL, 2002, 2011). Yet, it can be observed the consumption of raw milk and its products is common in distinct Brazilian regions (CRUZ; MENASCHE, 2014; NERO et al., 2003; OLIVAL et al., 2002). Previous studies isolated 56 (6.6%) *S. aureus* strains from 849 dairy farm samples (LEE et al., 2012). Veh et al. (2015) characterized genotypically and phenotypically a total of 285 *S. aureus* strains isolated from subclinical intramammary infection (IMI). Ângelo, Arcuri, Andrade (2014) studied the toxic shock syndrome toxin gene in 221 *S. aureus* isolated from milk of cows with subclinical IMI. In Northern Italy, 85 out of 197 samples (43.1%) were tested positive for *S. aureus* isolated from bulk tank milk samples from dairy goat farms (CORTIMIGLIA et al.,

2015). Regarding pasteurized milk, the occurrence of *S. aureus* is usually associated with post-processing contamination (ABDEL; DARDIR, 2009).

In Brazil the exact incidence rate of food poisoning caused by *S. aureus* is unknown, previous studies indicate that the raw milk and dairy products have great contribution in foodborne disease in humans (TONDO et al., 2000). Additionally, the reporting of foodborne disease outbreaks in Brazil only became compulsory in outbreaks occurring on ships or aircraft (BRASIL, 2011).

Sabioni et al. (1988) investigated an outbreak occurred in 1987 in Minas Gerais, suspected of cheese Minas consumed by a family. Microbiological analysis resulted in isolation of *Staphylococcus* coagulase positive and thermonuclease positive of $9{,}3x10^7$ CFU/g. Carmo et al. (2002) described two poisoning outbreaks in Minas Gerais, in 1999. In the first outbreak, 50 people showed symptoms of vomiting, diarrhea, cramps, chills and headache after consuming Minas cheese. In another outbreak investigated, involving 328 people who reported symptoms of diarrhea and vomiting, were collected samples of raw milk from bulk tanks and tap water. The analysis resulted in coagulase negative *Staphylococcus*, producer of SEC and SED.

2.2 *Listeria monocytogenes*

The genus *Listeria* has fifteen species (BERTSCH et al., 2013; DEN BAKKER et al., 2014) being *L. monocytogenes* and *L. ivanovii* considered pathogenic to humans and animals (LIU, 2006). *L. monocytogenes* is a rod-shaped Gram-positive, non-spore bacterium that can be widely distributed in the environment, including soil and surface water used for agricultural purposes, wildlife feces, and other environment (IVANEK et al., 2009; SAUDERS et al., 2012, STRAWN et al., 2013) and food (CASARIN et al., 2014). *L. monocytogenes* can survive in wide ranges of pH (4.0–

9.5), temperature (1–45 °C) and can survive at a water activity as low as 0.92 (CARPENTIER; CERF, 2011, LIANOU; KOUTSOUMANIS, 2013).

Listeriosis, which is caused by *L. monocytogenes,* primarily affects pregnant women, newborns and immunocompromised adults may cause sepsis, abortion and infection of the central nervous system and resulting in high morbidity and mortality (ALLENBERGER; WAGNER, 2009). Although having a low incidence compared to other foodborne diseases, such as salmonellosis or campylobacteriosis (CDC, 2011), it is considered as a major public health issue due to its high hospitalization rate (94%) and a high case-fatality rate (12.8 to 17% of cases) (CDC, 2011; ECDPC, 2013).

Both pathogenic and nonpathogenic species of *Listeria* have been isolated from raw milk samples (AURORA et al., 2009; JAMALI et al., 2013; JAMI et al., 2010; NERO et al., 2008; RAHIMI et al., 2010; SARANGI et al., 2009; SONI et al., 2013; VANEGAS et al., 2009), milk products (BARANCELLI et al., 2011; MOLLA et al., 2004) and its processing environments (BARANCELLI et al., 2011; CHAMBEL et al., 2007; DOIJAD et al., 2011). Temporal breakdowns in hygiene barrier efficiency, poor hygiene practices and unhygienic design of equipment may trigger *L. monocytogenes* food plant contamination (ALMEIDA et al., 2013; CARPENTIER; CERF, 2011; FOX et al., 2011a; IBBA et al., 2013).

The presence of *L. monocytogenes* in the environment of the dairy plants can be a potential source of contamination of the final product, especially when it is protected by milk solids and spread from the processing environment to the final product through the ventilation system, dripping and splashing, or by workers (KELLS; GILMOUR, 2004).

Pasteurization does not eliminate further risks of dairy products contamination by *L. monocytogenes* (GOULD et al., 2014; OLIVER; JAYARAO; ALMEIDA,

2005). Contamination with *L. monocytogenes* may occur at several stages of cheese production and originate from multiple sources, including starter cultures, brine, drains, floor, packaging material, cheese vat, shelves, cheese cloth, curd cutting knife, brushes and coolers (ALMEIDA et al., 2013; BARANCELLI et al., 2011; BRITO et al., 2008; CHAMBEL et al., 2007; LOMONACO et al., 2009; LONGHI et al., 2003; PARISI et al., 2013).

Cheeses are mostly ready to eat products, which do not have any step of thermal treatment before consumption and are usually conserved at refrigeration temperatures that allow the survival and growth of psycrothrophic bacteria, such as *L. monocytogenes* (JASSON et al., 2010). Different *L. monocytogenes* strains have been recurrently found on surfaces of food industry equipment despite regular cleaning and disinfection practices (ALMEIDA et al., 2013; BARANCELLI et al., 2011; LATORRE et al., 2009; PARISI et al., 2013).

Notably, many *L. monocytogenes* strains are capable of adhering to various both biotic (e.g. animal tissues) and abiotic (e.g. stainless steel, plastic) surfaces and produce biofilm (RENIER; HÉBRAUD; DESVAUX, 2011). Attachment to surfaces is believed to be important for survival and persistence of this pathogen in food processing environments, with some strains being able to remain on equipment surfaces even for several years (CARPENTIER; CERF, 2011; HOLLAL; BIRD; HALL, 2004; MØRETRØ; LANGSRUD, 2004).

In Brazil, human listeriosis is underdiagnosed and underreported (SILVA et al., 2010), and there is no evidence of foodborne transmission (BLUM-MENEZES et al., 2013; BRITO et al., 2008; MARTINS; GERMANO, 2011). In most countries, including Brazil, risk assessments are difficult to undertake due to numerous knowledge gaps (RISTORI et al., 2014).

2.3 Biofilm production by pathogenic bacteria

Biofilms are large, complex, and organized bacterial ecosystems in which water channels are dispersed providing passages for nutrient, metabolite, and waste product exchange (SAUER et al., 2007). These architecturally complex assemblies of microorganisms can be form on biotic or abiotic surfaces or at interfaces (JAHID et al., 2013). When the contamination of food products occurs, the biofilms are the major source of contamination the persistence of biofilm on food contact surfaces, and equipment, may constitute a continuous source of contamination (ABDALLAH et al., 2014a). Many pathogenic or spoilage bacteria can be found attached to surfaces in the form of planktonic cells or sessile cells or in a biofilm (BRAGA et al., 2005).

Several studies have shown the ability of *S. aureus* to attach on food contact surfaces such as metal, rubber, polypropylene, glass, wood and food products (SIMÕES et al., 2010). The persistence of biofilms on these surfaces presents a continuous source of food contamination resulting in food poisoning (ABDALLAH et al., 2014a) and is believed to have a significant public health impact (BRIDIER et al., 2015).

