GABRIEL SILVA OLIVEIRA

DIVERSIDADE E CONTROLE DE BIOFILME FORMADO PELA MICROBIOTA DO LEITE CRU

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Maria Cristina Dantas Vanetti

Coorientadoras: Cynthia Canêdo da Silva

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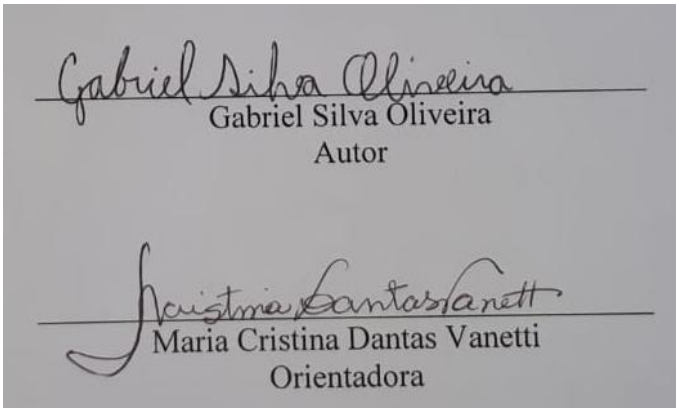

Dedico

A Deus, família e amigos.

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“Tudo posso naquele que me fortalece.”

(Filipenses 4:12)

BIOGRAFIA

Gabriel Silva Oliveira, filho de Imaculada de Souza Silva Oliveira e Antônio Carlos de Oliveira, nasceu em Viçosa, Minas Gerais no dia 25 de junho de 1992.

Em janeiro de 2015, graduou-se em Licenciatura em Ciências Biológicas pela Universidade Federal de Viçosa, Viçosa – Minas Gerais.

Em março de 2015, iniciou o curso de Mestrado em Microbiologia Agrícola, no Departamento de Microbiologia, da Universidade Federal de Viçosa, Minas Gerais, concentrando seus estudos na área de Microbiologia de Alimentos, tendo recebido o título de *Magister Scientiae* em Microbiologia Agrícola no dia 23 de fevereiro de 2017.

Em março de 2017, iniciou o curso de Doutorado em Microbiologia Agrícola na Universidade Federal de Viçosa, concentrando seus estudos na mesma área. Submeteu-se a defesa de tese em 16 de setembro de 2021.

RESUMO

OLIVEIRA, Gabriel Silva, D.Sc., Universidade Federal de Viçosa, setembro de 2021. **Diversidade e controle de biofilme formado pela microbiota do leite cru**. Orientadora: Maria Cristina Dantas Vanetti. Coorientadoras: Cynthia Canêdo da Silva e Solimar Gonçalves Machado.

O leite cru apresenta uma microbiota diversificada capaz de formar biofilmes em diferentes superfícies da indústria de laticínios. Devido a essa ampla diversidade, as comunidades dos biofilmes associadas às superfícies industriais são geralmente complexas e constituídas por diferentes espécies. A fim de se obter produtos seguros e de qualidade, a prevenção da formação e a remoção dos biofilmes, bem como a inibição de suas células são necessárias em razão dos muitos problemas causados nas indústrias, como bioincrustação, corrosão, resistência microbiana aumentada a agentes sanitizantes e contaminação dos alimentos. A caracterização da microbiota formadora de biofilme multiespécie pode contribuir para o desenvolvimento de estratégias de controle e, ou eliminação dos biofilmes no setor industrial onde estas estruturas são consideradas problemas. Assim, este trabalho teve como objetivo avaliar a influência das moléculas nisina e furanonas sobre a formação de biofilme multiespécie pela microbiota do leite cru refrigerado. Foi feito também o isolamento e identificação de bactérias desses biofilmes e avaliada a capacidade de formação de biofilme monoespécie de isolados selecionados. A resistência do biofilme multiespécie formado por quatro isolados ao sanitizante ácido peracético foi avaliada usando a metodologia de superfície de resposta (MSR). Após incubação por 10 dias a 4 ºC, o número de células sésseis no cupom de aço inoxidável mantido imerso em leite cru variou de 5,5 a 6,2 log UFC/cm^2 de psicrotróficos. A adição de nisina ou furanonas ao leite não influenciou no número de células viáveis no biofilme, mas alterou a diversidade microbiana. Os principais gêneros encontrados nos biofilmes foram *Acinetobacter*, *Serratia*, *Lactococcus*, *Pseudomonas* e *Bacillus*, os quais são contaminantes de grande importância para a indústria de laticínios. A partir dos biofilmes multiespécies, foram obtidos 36 isolados e identificados pelo sequenciamento do gene 16S rRNA. Dentre os isolados, 69,5% (n=25) foram capazes de formar biofilme monoespécie, sendo classificados como formadores de biofilme fracos a fortes. *Rahnella inusitata* F083, *Lactococcus garvieae* C081 e *Lactococcus laudensis* F103 foram os que apresentaram o maior capacidade de produção de biofilme. A atividade proteolítica foi detectada em 63,8% (n=23) dos isolados e as moléculas sinalizadoras acil homoserina lactonas (AHLs) de cadeia curta a média (C_4-C_8) foram detectadas em cinco isolados identificados como *R. inusitata*.

Para a formação de um biofilme multiespécie, quatro isolados que apresentaram capacidade moderada a forte de formação de biofilme, identificados como *Pseudomonas fluorescens*, *R. inusitata*, *Staphylococcus aureus* e *Micrococcus aloeverae* foram utilizados. Após 10 dias de incubação a 4 ºC, o biofilme multiespécie atingiu em torno de 10^8 UFC/cm^2. A otimização das condições de inativação de células desse biofilme por MSR consistiu na avaliação de diferentes concentrações de ácido peracético (0,05-0,5%), tempos de tratamento (5-30 min) e temperaturas (25-60 ºC) como variáveis independentes. As três variáveis independentes apresentaram efeito positivo na inativação das células dos biofilmes e a inativação máxima de, aproximadamente, 6,0 ciclos log UFC/cm^2, foi obtida quando os três fatores foram utilizados nos maiores valores. As evidências da complexidade dos biofilmes multiespécies formados em superfícies comumente utilizadas nas indústrias de laticínios reforçam a necessidade de compreender essa microbiota, as possíveis alterações em sua composição e resistência a tratamentos com sanitizantes, a fim de melhorar as estratégias de inativação desses biofilmes nas indústrias.

Palavras-chave: Biofilme multiespécie. Diversidade microbiana. Nisina. Furanona. Resistência a sanitizantes.

ABSTRACT

OLIVEIRA, Gabriel Silva, D.Sc., Universidade Federal de Viçosa, September, 2021. **Diversity and control of biofilm formed by raw milk microbiota**. Adviser: Maria Cristina Dantas Vanetti. Co-advisers: Cynthia Canêdo da Silva and Solimar Gonçalves Machado.

Raw milk has a diverse microbiota capable of forming biofilm on different surfaces of the dairy industry. Due to this wide diversity, biofilm communities associated with industrial surfaces are often complex and made up of different species. In order to obtain safe and quality products, the prevention of the formation and removal of biofilms, as well as the inhibition of their cells are necessary due to the many problems caused in industries, such as biofouling, corrosion, increased microbial resistance to sanitizing agents, in addition to being a source of food contamination. The characterization of multispecies biofilm-forming microbiota can contribute to the development of control strategies and/or elimination of biofilms in the industrial sector where these structures are considered problems. Thus, this work aimed to evaluate the influence of nisin and furanones molecules, on the formation of multispecies biofilm by the microbiota of refrigerated raw milk. The isolation of bacteria from these biofilms was also carried out and the capacity to form monospecies biofilm of the selected isolates was evaluated. In this study, the resistance of multispecies biofilm formed by four isolates to peracetic acid sanitizer was evaluated using response surface methodology (RSM). After incubation for 10 days at 4 ºC, the number of sessile cells in the stainless steel coupon kept immersed in raw milk ranged from 5.5 to 6.2 log CFU/cm^2 of psychrotrophics. The addition of nisin or furanones to milk did not influence the number of viable cells in the biofilm, but changed the microbial diversity. The main genera found in biofilms were *Acinetobacter*, *Serratia*, *Lactococcus*, *Pseudomonas* and *Bacillus*, which are very important contaminants for the dairy industry. From the multispecies biofilms, 36 isolates were obtained and identified by sequencing the 16S rRNA gene. Among the isolates, 69.5% (n=25) were able to form individual biofilms, being classified as weak to strong biofilm formers. *Rahnella inusitata* F083, *Lactococcus garvieae* C081 and *Lactococcus laudensis* F103 were the ones with the highest ability to form biofilm. Proteolytic activity was detected in 63.8% (n=23) of the isolates and short to medium chain acyl homoserine lactones (AHLs) signaling molecules (C4-C8) were detected in five isolates identified as *R. inusitata*. For the formation of a multispecies biofilm, four isolates that showed moderate to strong biofilm forming ability, identified as *Pseudomonas fluorescens*, *R. inusitata*, *Staphylococcus aureus* and *Micrococcus aloeverae* were used. After 10 days of incubation at 4 ºC, the multispecies biofilm reached

around 10^8 CFU/cm^2. The optimization of cell inactivation conditions of this biofilm by RSM used different concentrations of peracetic acid (0.05-0.5%), treatment times (5-30 min) and temperatures (25-60 ºC) as independent variables. The three independent variables had a positive effect on the inactivation of biofilm cells and the maximum inactivation of approximately 6.0 log cycles CFU/cm^2 was obtained when the three factors were used at their highest values. Evidence of the complexity of multispecies biofilms formed on surfaces commonly used in dairy industries reinforces the need to understand this microbiota, the possible changes in its composition and resistance to treatments with sanitizers, in order to improve the inactivation strategies of these biofilms in industries.

Keywords: Multispecies biofilm. Microbial diversity. Nisin. Furanone. Resistance to sanitizers.

SUMÁRIO

INTRODUÇÃO GERAL

Os biofilmes constituem uma preocupação frequente para a indústria de alimentos por exibirem resistência aumentada à desinfecção química, calor, antimicrobianos, radiação, respostas imunes, dentre outros estresses ambientais. Células presentes no biofilme podem ser liberadas e contaminar produtos, causando danos à qualidade e à segurança dos alimentos. Além disso, os biofilmes resultam em outras consequências danosas nas indústrias, tais como bioincrustação e corrosão de equipamentos e superfícies.

O leite apresenta uma microbiota diversificada, compreendendo diferentes gêneros de bactérias, archaeas, leveduras e fungos filamentosos. Muitos estudos sobre formação de biofilmes por espécies contaminantes do leite cru refrigerado têm se concentrado em isolar, cultivar individualmente os microrganismos e avaliar a formação de biofilme monoespécie. Porém, em razão da grande diversidade microbiana presente no leite, o biofilme formado nas indústrias laticinistas geralmente compreende mais de uma espécie microbiana, sendo denominados de biofilmes multiespécies. É comum a colonização inicial de superfícies por um tipo de bactéria promover a adesão de outros microrganismos na mesma superfície, favorecendo o desenvolvimento de comunidades multiespécies. Biofilmes multiespécies geralmente apresentam maior resistência a desinfetantes e a antimicrobianos do que os monoespécies, além de poderem abrigar e proteger uma diversidade de microrganismos deteriorantes e, ou patogênicos.

Nas indústrias, os principais métodos utilizados para inativar e remover biofilmes são agentes químicos, como soluções de hipoclorito de sódio, hidróxido de sódio, peróxido de hidrogênio e ácido peracético, e métodos físicos, por exemplo, temperatura elevada, ultrassom e força mecânica. Alternativas que promovam a inibição da formação de biofilme, como modificações das propriedades físico-químicas das superfícies, fagoterapia, utilização de

enzimas e bacteriocinas, têm demonstrado potencial para a mitigação dos mesmos. A bacteriocina nisina inibe principalmente bactérias Gram-positivas e é autorizada como bioconservante natural em alimentos e bebidas, incluindo alguns produtos lácteos. Nisina pode reduzir a adesão de algumas bactérias a superfícies, além de exercer atividade antibacteriana em células aderidas ao biofilme e por isso, o interesse em explorá-la neste estudo. Seu potencial anti-biofilme têm sido relatado em muitos estudos.

A formação do biofilme em muitas espécies microbianas é regulada pelo mecanismo de comunicação célula-célula, denominado *quorum sensing* (*QS*), que altera a expressão gênica das células e é dependente da densidade populacional. A prevenção da formação de biofilmes pela inibição do *QS* é, portanto, outra estratégia que se tem buscado para minimizar a formação dessas estruturas bacterianas. O processo de inibição do *QS* é denominado de *quorum quenching* (*QQ*) e compostos antagonistas do receptor de auto-indutores (AIs) são preferencialmente explorados para exercerem esta atividade inibitória. Furanonas estão entre as substâncias que têm apresentado resultados positivos na inibição da formação de biofilme de diferentes espécies de bactérias Gram-negativas. Ao contrário das estratégias bactericidas, a inibição do *QS* exerce menor pressão seletiva e, eventualmente, reduz a evolução da resistência aos antimicrobianos.

Considerando a importância dos biofilmes multiespécies para o setor industrial, este trabalho teve como objetivo avaliar a influência da bacteriocina nisina e de furanonas, substância com reconhecido efeito inibidor de *QS*, sobre a formação de biofilme pela microbiota do leite cru refrigerado. Objetivou-se também, isolar e identificar microrganismos a partir desses biofilmes multiespécies e avaliar a capacidade individual de formação de biofilme monoespécie desses isolados, além de determinar a formação de biofilme multiespécie por quatro isolados e sua resistência ao sanitizante ácido peracético.

CHAPTER 1

Nisin and furanone molecules alter the microbial community of multispecies biofilms formed in refrigerated raw milk

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Nisin and furanone molecules alter the microbial community of multispecies biofilms formed in refrigerated raw milk

Abstract

This study evaluated the influence of the bacteriocin nisin and the quorum quenching compound furanones on the diversity of multispecies biofilms formed on stainless steel from the contaminating microbiota of raw milk. Stainless steel coupons were kept immersed in raw milk at 4 ± 1 ºC and, every two days, the raw milk was exchanged for samples collected on the current day. The treatments involved raw milk samples as a control; raw milk added 4 µM nisin, or 50 nM furanones. After incubation for 10 days, the sessile cells number in the control treatment and in the presence of nisin and furanones ranged from 5.5 to 6.2 log CFU/cm^2 of psychrotrophic. The presence of nisin and furanones in milk did not influence the number of viable cells in the biofilm but altered its microbial diversity. The Chao-1 richness, Simpson and Shannon diversity indices showed a slight fluctuation in the biofilm over the days in the control treatment. In biofilm formed in the presence of nisin and furanones, a decrease was observed in the Chao-1 index. In contrast, the Simpson and Shannon diversity indices presented small fluctuations over the 10 days. Gammaproteobacteria was the dominant class almost every day of the formed biofilms. The main bacterial genera found in the biofilms were *Acinetobacter*, *Serratia*, *Lactococcus*, *Pseudomonas* and *Bacillus*, which are recognized contaminants of great importance for the dairy industry. The addition of nisin and furanones to the raw milk altered the microbial community of the biofilm, mainly in the abundance of the genera *Acinetobacter*, *Lactococcus* and *Serratia*. Characterizing and understanding the bacterial communities that form multispecies biofilms can help search for efficient prevention and control strategies for these structures for the dairy industry.

Key-words: Psychrotrophic. 16S rDNA amplicon sequencing. Gammaproteobacteria. Bacilli.

1. Introduction

Bacterial biofilms are aggregates of microorganisms in which cells are embedded in a self-produced matrix of extracellular polymeric substances (EPS) composed mainly of polysaccharides, proteins, nucleic acids and other biopolymers. The EPS are fundamental for microbial life in community and confer adhesion to surfaces and mechanical stability of the biofilms (Costerton et al. 1995; Wingender et al. 1999; Flemming & Wingender 2010; Røder et al. 2016). Biofilms are of great concern in industries, especially in the food industry, and the effects associated with their presence can result in risks to food safety and quality.

Due to the high microbial diversity in raw milk, biofilms on the surfaces of equipment and pipes in the dairy industries can be formed by more than one species interacting in a microbial community (Latorre et al. 2010; Oliveira et al. 2019; Weber et al. 2019). It is known that the Gram-negative bacteria represent more than 75% of the microbiota of refrigerated raw milk (Pinto et al. 2015; Vithanage et al. 2016; Yuan et al. 2017). However, cells adhered to stainless steel (SS) surfaces in the pre-pasteurization and post-pasteurization ducts of a milk processing plant were identified as 56% Gram-positive, while the other 44% were Gram-negative (Cherif-Antar et al. 2016). Oliveira et al. (2019) also observed that multispecies biofilms formed from the refrigerated raw milk microbiota were composed of about 50% of microorganisms belonging to the Gram-positive group. The results suggest that, although present in smaller numbers in the planktonic state in raw milk, Gram-positive bacteria may be present in significant proportions in the sessile state. A better knowledge of the composition of the biofilm-forming microbiota in the presence of raw milk can allow the development of strategies to mitigate them.

The main methods used by industries to inhibit and remove biofilms involve chemical agents, such as sodium hypochlorite solutions, sodium hydroxide, hydrogen peroxide and peracetic acid, and physical methods, for example, hot steam, ultrasound and mechanicals

(Cloete et al. 1998; Simões et al. 2005; Srey et al. 2013). In addition to these methods, natural molecules, such as bacteriocins and furanones, have aroused interest in preventing biofilm formation (Pimentel-Filho et al. 2014; Field et al. 2016; Mathur et al. 2018).

Bacteriocins are peptides or proteins ribosomally produced by bacteria and have bacteriostatic and/or bactericidal properties against other related or unrelated microorganisms (Cotter et al. 2013; Silva et al. 2018). Among bacteriocins, nisin is one of the most studied and has shown great industrial potential in several areas (de Arauz et al. 2009; Jung et al. 2018; Santos et al. 2018; Bahrami et al. 2019; Cunha et al. 2020). Nisin effectively inhibits Gram-positive bacteria and has been authorized to be used in the food industry as a natural biopreservative in several countries since 1988 (Deegan et al. 2006; Sobrino-López & Martín-Belloso 2008; de Arauz et al. 2009; Singh 2018). The nisin effect against Gram-positive bacteria was well documented, but the inhibitory action on Gram-negative bacteria was less explored and occurs when their outer membranes are unstructured (Zou et al. 2013; Prudêncio et al. 2016; Freitas et al. 2019).

Studies have shown the capacity of nisin to reduce the process of cell adhesion to the surface of certain microorganisms (Nostro et al. 2010; Pimentel-Filho et al. 2014), in addition to causing a reduction in biovolume and biofilm thickness (Kajwadkar et al. 2017; Andre et al. 2019). Nisin has been shown to have a significant effect against biofilms, as demonstrated by Godoy-Santos et al. (2019) that this bacteriocin was efficient against *Staphylococcus aureus* cells in biofilm, including the inner layers. However, its effect on multispecies biofilms is still poorly understood. One of the few studies in the area indicates that a nisin-based biogel was effective in inhibiting and eradicating periodontal multispecies biofilms, reinforcing its potential in controlling biofilms (Cunha et al. 2020).

The formation of biofilm by some microorganisms can be regulated by a signaling system based on auto-inducers molecules (AIs) (Ding et al. 2011; Li et al. 2018). This system

is called quorum sensing (QS), which depends on the synthesis, release and uptake of AIs (Miller & Bassler 2001; Mukherjee & Bassler 2019). These molecules are used for intraspecific and interspecific intercellular communication and can coordinate various physiological processes, including biofilms formation (Parsek & Greenberg 2005; Campos-Galvão et al. 2016; Almeida et al. 2017; Mukherjee & Bassler 2019). The QS inhibition is called quorum quenching (QQ), and this strategy has been used to prevent the formation of biofilms (Coughlan et al. 2016; Paluch et al. 2020). Furanone is one of those inhibitors that shown to be effective as QQ in Gram-negative bacteria since it has a structural similarity to AI-1, functioning as an analog molecule, thus preventing transcription of target genes (Almeida et al. 2016; Proctor et al. 2020). Özcan et al. (2019) observed that using furanone C-30 reduced the biofilm formation of *Pseudomonas aeruginosa* by 76%. Furanone was also able to reduce biofilm formation by *Serratia liquefaciens*, isolated from raw milk, and the amount of polysaccharides, proteins and extracellular DNA in the biofilms, in addition to making them more susceptible to kanamycin (Rodrigues et al. 2020).

Considering that biofilms are identified as one of the significant sources of contamination of products through the release of microorganisms and enzymes into dairy products, the prevention of biofilm formation is an effective strategy to ensure the product's quality. As biofilms formed in the presence of raw milk can be constituted by both Gram-negative and Gram-positive bacteria, evaluating the impact of inhibitors from each of these bacterial groups on biofilms multispecies can contribute to the understanding of the interaction between the contaminating microbiota of refrigerated raw milk. Thus, the objective of this work was to evaluate the influence of the nisin and furanones molecules on the diversity of multispecies biofilms formed in SS from the refrigerated raw milk microbiota. In addition, the biofilm was analyzed by sessile cell count and epifluorescence microscopy over the days.