In the food industry, factors such as pH, temperature changes, surface type and nutrient availability and among other factors influence the biofilm formation of *S. aureus* (HERRERA et al., 2007; MARQUES et al., 2007; MEIRA et al., 2012). These factors may also enhance the resistance of sessile cells to disinfecting agents (BELESSI et al., 2011; BRIDIER et al., 2011a; MEIRA et al., 2012) which makes biofilm elimination from food processing facilities a big challenge (GILBERT; ALLISON; McBAIN, 2002).

Foodborne pathogens can enter the milk processing equipment by direct contact with contaminants in the dairy farm environment (SIMÕES et al., 2010) and also through the water used in the milking machines (OLIVER; JAYARAO; ALMEIDA, 2005). Several bacteria are known to form biofilms on different materials (DI CICCIO et al., 2012). *Bacillus cereus, Escherichia coli, Shigella* spp. and *Staphylococcus aureus* have been detected in biofilms developing in the dairy and egg processing industries (JAN et al., 2011; SHI, ZHU, 2009). The presence of *Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Salmonella* spp. *Staphylococcus* spp. and *Escherichia coli* O157:H7 have been related to outbreaks associated with biofilms in dairy industry (AARNELA et al., 2007; DANIELSSON-THAM, 2002; DYKES; SAMPATHKUMAR; KORBER, 2003; ROMLING; YARON, 2006; SHARMA; ANAND, 2002a; WAAK; THAM; LAPIDOT).

Besides bacteria genus and species, extrinsic factors also play a major role influencing the degree of attachment and biofilm formation. Contact surfaces with food were postulated to have a large effect on the level of attachment and biofilm formation (SREY et al., 2013) due to the interaction between the microbial cell and the surfaces. The attachment of microbial cells on the substratum surface can be influenced by the electrostatic charge, hydrophobicity, composition, surface roughness and surface conditioning of the surface (TEH et al., 2014). Several reports have already shown the ability of bacteria to produce biofilms on materials commonly used in food industry, such as the stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, titanium, aluminum, and ceramic. (SIMÕES et al. 2010, VÁZQUEZ-SÁNCHES et al., 2013; HAMADI et al., 2014).

Biofilm growth on surfaces in the dairy environment can result in economic losses (BREMER, FILLERY, McQUILLAN, 2006; GRAM et al., 2007) because of the

release of bacteria into the dairy product, issues related to food spoilage and safety, and difficulties in cleaning and maintaining hygiene on the dairy farm or in dairy manufacturing plants (THE et al., 2014). Also, the existence of biofilms on heat transfer surfaces reduces the performance of heat exchangers, is a serious problem in many industrial and smaller scale processes (POGIATZIS et al., 2015).

Simões et al. (2010) affirm that ideally, preventing biofilm formation would be more logical than treating it. However, current biofilm control methods including high temperature and chemical methods have their own limitations due to sensitive substances that may be damaged by the heat and hydrolytically and also some chemically control methods may represent an environmental hazard (WHITE et al., 2006; MUN et al., 2009; SIMÕES et al., 2010).

2.4 Methodologies for assessing biofilms

Besides the food industry, biofilms also cause severe problems in many other industries, such as paper production, petroleum, nuclear power plants, and marine industries (BIXLER; BHUSHAN 2012; FLEMMING et al., 2013). The settlement and persistence of food-borne pathogens in food processing environment is closely related to their response to both biotic and abiotic factors (WINKELSTROTER et al., 2014).

Most of the pathogens involved in food-borne diseases are able to adhere to and produce biofilms in the majority of materials encountered in food production plants. Polystyrene and stainless steel are the most widely used materials in the food sector (DI CICCIO et al., 2015). Stainless steel material is traditionally selected for food equipment because it is durable, resistant to corrosion, and it is easily cleaned (SHI; ZHU, 2009).

Static biofilm systems may be preferable to continuous flow methods for a number of reasons which are the simplicity of experimental procedures, the adaptation to a variety of conditions, and a high screening capacity (ABDALLAH et al., 2014a). Microtiter plate (MTP)-based assays are among the most common systems used for the study of biofilm formation and resistance toward disinfectants (THERAUD et al., 2004). In MTP assay the biofilm is either grown on the bottom or on the walls of wells. Crystal violet (CV) staining is a colorimetric method that has been used widely to measure biofilm formation in part because of its amenability to large screening procedures (O'TOOLE; KOLTER, 1998; PITTS et al., 2003) and this staining is one of the first methods adopted for biofilm biomass quantification (CHRISTENSEN et al., 1985; STEPANOVIĆ et al., 2000). The advantage of this method is the high number of conditions that can be analyzed in one experiment and the low cost of experiments (ABDALLAH et al., 2014a). Despite the advantages of the above-described, static biofilm systems are limited by nutrient availability, due to this limitation continuous flow systems were developed in order create a continuous or semi-continuous medium flow (ABDALLAH et al., 2014a).

A number of microscopic techniques have been used for examination of biofilm, such as scanning electron microscopy (BUI et al., 2015; CASARIN et al., 2014; DI CICCIO et al., 2015; JONGSMA et al., 2015; SOUZA et al., 2014), fluorescence microscopy (JUNG et al., 2015; LUTSKIY et al., 2015; SAKIMURA et al., 2015; SEYEUX et al., 2015) and confocal laser scanning (ARIAS-MOLIZ et al., 2014; CÁRCAMO-OYARCE et al., 2015; JIN et al., 2005; KAMJUNKE, HERZSPRUNG; NEU, 2015; KHALIFA 2015; LI et al., 2014; QUINTAS et al., 2015; TAMURA et al., 2015; TIMMERMANS et al., 2015; ZHANG et al., 2014).

LIVE/DEAD® BacLight assay is method based on two different nucleic acid binding stains. The first dye is Syto9 (green fluorescent) is able to cross bacterial membranes and bind to DNA of both Gram-positive and Gram-negative bacteria, the second dye is propidium-iodide (red fluorescent) that crosses only damaged bacterial membranes (PANTANELLA et al., 2013). The stained samples are observed using a fluorescent optical microscopy to evaluate live and dead bacterial population. The main drawback of this method is the need of observing statistically relevant portion of the sample to be representative (PANTANELLA et al., 2013). In any case the method provides only semi-quantitative results (BOULOS et al., 1999; JIN et al., 2005).

2.5 Cleaning and disinfection of surfaces in the dairy plant

Food-borne disease can be transmitted by surface contamination of equipment and utensils, this risk can be effectively reduced by washing and sterilization with a disinfection agent (LI, KUDA, YANO, 2014). The persistence of pathogenic bacteria in food represents a great risk for public health. In fact, infections with pathogens may lead to serious human diseases worldwide (HOTA 2004; NEWELL et al., 2010). The regular action of cleaning and disinfection procedures is a common strategy used to control pathogen implantation on either industrial equipment or the products themselves (JAHID; HA, 2012). The effectiveness of a cleaning method is often determined by the state of the fouling layer (POGIATZIS et al., 2015). Prolonging cleaning can result in aging, which may convert the fouling layer from a readily removable form to one harder to remove (SOMMER; MARTIN-ROUAS; METTLER, 1999).

Most of the clinical guidelines of biocides have been developed for planktonic microorganisms (CERF; CARPENTIER; SANDERS, 2010). However, most of the

microorganisms live as surface-adherent communities. In addition, considerable works have already shown that the cells living under a biofilm state can be up to 1,000-fold more resistant to disinfectant products than their planktonic counterparts (BELESSI et al., 2011; BONEZ et al., 2013). Also these procedures are not fully effective on biofilm structures and can induce the selection of resistant phenotypes (SIMÕES et al., 2010).