2. Material and Methods

2.1. Sampling

The raw milk samples were collected from August to November 2019, in the dairy industry in the region of Viçosa, Minas Gerais, Brazil, that receives bulk milk, i.e., milk from several producers stored in tanks at an average temperature of 4 °C on the farm or in unit collectors for up to 48 h after milking. Approximately 3.0 L of raw milk were collected in sterile glass bottles and transported directly to the laboratory upon refrigeration. The experiment was repeated three times, and five samplings of raw milk were collected for each of the three repetitions.

2.2. Preparation of nisin and mixture of furanones

A stock solution of nisin (Nisaplin; 2.5% nisin; Danisco Brasil) at 1,500 µM was prepared in sodium phosphate buffer (10 mM, pH 2.0). The stock solution was stored at 4 °C. A mixture of four synthetic furanones (Sigma-Aldrich, Germany) was prepared by suspending 3-methyl-2(5H)-furanone, 2-methyltetrahydro-3-furanone, 2(5H)-furanone, and 2,2-dimethyl-3(2H)-furanone in acetonitrile at a concentration of 100 mM.

2.3. Preparation of the biofilm formation surface and cultivation condition

Stainless steel (SS) AISI 304 (#4) coupons with dimensions of 1 x 1 x 0.2 cm and 4.8 x 1.5 x 0.2 cm were used as a surface for biofilm formation. The coupons were washed and sanitized, as Oliveira et al. (2019) described.

Three different cultivation conditions were carried out to form biofilm from the raw milk microbiota. The control treatment consisted of only raw milk samples. At the same time, the second condition was raw milk with the addition of nisin to a final concentration of 4 µM, and the third condition was raw milk with the addition of a mixture of furanones to a final

concentration of 50 nM. For each culture condition, five 250 mL Erlenmeyer flasks were used and 210 mL of raw milk was added; each flask corresponded to one day of analysis (2nd, 4th, 6th, 8th and 10th day). Eleven coupons were hung in each flask, as previously described by Oliveira et al. (2019). The raw milk samples were incubated at 4 ± 1 ºC for up to 10 days. Every 48 h, the coupons for one of the flasks for each treatment were removed for sessile cell count, epifluorescence microscopy observation and DNA extraction for metataxonomic analyses. The coupons of the other flasks of each treatment were washed with sterilized water, and the milk samples were replaced by another sample of the same origin and with the same concentrations of nisin and furanones used in the treatments.

2.4. Planktonic and sessile cells count

The total psychrotrophic aerobic bacteria counts were determined in each raw milk sample collected. For this, decimal dilutions were prepared and then spread onto Plate Count Agar (PCA) (Himedia, Mumbai, India). The incubation was at 7 °C for 7 days (Cousin et al. 2001). The planktonic microbiota of milk after incubation for 48 h at 4 ºC was also performed, as described above.

The sessile cells were quantified from 4.8 x 1.5 x 0.2 cm coupons removed at different sampling times (2, 4, 6, 8, and 10 days) and washed with sterile phosphate-buffered saline (PBS, 0.2 M, pH 7.4) to remove planktonic cells. Then, each coupon was transferred to a test tube containing 13 mL of PBS and was subjected to ultrasound (Vibra Cell, Newtown, USA) for 1 min continuous mode operating (130 W, 20 kHz) and then vortexed for 30 s to remove the sessile cells (Bonkat et al. 2012). Subsequently, decimal dilutions were prepared and then spread onto PCA. Plates were incubated at 7 °C for 7 days.

Plating for quantification of the planktonic microbiota of milk and sessile cells of the coupons was performed in two replicates in three repetitions. The cell suspensions obtained

from the eight coupons of each analysis day were centrifuged at 5,000 *g* for 15 min, concentrated in a single bottle for each treatment, and stored at - 80 °C for further DNA extraction.

2.5. Epifluorescence microscopy

On the 2nd, 6th, and 10th day, 1 x 1 x 0.2 cm coupons incubated at 4 ºC under the three different conditions were collected, washed with PBS to remove planktonic cells and subsequently incubated for 20 min in the absence of light with the Live/Dead kit reagents (Invitrogen, Sweden). Then, the coupons were washed with PBS and placed on a glass coverslip to be observed under an epifluorescence microscope (Thermo Fisher, model EVOS M5000, USA), using GFP (green fluorescent protein) and RFP (red fluorescent protein) filters with excitement wavelength of 470 nm and 531 nm, respectively. The cells stained red or yellow were considered dead and those stained green were considered viable (Wang et al. 2012).

2.6. Biofilm DNA extraction and 16S rDNA amplicon sequencing

Cell pellets obtained from biofilms (section 2.4) were used to perform total DNA extraction using the DNeasy PowerBiofilm Kit (Qiagen, Germany) according to the manufacturer's protocol. The measurements of DNA quantity were performed using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). The extracted DNA was sent to Novogene (Novogene Bioinformatics Technology, Beijing) for amplicon V3-V4 bacterial sequencing (Forward primer: 3′-CCTAYGGGRBGCASCAG-5′, reverse primer: 3′-GGACTACNNGGGTATCTAAT-5′) by the Illumina HiSeq2500 platform, generating 250-bp paired-end reads. The sequences obtained in this study were deposited into the NCBI short reading file in BioProject PRJNA 757774.

2.7. Sequence data processing

Sequence quality analysis was tested using FastQC software, contigs assembly process and adapter removal, demultiplexing, timmage (Q < 25), OTU grouping (97% identity) and annotations were performed using the Qiime platform (Quantitative Insight in Microbial Ecology) version 1.8 (Caporaso et al. 2011) and the SILVA database version 132 (http://www.arb-silva.de). The normalized OTU tables were used to determine the alpha diversity (Chao1, Shannon, and Simpson) indexes and the relative abundance (reads/total reads in a sample) of the OTUs.

To characterize the overlap of OTUs present among broad multispecies biofilm formed in the control and treated milk, Venn diagrams were constructed in jvenn (Bardou et al. 2014).

Using Past 4.03 software (Hammer et al. 2001), a non-metric multidimensional scaling (n-MDS) ordination, based on the Bray-Curtis dissimilarity index, the OTU composition data were used to visualize possible differences in the community between the biofilms of the control treatment, nisin and furanones, during days 2, 4, 6 , 8 and 10 of incubation.

3. Results and Discussion

3.1. Microbiological counts

The psychrotrophic count of the milk samples ranged from 5.0 to 6.6 log CFU/mL, having a mean value of 5.9 log CFU/mL (Supplementary Figure S1). The milk reached these high counts probably because it constituted a milk mixture from several dairy producers. Besides, it had already spent about 48 h in storage tanks on farms before reaching the dairy industry for processing. The raw milk microbiota originates from multiple sources of contamination and this microbiota is composed of different groups of microorganisms, such as belonging to the genera *Pseudomonas*, *Bacillus*, *Lactococcus*, *Macrococcus*, *Serratia*, *Streptococcus*, among others (Machado et al. 2015; Skeie et al. 2019; Porcellato et al. 2021). Contamination of raw milk can increase milk and dairy products' spoilage, as many of the contaminating groups can grow at low temperatures and produce proteolytic and lipolytic enzymes (Samaržija et al. 2012; Machado et al. 2017).

After incubating the milk at 4 ºC for 48 h to form a biofilm, the planktonic microbiota was counted and an increase in cell density was observed. Psychrotrophics microbiota presented an average count of 7.2 log CFU/mL in the control treatment, with an average increase of 1.2 log CFU/mL cycles after 48 h incubation (Figure 1A). This group of microorganisms is of great concern to the dairy industry, as the raw material and many dairy products are stored and distributed at temperatures that favor its growth.

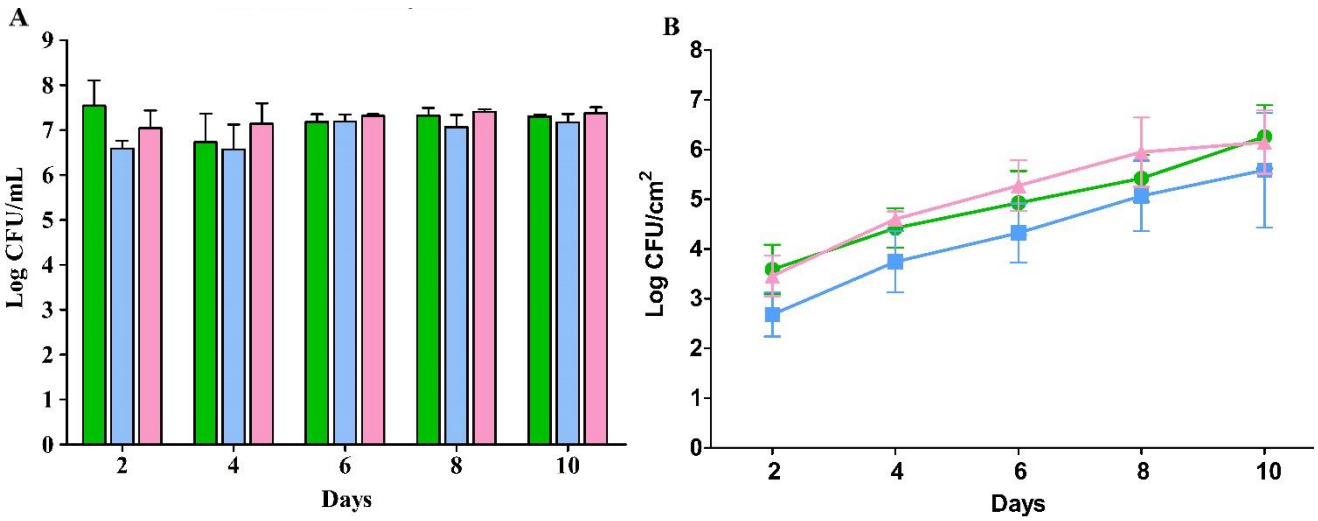

Figure 1- Logarithm of the number of colony-forming units per mL (CFU/mL) of the planktonic psychrotrophic milk samples after incubation at 4 ºC for 48 h (A). Logarithm of the CFU/cm^2 of psychrotrophics of the biofilm (B). Raw milk (control treatment) (■), raw milk with nisin (4 µM) (■), and raw milk with furanones (50 nM) (■). The bars mean the average of the treatments (mean and standard deviation, n=3).

The addition of nisin or furanones to the raw milk samples did not alter ($p > 0.05$) the number of planktonic bacteria during incubation at 4 ºC for 48 h (Figure 1A). After incubating the milk with nisin, the average of 6.9 log CFU/mL of psychrotrophics was registered, with an increase in 0.9 log CFU/mL cycle. This result agrees with Bhatti et al. (2004), who reported that the addition of nisin to pasteurized milk samples did not affect cell numbers during refrigerated storage, compared to the number of cells in milk without milk nisin. The mean observed in samples of milk added with furanones was 7.2 log CFU/mL of psychrotrophics, with a mean increase of 1.2 log CFU/mL cycle (Figure 1A).