The effectiveness of sanitizers on bacterial reduction has many factors depending on the product type, target microorganisms, time interval between contamination and washing, and treatment time (GIL et al., 2009). Effective control of biofilms can be achieved by understanding the type and nature of the contaminating residue materials (carbohydrates, fat, proteins, mineral salts) and the microorganisms to be removed from the surfaces (SIMÕES et al., 2010). Furthermore, the selection of detergents and disinfectants depends on their efficacy, safety and ease of removal; specifically relating to the corrosive nature of the chemical treatments and the subsequent sensory value effects on the final products (WIRTANEN; SAARELA; MATTILA-SANDHOLM, 2000). Usually the efficacy of disinfectants against biofilms increases with the increase in both biocide concentration and time of treatment (BELESSI et al., 2011; SURDEAU et al., 2006). Sanitizers are usually regarded as effective when they can reduce the targeted organism by at least 3 log (99.9%) in their numbers (PARK et al., 2013).

Peracetic acid (CH_3CO_3H) (PAA) is known as sanitizer and has proven to be effective on vegetative bacteria (FRÖHLING, 2011). PAA is a strong disinfectant with a wide spectrum of antimicrobial activity that was introduced to wastewater treatment several years ago (ANTONELLI et al., 2006; FALSANISI et al., 2006; KITIS, 2004; KOIVUNEN; HEINONEN-TANSKI, 2005).

PAA is active at low temperatures (0–25 °C), in a pH range of 3–7.5, and it does not produce mutagenic by-products, as it happens with chlorine and its derivate (ÖLMEZ; KRETZSCHMAR, 2009). The mechanism of action of PAA against micro-organisms is based in the denaturation of proteins and enzymes and increased cell wall permeability by oxidizing sulfhydril and disulfide bonds (KITIS, 2004).

PAA may be used as a sanitizer because this agent decomposes into safe and environmental friendly residues (acetic acid and hydrogen peroxide), hence it can be applied without rinsing risks of food contamination by toxic residues and its efficacy is not affected by protein residues (SOUZA et al., 2014). In addition, there are no reports in the literature pointing PAA as carcinogenic or that it presents toxicity in the reproduction and human development (SOUZA et al., 2014). PAA has been used as a disinfectant for many years in the food, beverage, and paper industries (RASIMUS et al., 2011).

In addition to biological and/or chemical alternatives, new physical processes (e.g. pulsed-light or laser decontamination devices) are a promising option for industrial applications. Plasma, the fourth state of matter, is an ionized gas and can be generated using a range of gases or gas mixtures, typically argon, helium, nitrogen, air or oxygen (MAI-PROCHNOW et al., 2014). Plasmas can be divided into thermal plasma and non-thermal plasma (KEENER, 2008; MOREAU; ORANGE; FEUILLOLEY, 2008). Cold plasma (CP) is an emerging nonthermal technology that could potentially decontaminate the surfaces of fresh produce (LACOMBE et al., 2015). This emerging technology offers the advantage of being chemical and water-free, also the possibility of being operated openly and continuously at atmospheric pressure (NIEMIRA, 2012a; NIEMIRA; SITES, 2008; NORIEGA et al., 2011). CP can efficiently kill or inactivate bacteria, yeasts and molds and other hazardous

microorganisms, as well as spores and biofilms that are generally very difficult to inactivate (KAMGANG et al., 2007; KAMGANG-YOUBI et al., 2009; NIEMIRA, 2012a). Previous studies have been done with the treatment of food surfaces associated microbial contaminations (BAIER et al., 2013; FRÖHLING et al., 2012). By definition microorganism can be classified as resistant, intermediate and sensitive to antibiotics or sanitizers, this classification is based on an *in vitro* response of an organism to an antimicrobial agent. A microorganism is considered resistant when it shows continued growth, even in the presence of antibiotic.

The parameters for evaluation of antimicrobial susceptibility are proposed by the Clinical and Laboratory Standards Institute (CLSI, 2012). The CLSI consists of an interdisciplinary subcommittee, with one of the objectives to continuously improve the standards used to evaluate the antimicrobial susceptibility of microorganisms, detect emerging resistance mechanisms by reviewing and developing new methods, and promote the education of users by sharing standardized processes and manuals (CLSI, 2012). Either broth or agar dilution methods can be used to measure quantitatively the *in vitro* activity of an antimicrobial agent against a given bacterial. To perform the tests, a series of tubes or plates is prepared with a broth or agar medium to which various concentrations of the antimicrobial agents are added (CLSI, 2012).

Sanitizers are considered as agents or products that reduce the number of live bacteria in the environment or in a product, to safe levels, according to health standards (BRASIL, 2007). The ideal sanitizer should be approved by the competent organs, has a wide spectrum of antimicrobial activity, be able to destruct microorganisms within a short period time and be stable, and present low toxicity and corrosivity (ANDRADE; PINTO; ROSADO, 2008). In conclusion it’s important to

notice the presence of the pathogenic bacteria in the dairy industry, their ability to produce biofilm and the need to avoid this structure within the cooperation between the academic field and industry. Different types of inactivation technologies such as PAA treatment and CP treatment have to be established to fulfil the requirements of food safety.

2.6 References

AARNELA, K. et al. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. **LWT- Food Science and Technology**, London, v. 40, p. 1041-1048, 2007.

ABDALLAH, M. et al. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. **Archives of Microbiology**, Berlin, v. 196, p. 453-472, 2014.

ABDALLAH, M. et al. Effect of growth temperature, surface type and incubation time on the resistance of *Staphylococcus aureus* biofilms to disinfectants. **Applied Microbiology and Biotechnology**, Berlin, v. 98, p. 2597-2607, 2014b.

ABEER, A. A.; ABDEL, ALL; DARDIR, H. A. Hygienic quality of local traditional fermented skimmed milk (laban rayb) sold in Egypt. **World Journal of Dairy and Food Sciences**, Lancaster, v. 4, p. 205-209, 2009.

ALLENBERGER, F.; WAGNER, M. Listeriosis: a resurgent foodborne infection. **Clinical Microbiology and Infection**, Paris, v. 16, p. 16-23, 2009.

ALMEIDA, G. et al. Foci of contamination of *Listeria monocytogenes* in different cheese processing plants. **International Journal of Food Microbiology**, Amsterdam, v. 167, p. 303-309, 2013.

ANDRADE, N. J.; PINTO, C. L. O.; ROSADO, M. S. **Higiene na indústria de alimentos**: avaliação e controle da adesão e formação de biofilmes bacterianos. 2. ed. São Paulo: Varela, 2008.

ÂNGELO, F. F.; ARCURI, E. F.; ANDRADE, N. J. Ocorrência do gene da toxina da síndrome do choque tóxico em *Staphylococcus* spp. isolados de leite de vacas com mastite e de leite cru refrigerado. **Revista Brasileira de Ciência Veterinária**, Niterói, v. 21, p. 137-140, 2014.

ANTONELLI, M. et al. Secondary effluent disinfection: PAA long term efficiency, environmental science and technology. **Environmental Science and Technology**, Easton, v. 40, p. 4771-4775, 2006.

ARGUDIN, M. A.; MENDOZA, M. C.; RODICIO, M. R. Food poisoning and *Staphylococcus aureus* enterotoxins. **Toxins**, Basel, v. 2, p. 1751-1773, 2010.