3.2 Biofilm

The data in Figure 1B show the number of sessile cells during the development of biofilm formed by the contaminating microbiota of refrigerated raw milk in SS coupon at 4 °C during 10 days. The number of cells that adhered to the SS coupon on the 2nd day of incubation reached 3.6, 2.6 and 3.4 log CFU/cm^2 of psychrotrophics in the control, nisin and furanones treatments, respectively (Figure 1B). Only the treatment with nisin showed a

statistical difference in reduced sessile cell number on the 2nd day compared to the control ($p < 0.05$). In the following days, no statistical difference was observed in the sessile cells number between milk of the control treatments and those added of nisin and furanones. Following 10 days of incubation, the biofilms presented a significant increase in cell number searching at 6.2, 5.5 and 6.1 log CFU/cm^2 of psychrotrophs in the control, nisin and furanones treatments, respectively (Figure 1B). The ability of psychrotrophic isolates from the dairy industry to form biofilm has been demonstrated (Rossi et al. 2018; Yuan et al. 2018; Hahne et al. 2019; Oliveira et al. 2019; Rodrigues et al. 2020). The presence of proteolytic psychrotrophic microorganisms to about 29% of the biofilm cells formed from raw milk microbiota (Oliveira et al. 2019) concerns the quality of dairy products.

Representative epifluorescence microscopy images of the biofilms demonstrating its development over the 10 days are shown in Figure 2 and corroborate the sessile cells number data (Figure 1B). The lower cell number on the treatment with nisin on 2nd day incubation was also possible to visualize through microscopy with a smaller number of adhered cells. The biofilm structure was observed in its hydrated state, and cell viability could be differentiated, where green cells are considered alive while reddish or yellowish cells are dead. On the second day (Figure 2A, 2D, 2G), some cell aggregates were observed on the SS surface, indicating an adhesion process and initial biofilm formation in all treatments. The multispecies biofilm formation process can begin with the fixation of good biofilm formers, facilitating the future fixation of other species on the same surface (Simões et al. 2007). In the control and furanones treatments, dead cells in yellow or red were not observed. However, in the treatment with nisin, yellow to red-colored cells were present, indicating that the cells were injured or dead due to the antimicrobial action exerted by the bacteriocin. Nisin is effective against several Gram-positive microorganisms, causing pore formation in the cell membrane, and has a lesser effect on Gram-negative ones (Sahl et al. 1995, Prudêncio et al.

2015). However, under low-temperature conditions, it has shown a nisin effect on Gram-negative bacteria due to the disturbance of the stability of the outer membrane caused by the low temperature (Galvão et al. 2015; Prudêncio et al. 2016). However, according to the results obtained from the number of sessile cells in biofilm (Figure 1B), the low concentration of nisin was not able to cause a significant difference in the development of the biofilm concerning the other two treatments ($p > 0.05$) after 48 h incubation. Over the days, the cell aggregates became more extensive, as observed on the 10th day (Figure 2C, 2F, 2I).

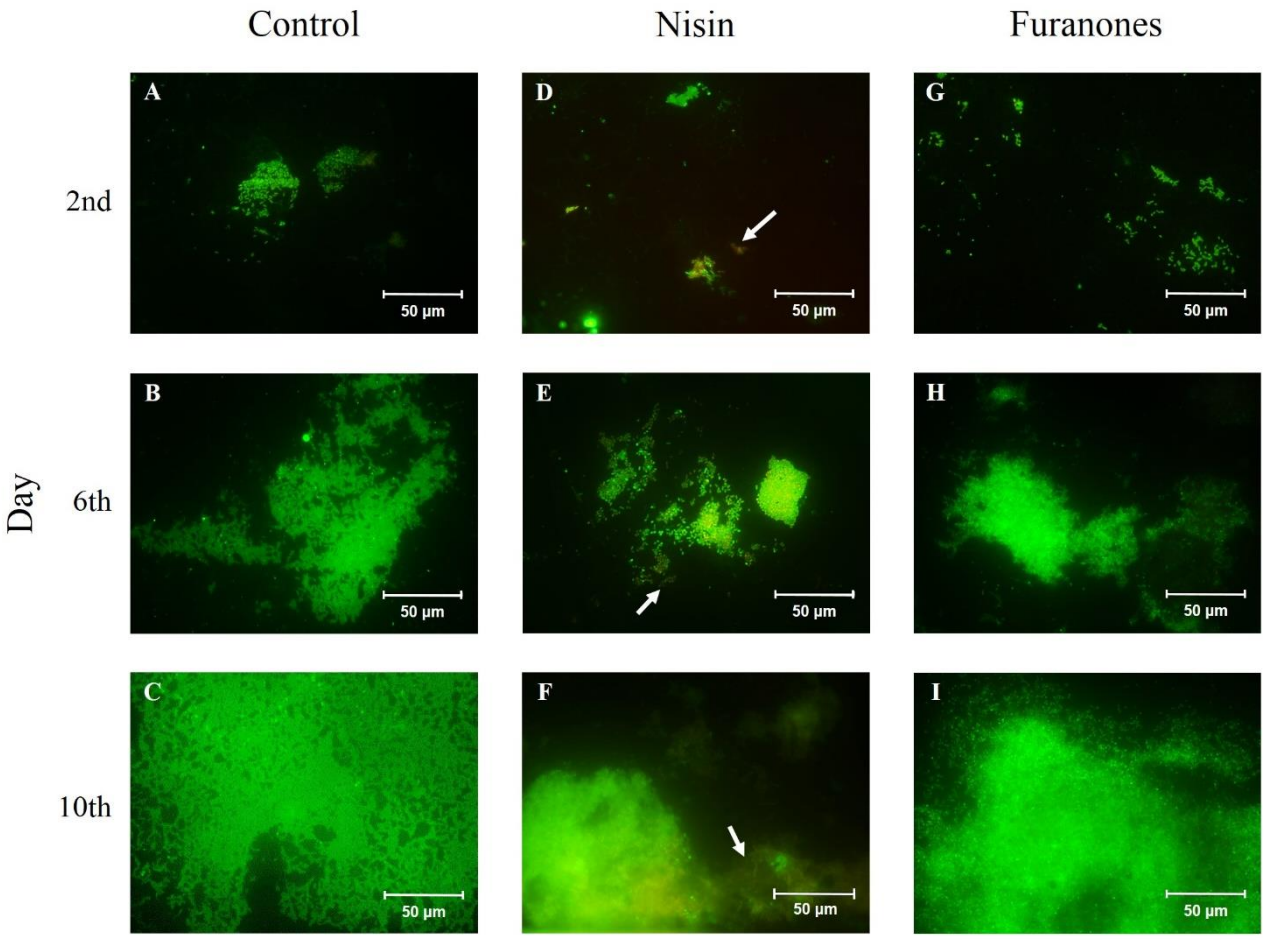

Figure 2- Epifluorescence microscopy of multispecies biofilm at a magnification of 600 x. A, B and C represent images of SS coupons that were immersed in raw milk at 4 °C on days 2, 6 and 10, respectively. D, E and F represent images of the SS coupons that were immersed in raw milk with nisin (4 µM) at 4 °C on days 2, 6 and 10, respectively. G, H and I represent images of the SS coupons that were immersed in raw milk with mixture of the furanones (50 nM) at 4 °C on days 2, 6 and 10, respectively. Arrows indicate cells in an injured or dead state, with yellow to red coloration.

3.3 Microbial diversity of biofilm

A total of 663,094 crude bacterial sequences were generated, with an average length of 250 pb. Among samples obtained of all biofilm, 230 different operational taxonomic units (OTUs) were detected at an identity threshold of 97%. Three indicators of alpha diversity within the samples were calculated to study the bacterial community; the Chao-1 index was used to estimate species richness, Simpson (1-D) and Shannon indices were used to estimate diversity. The results show that these indices varied throughout the biofilm formation and between treatments (Table 1).

Table1- Richness (Chao-1 index) and diversity (Simpson and Shannon index) of bacterial communities of biofilms from the control, nisin (4 μM) and furanones (50 nM) treatments.

Index	Control					Nisin					Furanones				
	2nd day	4th day	6th day	8th day	10th day	2nd day	4th day	6th day	8th day	10th day	2nd day	4th day	6th day	8th day	10th day
Chao-1	41	33	43	51	48	61	51	44	42	46	66	53	46	26	39
Simpson (1-D)	0.405	0.688	0.706	0.678	0.737	0.574	0.648	0.704	0.710	0.596	0.601	0.472	0.480	0.755	0.698
Shannon	0.921	1.395	1.576	1.455	1.539	1.299	1.359	1.395	1.461	1.116	1.527	1.185	1.064	1.519	1.393

On the 2nd day, lower species richness and diversity were observed in the control treatment (Table 1). The high contamination in raw milk samples probably imposes greater competition between species during the initial surface adhesion process, resulting in the initial adhesion of only good biofilm formers at low temperatures. On the other hand, nisin may have induced biofilm formation by different species as a response to the stress promoted by the presence of the bacteriocin. Bacteria exposure to sub-inhibitory concentrations to certain antimicrobials can cause physiological changes that stimulate biofilm formation (Townsley & Shank 2017; Ranieri et al. 2018).

Furanone treatment also showed a higher Chao-1 index on the 2nd day, suggesting that a possible inhibition of QS interfered with the presence of species sensitive to this

inhibitor and favored the adhesion of other species. Many Gram-negative bacteria present in milk have been identified as producers of AHL (Pinto et al. 2007; Viana et al. 2009) and have QS-regulated biofilm formation (Allison et al. 1998; Duanis-Assaf et al. 2016; Viana et al. 2009). These species may have been harmed by the presence of furanones, thus allowing other species to adhere to the surface. The importance of inhibiting QS in some milk contaminating bacteria goes beyond reducing biofilm formation. The QS may be related to the regulation of proteases production from different species, contributing to milk spoilage (Yuan et al. 2020). Bai & Vittal (2014) demonstrated that QS induced exoenzyme production and increased adhesion and biofilm formation of *Pseudomonas psychrophila* while the synthetic compound furanone C-30 showed an inhibitory effect on the production of protease, lipase and biofilm formation. However, the results of inhibition of biofilm formation by QQ are based on studies with biofilms formed by a single species and, in the present study, the effect of a quorum quencher on the behavior of a diverse microbiota is different.