ARIAS-MOLIZ, M. T. et al. Antimicrobial activity of chlorhexidine, peracetic acid and sodium hypochlorite/etidronate irrigant solutions against *Enterococcus faecalis* biofilms. **International Endodontic Journal**, Oxford, Dec. 2014. (In press).

AURORA, R.; PRAKASH, A.; PRAKASH, S. Genotypic characterization of *Listeria monocytogenes* isolated from milk and read to eat indigenous milk products. **Food Control**, Guildford, v. 20, p. 835-839, 2009.

BAIER, M. et al. Direct non-thermal plasma treatment for the sanitation of fresh corn salad leaves: evaluation of physical and physiological effects and antimicrobial efficacy. **Postharvest Biology and Technology**, Amsterdam, v. 84, p. 81-87, 2013.

DEN BAKKER, H. C. et al. Five new species of *Listeria* (*L. floridensis* sp. nov, *L. aquatica* sp. nov., *L. cornellensis* sp. nov. *L. riparia* sp. nov., and *L. grandensis* sp. nov.) from agricultural and natural environments in the United States. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 64, p. 1882-1889, 2014.

BARANCELLI, G. V. et al. Incidence of *Listeria monocytogenes* in cheese manufacturing plants from the northeast region of São Paulo, Brazil. **Journal of Food Protection**, Des Moines, v. 74, p. 816-819, 2011.

BELESSI, C. E. et al. Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. **International Journal of Food Microbiology**, Amsterdam, v. 1, p. S46-S52, 2011.

BERTSCH, D. et al. *Listeria fleischmannii* sp. nov., isolated from cheese. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 63, p. 526-532, 2013.

BIXLER, G. D.; BHUSHAN, B. Biofouling: lessons from nature. **Philosophical Transactions of the Royal Society A: Mathematical, Physical & Engineering Sciences**, London, v. 370, p. 2381-2417, 2012.

BLUM-MENEZES, D. et al. Listeriosis in the far south of Brazil: neglected infection? **Revista Sociedade Brasileira de Medicina Tropical**, Uberaba, v. 46, p. 381-383, 2013.

BONEZ, P. C. et al. Chlorhexidine activity against bacterial biofilms. **American Journal of Infection Control**, Saint Louis, v. 1, p. 854-857, 2013.

BOTREL, M. A. et al. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Alpes, France. **Foodborne Pathogens and Disease**, Larchmont, v. 7, p. 479-487, 2010.

BOULOS, L. et al. LIVE/DEAD® BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. **Journal of Microbiological Methods**, Amsterdam, v. 37, p. 77-86, 1999.

BRADLEY, A. J. et al. Survey of the incidence and etiology of mastitis on dairy farms in England and Wales. **Veterinary Record,** London, v. 160, p. 253-258, 2007.

BRAGA L. C. et al. Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production. **Journal of Ethnopharmacology,** Lausanne, v. 96, p. 335-339, 2005.

BRASIL. Agência Nacional de Vigilância Sanitária. Resolução da Diretoria Colegiada - RDC n.º 14, de 28 de fevereiro de 2007. Regulamento Técnico para Produtos Saneantes com Ação Antimicrobiana harmonizado no âmbito do Mercosul, e dá outras providências. **Diário Oficial da União**, Brasília, DF, 5 mar. 2007. Suplemento, p. 2.

BREMER, P. J.; FILLERY, S.; McQUILLAN, A. J. Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. **International Journal of Food Microbiology**, Amsterdam, v. 106, p. 254-262, 2006.

BRIDIER, A. et al. Comparative biocidal activity of peracetic acid, benzalkonium chloride and orthophthalaldehyde on bacterial strains. **Journal of Hospital Infection**, v. 78, p. 208-213, 2011b.

BRIDIER, A. et al. Biofilm-associated persistence of food-borne pathogens. **Food Microbiology**, London, v. 45, p. 167-178, 2015.

BRITO, J. R. et al. Retail survey of Brazilian milk and minas frescal cheese and a contaminated dairy plant to establish prevalence, relatedness and sources of *Listeria monocytogenes* isolates. **Applied and Environmental Microbiology**, Washington, v. 74, p. 4954-4961, 2008.

BUI, L. M. G. et al. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. **Infection and Immunity**, Washington, v. 83, p. 470-481, 2015.

BUNTHOF, C. J.; ABEE, T. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. **Applied Environmental Microbiology**, Washington, v. 68, p. 2934-2942, 2002.

CÁRCAMO-OYARCE1, G. et al. Quorum sensing triggers the stochastic escape of individual cells from *Pseudomonas putida* biofilms. **Nature Communications**, London, v. 6, art. 5945, 2015.

CARMO, L. S. et al. Food poisoning due to enterotoxigenic strains of *Staphylococcus* present in Minas cheese and raw milk in Brazil. **Food Microbiology,** London, v. 19, p. 9-14, 2002.

CARPENTIER, B.; CERF, O. Review persistence of *Listeria monocytogenes* in food industry equipment and premises. **International Journal of Food Microbiology**, Amsterdam, v. 145, p. 1-8, 2011.

CASARIN, L. S. et al. Adhesion of *Salmonella Enteritidis* and *Listeria monocytogenes* on stainless steel welds. **International Journal of Food Microbiology**, Amsterdam, v. 191, p. 103-108, 2014.

CERF, O.; CARPENTIER, B.; SANDERS, P. Tests for determining in use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. **International Journal of Food Microbiology**, Amsterdam, v. 136, p. 247-254, 2010.

CENTER FOR DISEASE CONTROL AND PREVENTION. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food-Foodborne diseases active surveillance network, 10 U.S. sites, 1996–2010. **Morbidity and Mortality Weekly Report**, Atlanta, v. 60, n. 22, p. 749-755, 2011.

CHAMBEL, L. et al. Occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal unveiled by an integrated analysis of identification, typing and spatial: temporal mapping along production cycle. **International Journal of Food Microbiology**, Amsterdam, v. 116, p. 52-63, 2007.

CHRISTENSEN, G. D. et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. **Journal of Clinical Microbiology,** Washington, v. 2, p. 996-1006, 1985.

ĆIRKOVIĆ, I. et al. Methicillin-resistant *Staphylococcus aureus* biofilm formation on dacryocystorhinostomy silicone tubes depends on the genetic lineage. **Graefe's Archive for Clinical and Experimental Ophthalmology**, Berlin, v. 253, p. 77-82, 2015.

CORTIMIGLIA, C. et al. Short communication: prevalence of *Staphylococcus aureus* and methicillin-resistant S. aureus in bulk tank milk from dairy goat farms in northern Italy. **Journal of Dairy Science**, Champaign, 2015. (In Press).

CRUZ, F. T.; MENASCHE, R. Tradition and diversity jeopardised by food safety regulations? The Serrano Cheese case, Campos de Cima da Serra region, Brazil. **Food Policy**, Guildford, v. 45, p. 116-124, 2014.

DI CICCIO, P. et al. Biofilm formation by *Staphylococcus aureus* on food contact surfaces: relationship with temperature and cell surface hydrophobicity. **Food Control**, Guildford, v. 50, p. 930-936, 2015.

DOIJAD, S. et al. Incidence and genetic variability of Listeria species from three milk processing plants. **Food Control**, Guildford, v. 22, p. 1900-1904, 2011.

DYKES, G. A.; SAMPATHKUMAR, B.; KORBER, D. R. Planktonic or biofilm growth affects survival, hydrophobicity and protein expression patterns of a pathogenic *Campylobacter jejuni* strain. **International Journal of Food Microbiology,** Amsterdam, v. 80, p. 1-10, 2003.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL (ECDC). **Annual epidemiological report 2013**: reporting on 2011 surveillance data and 2012 epidemic intelligence data. Stockholm: ECDC, 2012.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA). The community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. **EFSA Journal**, Parma, v. 8, p. 1496, 2010.