The Chao-1 index showed a tendency to increase over the days only in the control treatment (Table 1), indicating the aggregation of new species during the biofilm formation process. As the number of species increases, there is also an increase in the interactions within multispecies biofilms (Røder et al. 2016). Some good biofilm-forming microorganisms, such as *Pseudomonas* and *Acinetobacter*, may include other weak or non-biofilm forming microorganisms, thus increasing the total biofilm (Ibusquiza et al. 2012; Zupančič et al. 2018). This consequently increases the Simpson and Shannon diversity indices between the 2nd and the 10th day in the control treatment (Table 1). The increase in Simpson's index indicates a trend towards decreasing dominance by certain species in the biofilm community, and the increase in Shannon's diversity index was a consequence of the slight increase observed in species richness. This increase can be partly explained by the possible

exploitation of the different micro-niches formed in biofilms, such as anaerobic and aerobic regions (Flemming et al. 2016).

Unlike the control treatment, the Chao-1 index reduced in biofilm formed on treatments with nisin and furanones, indicating a reduction of species over the days (Table 1). The more significant number of adhered species on the 2nd day in both treatments probably led to an increase in competitive interactions, whether by space, nutrients, oxygen, or even by producing toxic compounds. In this way, many less competitive and more sensitive species were eliminated from the biofilm community, causing the Chao-1 index to decrease in these samples over the days. Due to this change in species richness over the days in treatments with nisin and furanones, the Simpson and Shannon diversity indices in these biofilms showed small fluctuations (Table 1). The oscillations observed over the days demonstrate that multispecies biofilm formation is a dynamic process, where species aggregate and others stand out due to the different interactions (Burmølle et al. 2014; Røder et al. 2016). The diversity in the microbial communities of multispecies biofilms leads to a variety of complex relationships involving inter- and intra-species interactions, and these interactions can be competitive, cooperative, or neutral (Hansen et al. 2007; Liu et al. 2016). Parijs & Steenackers (2018) demonstrated that competitive interactions are prevalent in multispecies biofilms, and these interactions have a strong influence on the increased tolerance of microorganisms to antimicrobial treatments.

From the total of OTUs obtained, a Venn diagram was assembled between treatments during the days of biofilm formation (Figure 3). The results showed that most OTUs were shared between treatments each day analyzed. On the 2nd day, 22 OTUs (34%) were common to the three treatments, indicating that these microorganisms are good biofilm formers and may have essential roles in the initiation of the formation of the multispecies biofilms. The treatments with nisin and furanones shared more OTUs (12 OTUs) than with the control (2

OTUs with each treatment), indicating that the addition of nisin and furanones somehow influenced or allowed the adhesion of other microorganisms to the SS surface. The control treatment showed fewer specific OTUs concerning both treatments on the 2nd day, indicating greater richness in the treatments, as seen in Table 1.

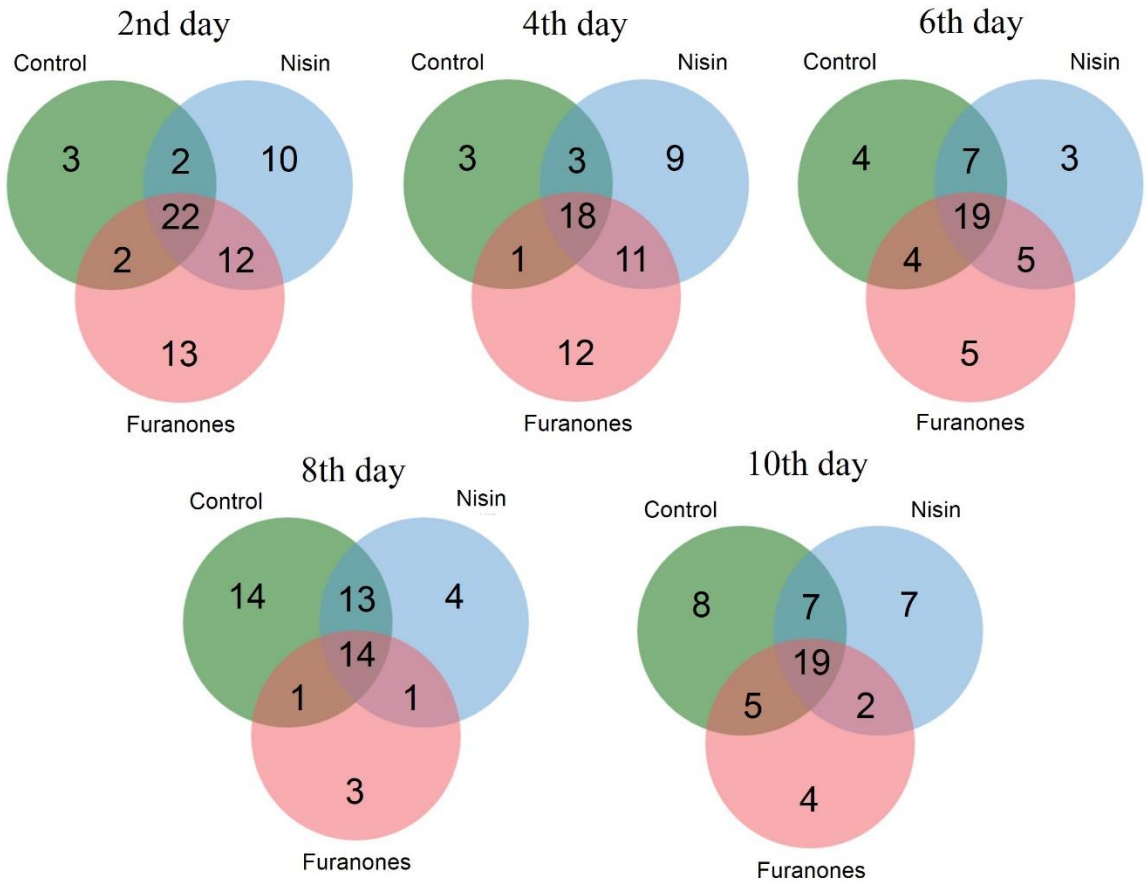

Figure 3- Venn diagram showing the number of bacterial OTUs shared over the days of multispecies biofilm formation, control treatment (■), with nisin (4 µM) (■) and with furanones (50 nM) (■).

Between the 2nd and 10th days, there was a change in the number of OTUs in biofilms formed in all treatments. The control treatment had a higher number of total OTUs (39) compared to treatments with nisin (35) and furanones (30) on the 10th day, and of this total, 19 OTUs (36%) were shared between treatments. Over the days, many species remained throughout the biofilm, indicating a persistent microbiota, which may be good competitors or those that positively interact with other species. In addition to competitive interactions,

cooperative interactions can be present in biofilms and promote benefits for one or all members (Liu et al. 2016).

The non-metric multidimensional scaling (n-MDS) ordination graph demonstrated that the composition of the biofilm community of the initial days was different from the final days for all treatments (Figure 4). Only on the 2nd day the community shows a similarity in composition between the three treatments, clustering in the upper first quadrant of the graph. This initial microbial community can be constituted by the strong biofilm formers that start the process. The lack of grouping over the days of each treatment demonstrates that biofilm formation is a dynamic process and that there is ecological succession. The n-MDS of the biofilm samples did not reveal significant differences between the communities. However, it was possible to observe that the addition of the inhibitory compounds nisin and furanones in milk influenced the structure of the bacterial community of the biofilm formed concerning the control treatment, mainly on the 6th, 8th, and 10th days.

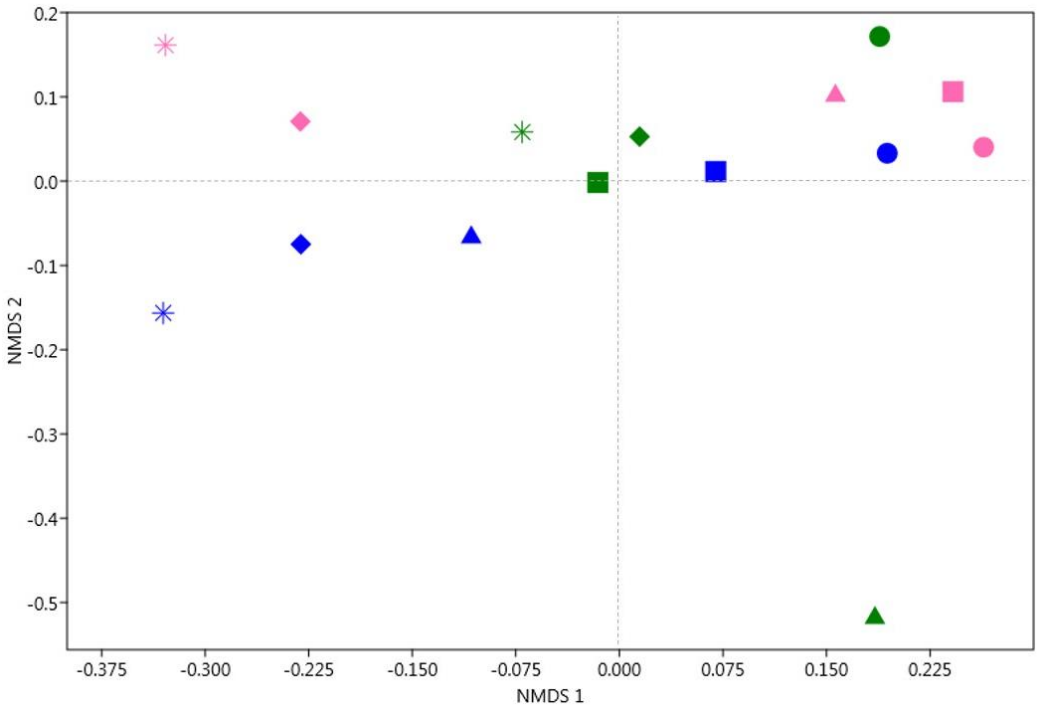

Figure 4- Non-metric multidimensional scaling (stress level: 0.0759) of the community composition of the multispecies biofilm from the control (■), nisin (4 µM) (■) and furanones (50nM) (■) treatment on days 2 (●), 4 (■), 6 (▲), 8 (♦) and 10 (⁎).

The OTUs obtained were classified into 9 phyla, 14 classes, 26 orders, 42 families and 71 bacterial genera. The dominant genera observed during the days of the three treatments belonged to two classes, Gammaproteobacteria and Bacilli, with the class Gammaproteobacteria being dominant in almost every day, with an approximate average of 85% of the relative abundance (Figure 5A). The contaminating microbiota of refrigerated raw milk includes both Gram-positive and Gram-negative microorganisms, but the latter represents more than 75%, among which a large part belong to the Gammaproteobacteria class (Nörnberg et al. 2011; Pinto et al. 2015). Oliveira et al. (2019) observed that in the biofilms formed by the microbiota of raw milk at 7 ºC, Gammaproteobacteria and Bacilli represented about 50% each of the microorganisms identified. The difference found between these studies may be due to the temperature used to form biofilms. In addition, possible variations occur in the milk microbiota according to seasons of the year, type of breed and feeding of the herd, which may have directly influenced the composition of these biofilms. Porcellato et al. (2021) demonstrated that the milk microbiota changed during long-term sampling analyses and factors such as herd feeding and climate could be related to these changes.

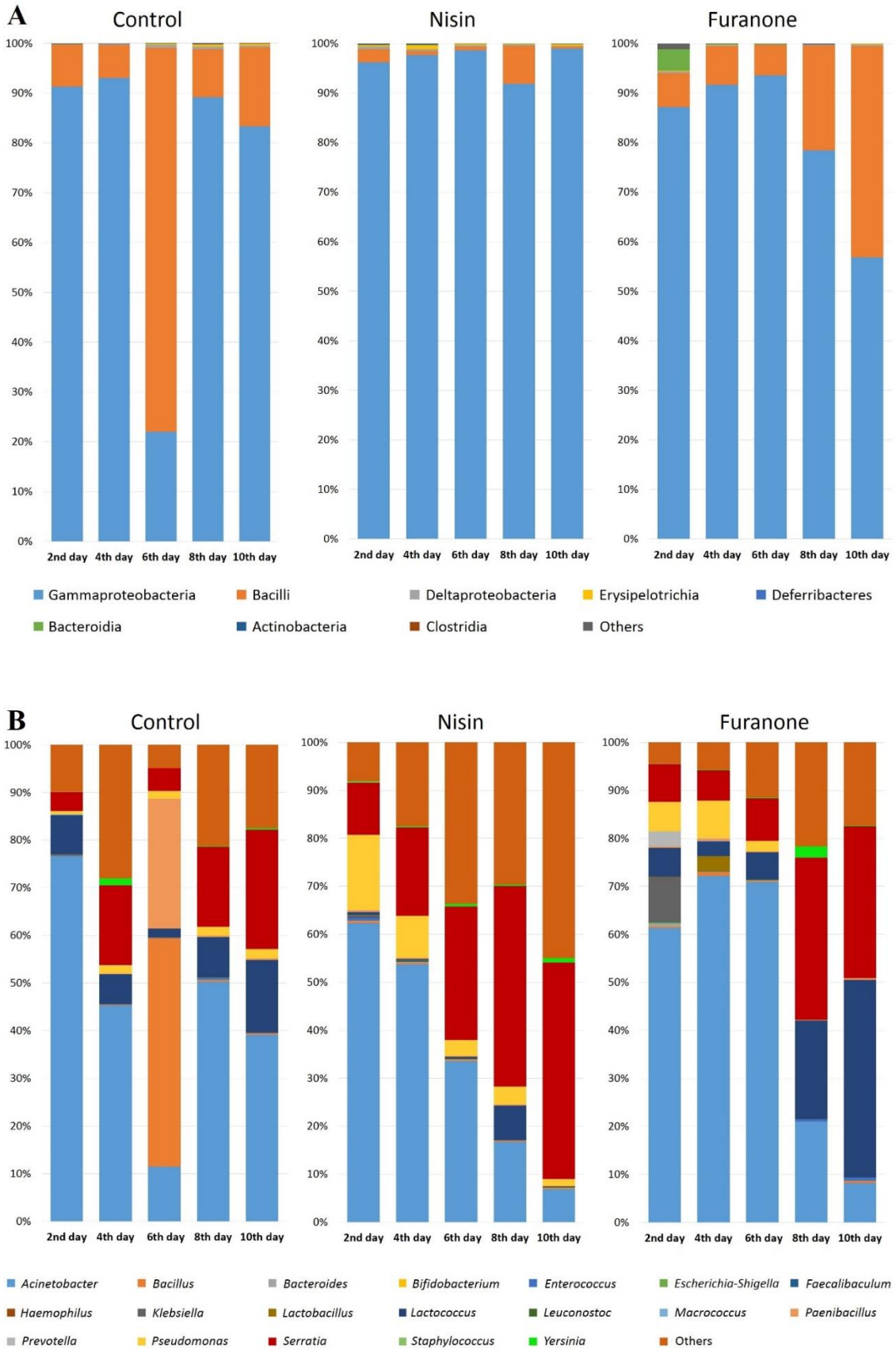

Figure 5- Bacterial composition of the biofilm at the class (A) and genus (B) level. Each bar represents the composition of the bacterial community over the biofilm days of the control, nisin (4 μM) and furanones (50 nM) treatments.

The most abundant genera of Gammaproteobacteria detected in the biofilm were *Acinetobacter*, *Serratia* and *Pseudomonas*, representing 41.9%, 19.9% and 3.8%, respectively (Figure 5B). *Acinetobacter* was the genus with the highest relative abundance on the biofilm on 2nd and 4th days in all treatments; from the 6th day onwards, its abundance decreased. This genus was the second most dominant in Chinese refrigerated raw milk samples, representing 13% of the psychrotrophic isolates (Yuan et al. 2017). The presence of *Acinetobacter* in great abundance in the early days of biofilm formation reinforces its great potential to form biofilm and interact with other species. Zupančič et al. (2018) observed that *Acinetobacter junii* had a greater number of positive correlations in mixed biofilms, suggesting a strong potential for coexistence with other bacterial genera, in addition to being able to include other poor or non-biofilm producing microorganisms, increasing overall biofilm formation within microbial consortia.

In treatments with nisin and furanones, the genus *Acinetobacter* showed a decrease in its relative abundance, mainly on the 8th and 10th days. In the treatment with nisin, this reduction was gradual over the days, while in the treatment with furanones, this reduction was abrupt after the 6th day. It is supposed that the addition of nisin and furanones may have led to changes in the interactions of the biofilm community, which may have impaired the permanence of *Acinetobacter* in a multispecies biofilm. Species belonging to the genus *Acinetobacter* have been identified as AHLs molecules producers (González et al. 2001; 2009), and the involvement of the QS system in the biofilm formation, mainly during maturation stages, was indicated by Niu et al. (2008). This genus brings great concerns to the dairy industries, as it can produce lipolytic and proteolytic enzymes (von Neubeck et al. 2015; Yuan et al. 2018).

The highest abundance of *Serratia* in relation to *Pseudomonas* in the multispecies biofilm was observed almost every day in all treatments. Cleto et al. (2012) demonstrated

that *Serratia* spp. isolated from a milk processing plant showed a general ability to form biofilm much greater than *Pseudomonas* spp.. Both genera were characterized as the main dominant psychrotrophic microorganisms in raw milk with a high potential for deterioration. Their enzymes can resist heat treatment, directly impacting the quality and shelf life of the final products (Machado et al. 2015). Multispecies biofilms formed by *P. fluorescens*, *Serratia liquefaciens* and *S. aureus* produced heat-resistant enzymes, which compromised the quality of UHT milk when raw milk was exposed to these biofilms (Teh et al. 2014). *Serratia* showed greater abundance in the treatment with nisin than in the control treatment, being the genus with the greatest abundance in the biofilm on the 8th and 10th days. The change in the multispecies biofilm community caused by the addition of nisin may have allowed the population of *Serratia* to increase, probably by reducing Gram-positive competitors or even the reduction of the genus *Acinetobacter*. *Pseudomonas,* on the other hand, showed greater abundance on the 2nd and 4th day in the nisin treatment than in the control treatment, but on the following days, they reduced to levels similar to the control. Species belonging to both genera have shown QS-regulated biofilm formation (Labbate et al. 2004; Van Houdt et al. 2007; Bai & Vittal 2014). The relative abundance of *Serratia* in multispecies biofilm did not reduce in the presence of furanones compared to control (Figure 5B). However, in monospecies biofilm, furanone reduced *S. liquefaciens* biofilm formation in polystyrene (Rodrigues et al. 2020). On the other hand, furanones promoted an increase in the relative abundance of *Serratia* and a reduction of *Pseudomonas* in the multispecie biofilm, reaching values lower than 1% on the 8th and 10th days for *Pseudomonas* (Figure 5B). This reducing effect of furanones was also observed in *P. psychrophila* monospecies biofilm formation (Bai & Vittal 2014).

The most abundant genera within the Bacilli class present in the biofilms were *Lactococcus* and *Bacillus*, representing 8.3 and 3.4% of the total sequences obtained,

respectively (Figure 5B). Both genera are often isolated from the dairy and biofilm from industries (Lafarge et al. 2004; Anand et al. 2012; Teh et al. 2012; Lin et al. 2017; Oliveira et al. 2019). The genus *Lactococcus* belongs to the group of lactic acid bacteria, which are technologically important to the dairy industry, although they can cause the deterioration of some dairy products. Nisin has promoted a reduction in the relative abundance of *Lactococcus* compared to the control treatment. Nisin is produced by *Lactococcus lactis* and is active against other strains of the same or related species (Khelissa et al. 2021). In the treatment with furanones, *Lactococcus* increased relative abundance compared to the control treatment, mainly on the 8th and 10th days. This constatation could be explained by the reduced presence of Gram-negative in biofilm due to the QQ effect of furanones. *Lactococcus* sp. has been shown to benefit from EPS produced by another species, reinforcing its ability to aggregate and form multispecies biofilms (Kives et al. 2005).

The genus *Bacillus* showed a great abundance only on the 6th day of the control treatment, while on the other days, its abundance was less than 1%. This result can be explained by possible high contamination of *Bacillus* in the milk sample used for the exchange that proceeded that day, causing a great adhesion of this genus to the biofilm only on that day. In treatments with nisin and furanones, this may not have occurred due to the presence of both compounds, which could affect the adhesion of this genus to the biofilm. Furanones have shown an inhibitory effect on the biofilm formation in some Gram-positive bacteria, but the mechanisms of activity against these bacteria are not fully elucidated (Kayumov et al. 2015; Sharafutdinov et al. 2019). Kayumov et al. (2015) demonstrated that furanones significantly affected the regulatory pathways involved in *Bacillus subtilis* biofilm formation, leading to low EPS production and repression of the *eps* operon and *yqxM* promoter. The presence of *Bacillus* on industrial processing surfaces has been reported, in addition to its formation of biofilm associated with other species (Salustiano et al.

2009; Kumari & Sarkar 2016; Abriat et al. 2019). Bacteria belonging to the *Bacillus* genus are of particular concern to dairy industries. They are spore-forming, able to survive industrial pasteurization, produce lipolytic and proteolytic enzymes, and form biofilms on stainless steel surfaces (Hantsis-Zacharov & Halpern 2007; Gopal et al. 2015).

4. Conclusion

The genera that form multispecies biofilms are important for the dairy industry, as many are spoilage and pathogenic microorganisms. Nisin and furanones did not decrease the formation of multispecies biofilm but changed its bacterial composition. The characterization of bacterial communities that form multispecies biofilms in the presence or absence of these molecules can help search for efficient prevention and control strategies for these structures for the dairy industry. Our study added additional knowledge regarding the ecology of the biofilm microbiota forming in the presence of refrigerated raw milk. Future studies are still needed to understand the effect caused by the compounds nisin and furanones, in changing the microbial composition and interactions and whether these changes alter the resistance of these biofilms to treatments with sanitizers. This information can also be exploited to improve biofilm inactivation strategies with other natural anti-biofilm compounds.