FALSANISI, D. et al. Kinetics of PAA demand and its implications on disinfection of wastewaters. **Water Quality Research Journal of Canada**, London, v. 41, p. 398-409, 2006.

FLEMMING, H. C.; MEIER, M.; SCHILD, T. Mini-review: microbial problems in paper production. **Biofouling**, Oxfordshire, v. 29, p. 683-696, 2013.

FOX, E. et al. *Listeria monocytogenes* in Irish Farmhouse cheese processing environments. **International Journal of Food Microbiology**, Amsterdam, v. 145, Suppl. 1, p. S39-45, 2011.

FRÖHLING, A. et al. Atmospheric pressure plasma treatment of *Listeria innocua* and *Escherichia coli* at polysaccharide surfaces: inactivation kinetics and flow cytometric characterization. **Innovative Food Science and Emerging Technologies**, Amsterdam, v. 13, p. 142-150, 2012.

GIL, M. I. et al. Fresh-cut product sanitation and wash water disinfection: problems and solutions. **International Journal of Food Microbiology**, Amsterdam, v. 134, p. 37-45, 2009.

GILBERT, P.; ALLISON, D. G.; McBAIN, A. J. Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? **Journal of Applied Microbiology**, Oxford, v. 92, p. 98-110, 2002.

GOULD, L. H.; MUNGAI, E.; BEHRAVESH, C. B. Outbreaks attributed to cheese: differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. **Foodborne Pathogens and Disease**, Larchmont, v. 11, p. 545-551, 2014.

GRAM, L. et al. Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. **Food Control,** Guildford, v. 18, p. 1165-1171, 2007.

GUSTAFSON, J. E. et al. *Staphylococcus aureus* and understanding the factors that impact enterotoxin production in foods: A review. **Food Control**, Guildford, p. 1-14, 2014. (In press*)*.

HAMADI, F. et al. Adhesion of *Staphylococcus aureus* on stainless steel treated with three types of milk. **Food Control,** Guildford, v. 38, p. 104-108, 2014.

HENNEKINNE, J. K.; De BUYSER, M. L.; DRAGACCI, S. *Staphylococcus aureus* and its food-poisoning toxins: characterization and outbreak investigation. **FEMS Microbiology Reviews**, Amsterdam, v. 36, p. 815-836, 2012.

HERRERA, J. J. R. et al. Adhesion and detachment kinetics of several strains of *Staphylococcus aureus* subsp. aureus under three different experimental conditions. **Food Microbiology**, Guildford, v. 24, p. 585-591, 2007.

HOLAH, J. T.; BIRD, J.; HALL, K. E. The microbial ecology of high-risk, chilled food factories: evidence for persistent *Listeria* spp. and *Escherichia coli* strains. **Journal of Applied Microbiology**, Oxford, v. 97, p. 68-77, 2004.

HOTA, B. Contamination, disinfection, and cross-colonization: are hospital surfaces reservoirs for nosocomial infection? **Clinical Infection Disease,** Oxford, v. 39, p. 1182-1189, 2004.

IBBA, M. et al. *Listeria monocytogenes* contamination in dairy plants: evaluation of *Listeria monocytogenes* environmental contamination in two cheese-making plants using sheeps milk. **Italian Journal of Food Safety**, Pavia, v. 2, p. 109-112, 2013.

IVANEK, R. et al. Modeling of spatially referenced environmental and meteorological factors influencing the probability of Listeria Species isolation from natural environments. **Applied and Environmental Microbiology,** Washington, v. 75, p. 5893-5909, 2009.

JAHID, I. K. et al. Influence of glucose concentrations on biofilm formation, motility, exoprotease production, and quorum sensing in *Aeromonas hydrophila*. **Journal of Food Protection**, Des Moines, v. 76, p. 239-247, 2013.

JAMALI, H.; RADMELIA, B.; THONG, K. L. Prevalence, characterization and antimicrobial resistance of *Listeria* species and *Listeria monocytogenes* isolates from raw milk in farm bulk tanks. **Food Control**, Guildford, v. 34, p. 121-125, 2013.

JAMI, S.; JAMSHIDI, A.; KHANZADI, S. The presence of *Listeria* spp. in raw milk samples in Mashhad, Iran. **World Applied Sciences Journal**, Dubai, v. 10, p. 249-253. 2010.

JAN, S. et al. Biodiversity of psychrotrophic bacteria of the *Bacillus cereus* group collected on farm and in egg product industry. **Food Microbiology**, London, v. 28, p. 261-265, 2011.

JASSON, V. et al. Alternative microbial methods: an overview and selection criteria. **Food Microbiology**, London, v. 27, p. 710-730, 2010.

JIN, Y. et al. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms. **Mycopathologia**, Den Haag, v. 159, p. 353-360, 2005.

JONGSMA, M. A. et al. In vivo biofilm formation on stainless steel bonded retainers during different oral health-care regimens. **International Journal of Oral Science**, Sichuan, v. 7, p. 1-7, 2015.

JUNG, Y. et al. Embedded biofilm, a new biofilm model based on the embedded growth of bacteria. **Journal of Applied Environmental Microbiology**, Oxford, v. 81, p. 211-219, 2015.

KELLS, J.; GILMOUR, A. Incidence of *Listeria monocytogenes* in two milk processing environments, and assessment of Listeria monocytogenes blood agar for isolation. **International Journal of Food Microbiology,** Amsterdam, v. 91, p. 167-174, 2004.

KITIS, M. Disinfection of wastewater with peracetic acid: a review. **Environment International**, New York, v. 30, p. 47-55, 2004.

KAMGANG, J. O. et al. Destruction of planktonic, adherent and biofilm cells of *Staphylococcus epidermidis* using a gliding discharge in humid air. **Journal of Applied Microbiology,** Oxford, v. 103, p. 621-628, 2007.

KAMGANG-YOUBI, G. et al. Microbial inactivation using plasma-activated water obtained by gliding electric discharges. **Letters in Applied Microbiology**, Oxford, v. 48, p. 13-18, 2009.

KAMJUNKE, N.; HERZSPRUNG, P.; NEU, T. R. Quality of dissolved organic matter affects planktonic but not biofilm bacterial production in streams. **Science of the Total Environment**, Amsterdam, v. 506-507, p. 353-360, 2015.

KHALIFA, L. et al. Targeting *Enterococcus faecalis* biofilm using phage therapy. **Applied and Environmental Microbiology**, Washington, 2015. (In press).

KOIVUNEN, J.; HEINONEN-TANSKI, H. Peracetic acid (PAA) disinfection of primary, secondary and tertiary treated municipal wastewaters. **Water Research**, New York, v. 39, p. 4445-4453, 2005.

MOLLA, B.; YILMA, R.; ALEMAYEHU, D. *Listeria monocytogenes* and other *Listeria* species in retail meat and milk products in Addis Ababa, Ethiopia. **Ethiopian Journal of Health Development**, Addis Ababa, v. 18, p. 208- 212, 2004.

LACOMBE, A. et al. Atmospheric cold plasma inactivation of aerobic microorganisms on blueberries and effects on quality attributes. **Food Microbiology**, London, v. 46, p. 479-484, 2015.

LAPIDOT, A.; ROMLING, U.; YARON, S. Biofilm formation and the survival of *Salmonella typhimurium* on parsley. **International Journal of Food Microbiology**, Amsterdam, v. 109, p. 229-233, 2006.