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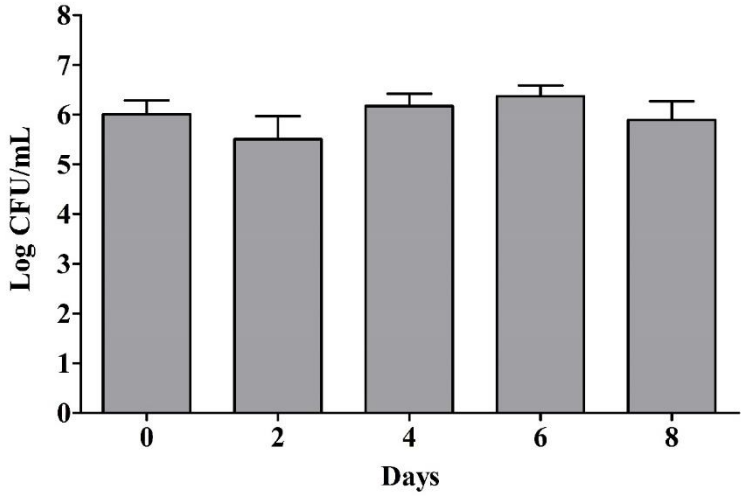
Suplementary material



Suplementary Figure 1- Logarithm of the number of colony-forming units per milliliter (CFU/mL) of the psychrotrophic at 7 ºC for seven days of refrigerated raw milk samples. Day 0 represents the day on which the experiment was set up, for the beginning of biofilm formation. Mean and SD, n=3.

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CHAPTER 2

Isolation, identification and evaluation of phenotypes of bacterial isolates from multispecies biofilms formed by the microbiota of refrigerated raw milk

Article formatted according to Biofouling

Isolation, identification and evaluation of phenotypes of bacterial isolates from multispecies biofilms formed by the microbiota of refrigerated raw milk

Abstract

Biofilms in dairy processing environments are of concern and can increase microbial contamination of products. This study aimed to evaluate the individual biofilm formation ability of 36 isolates obtained from multispecies biofilm formed by the contaminating microbiota of refrigerated raw milk. The isolates were identified by partial sequencing of the 16S rRNA gene. Biofilm formation and proteolytic enzyme activity were determined. The quorum sensing signaling molecules *N*-acyl homoserine lactones (AHL) of medium and short-chains (C4-C8) were evaluated with the biomonitor *Chromobacterium violaceum* CV026 only with Gram-negative isolates. Most of the isolates (69.5%) were able to form an individual biofilm, being classified as weak to strong biofilm formers. *Rahnella inusitata* F083, *Lactococcus garvieae* C081, *Lactococcus laudensis* F013 and *Lactococcus raffinolactis* F083 have the highest biofilm production values. Proteolytic activity was detected in 63.8% of the isolates and AHLs were detected in five isolates identified as *R. inusitata*. The results reinforce the great importance of raw milk contaminant bacteria for the dairy industry, as many were classified as biofilms formers and proteolytic bacteria.

Keywords: Biofilm; milk isolates; proteolytic; AHL.

1. Introduction

Milk is a food rich in nutrients and minerals, has high water activity and a pH close to neutral, making it an ideal medium for microbial growth (Champagne et al. 1994; Quigley et al. 2013). The raw milk microbiota is characterized by a complex and diverse community, originates from multiple sources of contamination and can vary according to region, climate and seasons. Common genera of bacteria found in raw milk include *Acinetobacter*, *Bacillus*, *Enterococcus*, *Lactococcus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, among others (Vithanage et al. 2016; Skeie et al. 2019; Oikonomou et al. 2020; Porcellato et al. 2021). Many of the members of this microbiota play an important role in the deterioration of milk and dairy products, as the vast majority can produce proteolytic and lipolytic enzymes (Teh et al. 2012; Machado et al. 2017; Júnior et al. 2018; Yuan et al. 2018b).

Another relevant characteristic of the spoiling milk microbiota is adhering and colonizing food contact surfaces in processing environments, forming complex layered structures known as biofilm (Yuan et al. 2018a; Banda et al. 2020). Biofilm is a cluster of microbial cells involved in a self-produced matrix composed of extracellular polymeric substances (EPS) (Flemming & Wingender 2010). Biofilm formation in some microorganisms is usually coordinated through cell-to-cell communication, known as quorum sensing (QS). This system involves the detection of small signal molecules secreted in the medium, the autoinducers (AIs), and response according to the density of these molecules, regulating the expression of different genes, such as those related to steps of biofilm formation (Papenfort & Bassler 2016; Prescott & Decho 2020). Gram-negative bacteria use *N*-acyl homoserine lactones (AHL) as AIs molecule, which has been shown to regulate biofilm formation.

Biofilm is a predominant lifestyle for bacteria in many environments. In food industries, the presence of residues of food matrix components on processing surfaces, such

as fats, carbohydrates and proteins, can favor the attachment and multiplication of bacteria (Mittelman 1998; Dutra et al. 2018). The presence of biofilm in industries is of great concern, as the cells embedded in the EPS layer are more resistant to standard cleaning procedures, making them difficult to eliminate (Milan et al. 2015; Yuan et al. 2020). Thus, biofilm functions as a source of food contamination, causing significant economic losses and serious problems of health (Srey et al. 2013). Furthermore, spoilage-associated microorganisms belonging to the genera *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, and *Serratia* have been described as attaching to food contact surfaces and forming biofilms (Teh et al. 2012; Yuan et al. 2018a; Oliveira et al. 2019).

Biofilm formation by species associated with refrigerated raw milk has focused on isolating and culturing the contaminating microorganisms individually. However, due to the abundant and diverse contaminating microbiota found in milk, most biofilms formed on the surfaces of dairy industries may comprise more than one species, thus forming multispecies biofilms (Oliveira et al. 2019; Weber et al. 2019). During multispecies biofilm formation, interactions between different bacteria can regulate the initial adhesion process, and biofilm formation of certain species can facilitate the adhesion of poorly biofilm-forming species, taking advantage of the already formed EPS matrix (Burmølle et al. 2014; Maggio et al. 2021).

Therefore, knowing the biofilm-forming microbiota in the dairy industries can be a step towards finding better strategies for its prevention and elimination. Thus, this study aimed to isolate, identify, evaluate the biofilm formation ability and proteolytic activity to isolates obtained from multispecies biofilm formed by the contaminating microbiota of refrigerated raw milk. Furthermore, the production of AHL in Gram-negative isolates was evaluated.

2. Material and Methods

2.1. Bacterial isolates

Biofilms formed by Oliveira et al. (Chapter 1) from raw milk microbiota in the presence of nisin, furanones, and in the absence of these molecules, in stainless steel for 2, 4, 6, 8 and 10 days at 4 ºC, were used to isolate the bacteria. The 36 bacterial isolates were obtained from the selection of colonies from plating on Plate Count Agar (PCA) (Himedia, India) of the cell suspension of the formed multispecies biofilms. The colonies were selected according to morphological characteristics distinct from the plates with counts from 25 to 300 colonies. Each morphotype was purified by streaking twice on PCA to obtain pure cultures. These isolates were preserved at -80 ºC in Tryptone Soy Broth (TSB) (Himedia, India), added with 20% glycerol.

2.2. Taxonomic identification of isolates

The DNA from the 36 bacterial isolates was extracted using the Wizard® Genomic DNA Purification Extraction Kit (Promega, USA) according to the manufacturer's protocol. After extraction, the DNA was subjected to a polymerase chain reaction (PCR) using oligonucleotides 10f and 1100r (Lane 1991), homologous to conserved regions of the 16S rRNA gene of the Bacteria Domain. The amplified 16S rDNA fragments were purified using the Wizard® SV Gel and PCR Clean-up System kit (Promega, USA) and sequenced on the SeqStudio™ Genetic Analyzer sequencer (Thermo Fisher Scientific, USA). The 16S rDNA sequences obtained were compared with those deposited in the Genbank database (http://www.ncbi.nem.nih.gov) using BLASTn. The 16S rDNA sequences of the isolates identified in this work were deposited in GenBank. Each bacterial isolate received an identification, which contained a letter C (control), N (nisin), or F (furanone), to identify the origin of the treatment used in biofilm formation, and three numbers, which the first two refer

to the day of the formed biofilm (02, 04, 06, 08 and 10 days) and the last number refers to which experiment repetition (repeat 1, 2 or 3) the bacterial isolate was obtained.

Sequences of reference organisms related to the bacteria identified and described in the literature were then selected for phylogenetic analyses. The sequences were aligned and analyzed using MEGA 7.0.18 software (Tamura et al. 2007). The construction of a phylogenetic tree to reveal the taxonomic identification was performed using the Neighbor-Joining method (Saitou & Nei 1987), with bootstrap values calculated from 1,000 replicates.

2.3. Evaluation of biofilm formation

The 36 bacterial isolates were tested for biofilm formation in 96-well polystyrene microplate in TSB supplemented with 1% (p/v) glucose (Stepanovic et al. 2007). Each isolate was growing in TSB at 25 °C for 24 h and 20 µL aliquot was used to inoculate four wells filled with 200 µL of the medium. Negative controls were carried out with non inoculated medium. The microplates were incubated at 25 °C for 48 h. After incubation, the culture supernatant was discarded, each well was rinsed with sterile Phosphate-Buffered Saline (PBS, 0.2 M, pH 7.4) three times, and the cells adhered to the surface were stained with 200 µL of 0.1% (p/v) crystal violet for 30 min. Then, the crystal violet was removed, and the plates were washed three times with PBS and air-dried for 15 min at 37 °C. Then, the crystal violet attached to the biofilm mass was recovered by adding 200 µL of 95% ethanol (v/v) to each well and the optical density (OD) was determined in a Multiskan® microplate spectrophotometer (Thermo Fisher Scientific, USA) at 590 nm. Biofilm formation was performed in three repetitions.

The formed biofilms were classified according to Stepanovic et al. (2007). In brief, the average OD values for each microorganism and negative control were calculated. Then, the breakpoint value, ODc (Optical Density of control) was established. The ODc was defined as

three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + (3×SD of negative control). Lastly, the OD value of a tested microorganism was expressed as the average OD value of the microorganism, reduced by ODc value (OD = average OD of a microorganism − ODc). The ODc value was calculated for each microplate separately. After calculations, the microorganisms were classified based in the calculated of OD values: OD < ODc = non biofilm producer; ODc < OD ≤ 2xODc = weak biofilm producer; 2xODc < OD ≤ 4xODc = moderate biofilm producer; 4xODc < OD = strong biofilm producer.