LARSEN, H. D. et al. Differences between Danish bovine and human *Staphylococcus aureus* isolates in possession of superantigens**. Veterinary Microbiology**, Amsterdam,v. 76, p. 153-162, 2000a.

LARSEN, H. D. et al. The dynamics of *Staphylococcus aureus* intramammary infection
in nine Danish dairy herds. **Veterinary Microbiology**, Amsterdam, v. 71, p. 89-101, 2000b.

LATORRE, A. A. et al. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking on a dairy farm. **Applied and Environmental Microbiology**, Washington, v. 75, p. 1315-1323, 2009.

LI, R.; KUDA, T.; YANO, T. Effect of food residues on efficiency of surfactant disinfectants against food related pathogens adhered on polystyrene and ceramic surfaces. **LWT-Food Science and Technology**, London, v. 57, p. 200-206, 2014.

LIANOU, A.; KOUTSOUMANIS, K. P. Strain variability of the behavior of foodborne pathogens: A review. **International Journal of Food Microbiology**, Amsterdam, v. 167, p. 310-321, 2013.

LIU, D. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. **Journal of Medical Microbiology**, v. 55, p. 645-659, 2006.

LOMONACO, S. et al. *Listeria monocytogenes* in gorgonzola: subtypes, diversity and persistence over time. **International Journal of Food Microbiology**, Amsterdam, v. 128, p. 516-520, 2009.

LONGHI, C. et al. Detection of *Listeria monocytogenes* in Italian-style soft cheeses. **Journal of Applied Microbiology**, Oxford, v. 94, p. 879-885, 2003.

LUTSKIY, M. et al. A microbiology-based assay for quantification of bacterial early stage biofilm formation on reverse-osmosis and nanofiltration membranes. **Separation and Purification Technology**, Kidlington, v. 141, p. 214-220, 2015.

MARQUES, S. C. et al. Formation of biofilm by *Staphylococcus aureus* on stainless steel and glass surfaces and its resistance to some selected chemical sanitizers. **Brazilian Journal of Microbiology**, São Paulo, v. 38, p. 538-543, 2007.

MARTINS, E. A.; GERMANO, P. M. L. *Listeria monocytogenes* in ready-to- eat, sliced, cooked ham and salami products, marketed in the city of São Paulo, Brazil: occurrence, quantification, and serotyping. **Food Control**, Guildford, v. 22, p. 297-302, 2011.

MEIRA, Q. G. S. et al. Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food- contact surfaces and sensitivity to sanitizers. **Food Control**, Guildford, v. 25, p. 469-475, 2012.

MORETRO, T.; LANGSRUD, S. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. **Biofilms**, Cambridge, v.1, n. 2, p. 107-121, 2004.

MUN, S. et al. Inactivation of *Pseudomonas aeruginosa* biofilm by dense phase carbon dioxide. **Biofouling**, Oxfordshire, v. 25, p. 473-479, 2009.

NERO, L. A.; MAZIERO, D.; BEZERRA, M. M. S. Hábitos alimentares do consumidor de leite cru de Campo Mourão-PR. **Semina**, Londrina, v. 24, p. 21-26, 2003.

NERO, L. A. et al. *Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil: occurrence and interference of indigenous micro biota in their isolation and development. **Zoonoses Public Health**, Berlin, v. 55, p. 299-305, 2008.

NEWELL, D. G. et al. Food-borne diseases- the challenges of 20 years ago still persist while new ones continue to emerge. **International Journal of Food Microbiology,** Amsterdam, v. 30, n. 139, Suppl 1, p. S3-S15, 2010.

NIEMIRA, B. A. Cold plasma decontamination of foods. **Annual Review of Food Science and Technology**, Ohio, v. 3, p. 125-42, 2012a.

NIEMIRA, B. A.; SITES, J. Cold plasma inactivates salmonella stanley and Escherichia coli O157: H7 inoculated on golden delicious apples. **Journal of Food Protection**, Des Moines, v. 71, p. 1357-1365, 2008.

NORIEGA, E. et al. Cold atmospheric gas plasma disinfection of chicken meat and chicken skin contaminated with *Listeria innocua*. **Food Microbiology**, London, v. 28, p. 1293-1300, 2011.

OLIVER, S. P.; JAYARAO, B. M.; ALMEIDA, R. A. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. **Foodborne Pathogens and Disease**, Larchmont, v. 2, p. 115-129, 2005.

OLIVAL, A. A. et al. Hábitos de consumo do leite informal, associado ao risco de transmissão de doenças no município de Pirassununga, SP. **Higiene Alimentar**, São Paulo, v. 16, p. 35-40, 2002.

ÖLMEZ, H.; KRETZSCHMAR, U. Review: potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. **LWT- Food and Science Technology**, London, v. 42, p. 686-693, 2009.

O'TOOLE, G. A.; KOLTER, R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. **Molecular Microbiology**, Salem, v. 28, p. 449-461, 1998.

PANTANELLA, F. et al. Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. **Annals of Hygiene**, Rome, v. 25, p. 31-42, 2013.

PARISI, A. et al. Occurrence of *Listeria* spp. in dairy plants in southern Italy and molecular subtyping of isolates using AFLP. **Food Control**, Guildford, v. 29, p. 91-97, 2013.

PARK, E. et al. Efficiency of peracetic acid in inactivating bacteria, viruses, and spores in water determined with ATP bioluminescence, quantitative PCR, and culture-based methods. **Journal of Water and Health**, London, v. 12, p. 13-23, 2014.

PERILLO, J. et al. Molecular characterization of enterotoxigenic and borderline oxacillin resistant *Staphylococcus* strains from ovine milk. **Food Microbiology**, London, v. 32, p. 265-273, 2012.

PITTS, B. et al. A microtiterplate screening method for biofilm disinfection and removal. **Journal of Microbiological Methods**, Amsterdam, v. 54, p. 269-276, 2003.

POGIATZIS, T. A. et al. Choosing when to clean and how to clean biofilms in heat exchangers. **Heat Transfer Engineering**, London, v. 36, p. 676-684, 2015.

QUINTAS, V. et al. In situ antimicrobial activity on oral biofilm: essential oils vs. 0.2 % chlorhexidine. **Clinical Oral Investigations,** Berlin, v. 19, p. 97-107, 2015.

RAHIMI, E.; AMERI, M.; MOMTAZ, H. Prevalence and antimicrobial resistance of *Listeria* species isolated from milk and dairy products in Iran. **Food Control**, Guildford, v. 21, p. 1448-1452, 2010.

RASIMUS, S. et al. Biofilm-forming bacteria with varying tolerance to peracetic acid from a paper machine. **Journal of industrial microbiology and biotechnology**, Hampshire, v. 38 p. 1379-1390, 2011.

RENIER, S.; HÉBRAUD, M.; DESVAUX, M. Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen. **Environmental Microbiology**, Oxford, v. 13, p. 835-850, 2011.

RISTORI, C. A. et al. Prevalence and Populations of *Listeria monocytogenes* in meat products retailed in Sao Paulo, Brazil. **Foodborne Pathogens and Disease**, Larchmont, v. 11, p. 869-973, 2014.

SAKIMURA, T. et al. Biofilm-forming *Staphylococcus epidermidis* expressing vancomycin resistance early after adhesion to a metal surface. **BioMed Research International**, New York, v. 2015, art. 943056, 2015. (In press).

SARANGI, L. N. et al. Prevalence of *Listeria* species in milk samples of cattle of Odisha. **Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases**, Hisar, v. 30, p. 135-136, 2009.

SAUER, K.; RICKARD, A.; DAVIES, D. G. Biofilms and biocomplexity. **Microbe**, Washington, v. 2, p. 347-355, 2007.

SAUDERS, B. D. et al. Diversity of *Listeria* species in urban and natural environments. **Applied and Environmental Microbiology**, Washington, v. 78, p. 4420-4433, 2012.

SEYEUX, A. et al. The fate of the protective oxide film on stainless steel upon early stage growth of a biofilm. **Corrosion Science**, Oxford, v. 91, p. 352-356, 2015.

SHARMA, M.; ANAND, S. K. Characterization of constitutive microflora of biofilms in dairy processing lines. **Food Microbiology**, London, v. 19, p. 627-636, 2002a.

SILVA, N. et al. **Manual de métodos de análise microbiológica de alimentos e água**. 4. ed. São Paulo: Varela, 2010.

SIMÕES, M.; SIMÕES, L. C.; VIEIRA, M. J. A review of current and emergente biofilm control strategies. **LWT- Food Science and Technology**, London, v. 43, p. 573-583, 2010.

SHI, X.; ZHU, X. Biofilm formation and food safety in food industries. **Trends in Food Science & Technology**, Cambridge, v. 20, p. 407-413, 2009.

SOMMER, P.; MARTIN-ROUAS, C.; METTLER, E. Influence of the adherent population level on biofilm population, structure and resistance to chlorination. **Food Microbiology**, Amsterdam, v. 16, p. 503-515, 1999.

SONI, D. K. et al. Characterization of *Listeria monocytogenes* isolated from Ganges water, human clinical and milk samples of Varanasi, India. **Infection, Genetics and Evolution**, Amsterdam, v. 14, p. 83-91, 2013.

SOSPEDRA, I.; MAÑES, J.; SORIANO, J. M. Report of toxic shock syndrome toxin 1 (TSST-1) from *Staphylococcus aureus* isolated in food handlers and surfaces from foodservice establishments. **Ecotoxicology and Environmental Safety**, New York, v. 80, p. 288-290, 2012.

SPOOR, L. E. et al. Livestock origin for a human pandemic clone of community-associated methicillin-resistant *Staphylococcus aureus*. **mBio**, Washington ,v. 4, p. 356-313, 2013.

SOUZA, E. L. et al. Biofilm formation by *Staphylococcus aureus* from food contact surfaces in a meat-based broth and sensitivity to sanitizers. **Brazilian Journal of Microbiology**, São Paulo, v. 45, n. 1, p. 67-75, 2014.

SREY, S.; JAHID, I. K.; HA, S-Do. Biofilm formation in food industries: a food safety concern. **Food Control**, Guilford, v. 31, p. 572-585, 2013.

STEPANOVIĆ, S. et al. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. **Journal of Microbiological Methods,** Amsterdam, v. 40, p. 175-179, 2000.

STRAWN, L. K. et al. Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. **Environmental Microbiology**, Oxford, v. 79 p. 588-600, 2013.

SURDEAU, N. et al. Sensitivity of bacterial biofilms and planktonic cells to a new antimicrobial agent, Oxsil 320N. **The Journal of Hospital Infection**, London, v. 62, p.487-493, 2006.

TAMURA, N. K. et al. Adherence and biofilm formation of *Fusarium oxysporum* isolated from a corneal ulcer. **Global Advanced Research Journal of Medicine and Medical Science**, Nigeria, v. 4. p. 28-34, 2015

THE, K. H. et al. Biofilm: an unrecognised source of spoilage enzymes in dairy products? **International Dairy Journal**, Barking, v. 34, p. 32-40, 2014.

THERAUD, M. et al. Efficacy of antiseptics and disinfectants on clinical and environmental yeast isolates in planktonic and biofilm conditions. **Journal of Medical Microbiology**, London, v. 53, p. 1013-1018, 2004.

TIMMERMANS, A. J. et al. Biofilm formation on the Provox ActiValve: composition and ingrowth analyzed by Illumina paired-end RNA sequencing, fluorescence in situ hybridization and confocal laser scanning microscopy. **Head Neck**, New York, 2015. (In press).

TONDO, E. C. et al. Assessing and analysing contamination of a dairy products processing plant by *Staphylococcus aureus* using antibiotic resistance and PFGE. **Canadian Journal of Microbiology**, Ottawa, v. 46, p. 1108-1114, 2000.

VANEGAS, M. C. et al. Detection of *Listeria monocytogenes* in raw whole milk for human consumption in Columbia by real time PCR. **Food Control**, Guildford, v. 20, p. 430-432, 2009.

VANDERHAEGHEN, W. et al. Methicillin-resistant Staphylococcus aureus (MRSA) in food production animals. **Epidemiology and Infection,** Cambridge, v. 138, p. 606-625, 2010b.

VÁZQUEZ-SÁNCHEZ, D. et al. Biofilm-forming ability and resistance to industrial disinfectants of *Staphylococcus aureus* isolated from fishery products. **Food Control**, Guildford, v. 39, p. 8-16, 2014.

VEH, K. A. et al. Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and nonpersistent subclinical bovine intramammary infections during lactation or the dry period. **Journal of Dairy Science**, Champaign, v. 98, p. 155-168, 2015.

WAAK, E.; THAM, W.; DANIELSSON-THAM, M. L. Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm tanks and in dairy plant receiving tanks. **Applied and Environmental Microbiology**, Washington, v. 68, p. 3366-3370, 2002.

WHITE, A.; BURNS, D.; CHRISTENSEN, T. W. Effective terminal sterilization using supercritical carbon dioxide. **Journal of Biotechnology**, Amsterdam, v. 123, p. 504-515, 2006.

WINKELSTROTER, L. K. et al. Unraveling microbial biofilms of importance for food microbiology. **Microbial Ecology**, New York, v. 1, p. 35-46, 2014.

WIRTANEN, G.; SAARELA, M.; MATTILA-SANDHOLM, T. Biofilms impact on hygiene in food industries. In: BRYERS, J. D. (Ed.). **Biofilms II**: process analysis and applications. [S.l.:s.n.], 2000. p. 327-372.

ZECONI, A.; SCALI, F. *Staphylococcus aureus* virulence factors in evasion from innate immune defenses in human and animal diseases. **Immunology Letters**, Amsterdam, v. 150, p. 12-22, 2013.

ZHANG, R. Y. et al. Use of lectins to in situ visualize glycoconjugates of extracellular polymeric substances in acidophilic archaeal biofilms. **Microbial Biotechnology**, Hoboken, v.8, p. 448-61, 2014.

3. OBJECTIVES

3.1 General

To evaluate the occurrence of *S. aureus* and *L. monocytogenes* in dairy plants, the ability of strains to produce biofilms on polystyrene and stainless steel surface, and the efficiency of peracetic acid and cold plasma jet treatment to inactivate the isolates at different times.

3.2 Specifics

- To investigate the occurrence of *S. aureus* and *Listeria monocytogenes* in raw milk, pasteurized milk, cheeses, non-food contact surfaces, food contact surfaces in 3 dairy plants located in Southeast region of Brazil.
- To determine the efficiency of peracetic acid and cold plasma jet at different treatment times to inactivate planktonic cells of *S. aureus* and *L. monocytogenes* strains using flow cytometric measurements and plate counts.
- To determine the ability of *S. aureus* and *L. monocytogenes* strains to produce biofilms on polystyrene microplates and stainless steel surfaces, taking into account that they are commonly used in dairy equipment and utensils.
- To assess qualitatively, by epifluorescence microscope, the inactivation of mono-species biofilms of *S. aureus* or *L. monocytogenes* by peracetic acid at different treatment times.
- To determine the efficiency of peracetic acid to remove adherent cells of *S. aureus* or *L. monocytogenes* in polystyrene at different treatment times.

In summary, the experimental activities can be observed in Figure 1.

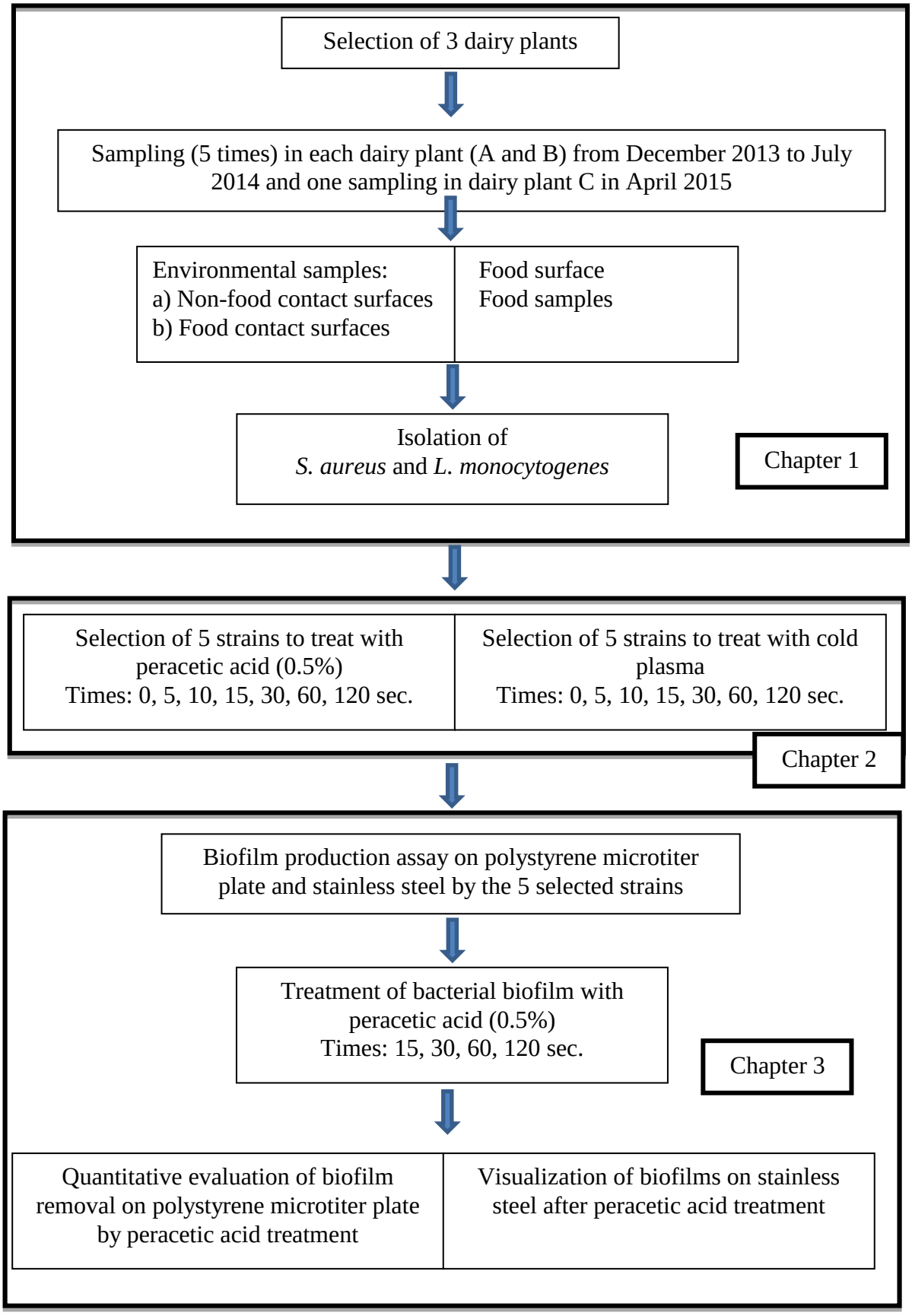

Figure 1. Flowchart with the organization of the experiment.

This thesis is divided in 3 independent experiments, which will be presented in three chapters formatted as scientific articles. The first experiment (Chapter 1) describes the incidence of *S. aureus* and *Listeria monocytogenes* in dairy plants from Southeast Brazil. From December 2013 to April 2015, samples were collected in 3 dairy plants, totaling 11 visits. During each sampling procedure, the following samples were collected in sterilized containers during cheese production: raw and pasteurized milk, brine, recently prepared cheese, and packaged Minas Frescal cheese from one batch. Swab samples from food contact utensils and of non-food contact surfaces were also collected. The lateral and upper surfaces of Minas Frescal cheese and other cheeses' surface were also collected.

The second experiment (Chapter 2) was conducted in the Leibniz Institute of Agricultural Engineering Potsdam-Bornim (ATB), Germany, from September 2014 to December 2014. Due to the low incidence of the pathogens as described in Chapter 1, two *S. aureus* previously isolated from dairy farms as described by Lee et al. (2012) were added to the experiment. In this study, peracetic acid treatment was performed directly on liquid phase (bacterium suspensions). Atmospheric plasma jet was tested on polysaccharide gel inoculated with the pathogens. Treatment times chosen were 5, 10, 15, 30, 60 and 120 sec. Results were obtained using plate count method and flow cytometry.

The third experiment (Chapter 3) was conducted in Brazil, aiming to evaluate the efficiency of peractic acid at different treatment times on biofilms prepared with *S. aureus* strains isolated as described in Chapter 1 and from dairy farms (LEE et al., 2012), and another *L. monocytogenes* strain previously isolated from brine as described by Barancelli et al. (2011).

7. GENERAL CONCLUSION

Taking into account the objectives and the results of this Thesis, the following conclusions can be drawn from the present study:

- The low incidence of *L. monocytogenes* and *S. aureus* in the dairy plants studied indicates a concern of the industry to improve quality, but still the presence of the pathogens shows the importance of the use cleaning and sanitizing.
- Results of PAA and plasma treatments can not be compared due the different surface tested. PAA presented higher effectiveness as a disinfectant against *S. aureu*s and *L. monocytogenes*, reaching almost 100% of inactivation of suspended microbial population within 120 sec. CP showed a reduction of up to 2 log cycles of *S. aureu*s and *L. monocytogenes* on gel discs after 120 sec.
- The high effectiveness of PAA (0.5%) as a sanitizer to inhibit the bacterial growth in suspension was confirmed by the MIC values (78.1-156.2 mg/L). PAA was also effective against the mono species biofilms formed by *S. aureus* and *L. monocytogenes* on stainless steel, with nearly 100% inactivation after 30 sec. Although PAA reduced significantly ($p<0.05$) the BFI of all *S. aureus* strains tested, it was not able to reduce the BFI of *L. monocytogenes* on polystyrene microtiter plate, which indicates that *L. monocytogenes* can attach more to polystyrene than *S. aureus*.
- PAA was able to remove and inactivate biofilms formed by all the *S. aureus* strains tested on stainless steel and polystyrene. Further studies are necessary to determinate the ideal PAA treatment for removing biofilms of *L. monocytogenes*.
- CP treatment has a potential for reducing the bacterial load on surfaces and could be used in the future in the industry as it has been used in hospital environment. However, experiments with longer CP treatment times should be conducted to fully describe the kinetics of this technology for inactivation of important food pathogens.