2.4. Evaluation of proteolytic activity

Each isolate was activated as described in section 2.3, then streaked in PCA (Himedia, Mumbai, India) supplemented with 10% (v/v) of reconstituted skim milk (PCA-milk). Plates were incubated at 25 °C and analyzed after 48 h of incubation. Isolates were classified as non-proteolytic (-), with no clear visible zone of proteolysis around colony growth, and proteolytic (+), with a clear zone of proteolysis around the colony growth.

2.5. Evaluation of N-acyl homoserine lactones (AHL) production

The 18 isolates identified as Gram-negative bacteria were activated as described in section 2.3, and then streaked in PCA (Himedia, Mumbai, India) parallel to the monitor strain *Chromobacterium violaceum* CV026. The presence of exogenous AHL with *N*-acyl side chains from C4 to C8 in length induces the production of the pigment violacein (McClean et al. 1997). The plates were incubated at 30 °C for 24 to 48 h and examined to observe the production of the pigment violacein by *C. violaceum* CV026.

3. Results and Discussion

3.1. Taxonomic identification of bacterial isolates

The taxonomic identification of the 36 selected bacterial isolates showed that they were affiliated with 15 different bacterial species (Figure 1). Of these 36 isolates obtained, 18 (50%) were Gram-negative bacteria and the other 18 (50%) were Gram-positive. Oliveira et al. (2019) showed that each of these groups represents approximately 50% of the multispecies biofilm communities formed by the microbiota of refrigerated raw milk through metagenomic analyses.

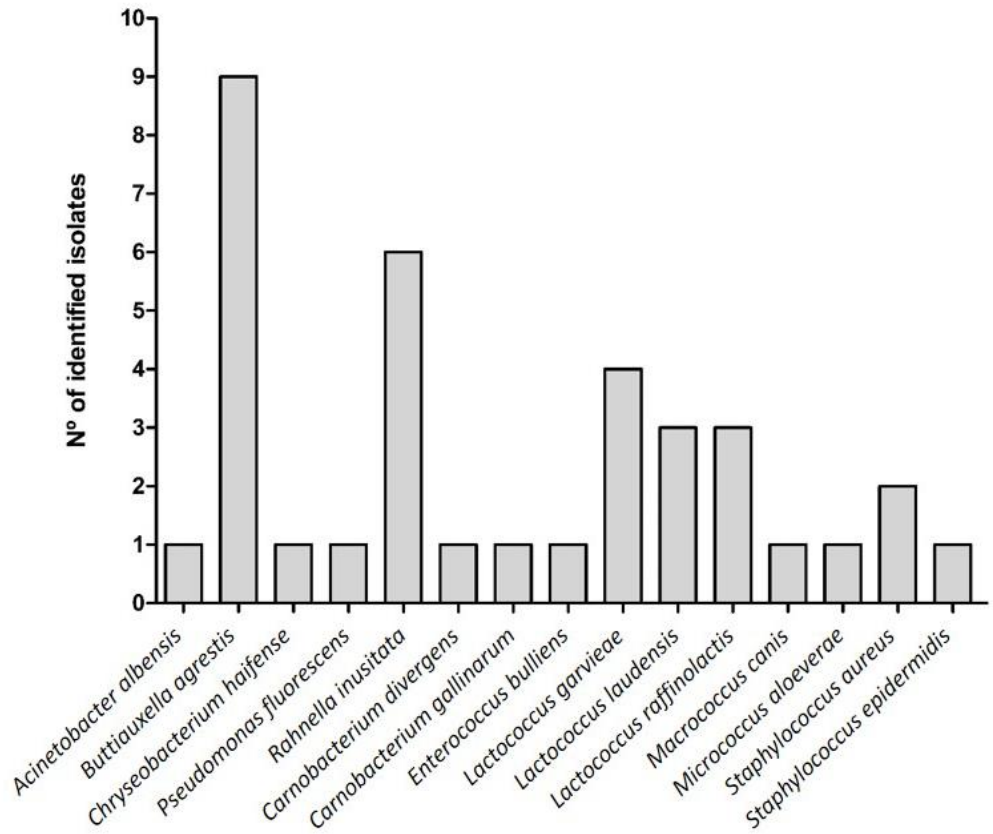


Figure 1- Number of identified bacterial isolates of each species isolated from multispecies biofilm formed from refrigerated raw milk.

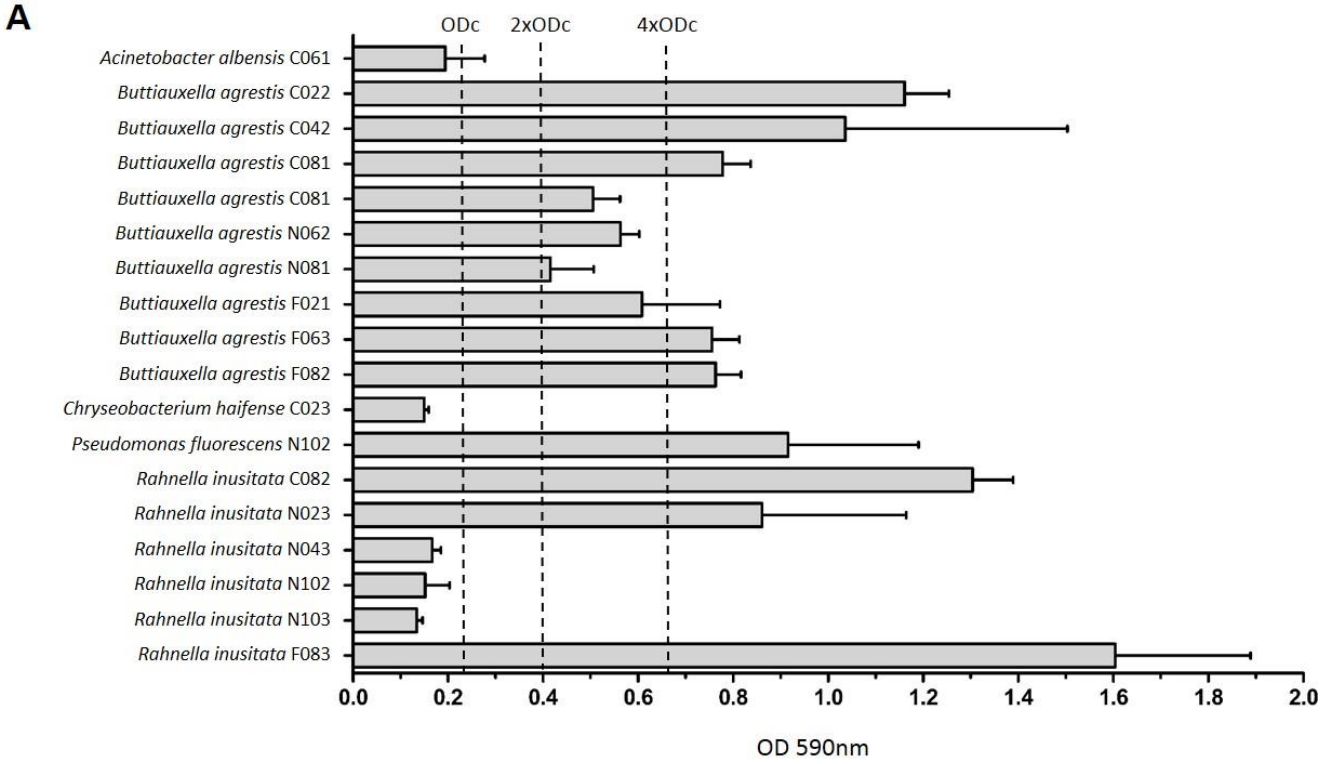
Buttiauxella agrestis and *Rahnella inusitata* were the representatives of the Gram-negative group, with the highest number of isolates, with nine and six isolates, respectively. *B. agrestis* has been commonly reported as a Gram-negative microbiota contaminating milk, with great importance due to its spoiling potential (Deperrois-Lafarge & Meheut 2012). Species belonging to the genus *Buttiauxella* were reported in cold-stored pasteurized milk, indicating its ability to survive after pasteurization (He et al. 2009). The genus *Rahnella* is commonly found in milk (Lindberg et al. 1998; Vithanage et al. 2014; 2016), including *R. inusitata* (Trmčić et al. 2015; Yuan et al. 2017). Only one isolate of *Pseudomonas fluorescens* was isolated, which is considered a frequent contaminant of milk due to its spoilage and biofilm formation potential (Machado et al. 2015; Martins et al. 2015; Rossi et al. 2018). This species has been isolated in biofilms on surfaces of tanks, pipes, and other surfaces in the dairy industry (Teh et al. 2011; Aswathanarayan & Vittal 2014).

Ten isolates belonging to the genus *Lactococcus* were obtained among the Gram-positive bacteria, with representatives of three different species, *Lactococcus garvieae*, *Lactococcus laudensis* and *Lactococcus raffinolactis*. *Lactococcus* was one of the most abundant genera isolated from milk samples, with *L. raffinolactis* being the second most frequent species in a study carried out by von Neubeck et al. (2015). Three isolates of the genus *Staphylococcus* were also obtained: two *Staphylococcus aureus* and one *Staphylococcus epidermidis*. *Staphylococcus* does not grow at low temperatures, but when present in the medium, it has shown its ability to adhere to industrial surfaces, which explains its isolation from the multispecies biofilm formed at 4 ºC (Lee et al. 2014; Teh et al. 2011). This genus is commonly found in milk, and its presence in biofilms in the industry raises significant concerns due to the pathogenicity of *S. aureus*, offering risks to the consumer's health (Ronco et al. 2018).

The other species isolated from the biofilms shown in Figure 1 are part of the microbiota that contaminate milk and are widely isolated in the dairy industries, and many have deteriorating potential, which worries their presence in multispecies biofilms (von Neubeck et al. 2015; Vithanage et al. 2016; Yuan et al. 2017; Ribeiro-Júnior et al. 2020).

3.2. Biofilm formation by the isolates

The isolates obtained showed different abilities to form biofilm on a polystyrene microplate (Figure 2). The 595nm OD values ranged from 0.128 to 1.603, with the highest value obtained by *R. inusitata* F083, followed by *L. garvieae* C081, *L. laudensis* F103 and *Lactococcus raffinolactis* F083. According to the classification proposed by Stepanovic et al. (2007), 25 (69.5%) isolates were able to form a biofilm, including 3 (12%), 8 (32%), 14 (56%), were weak, moderate and strong biofilm formers, respectively. Cherif-Antar et al. (2016) isolated different microorganisms from milk processing line surfaces and found that they had different biofilm-forming abilities, ranging from non-former to strong biofilm former.



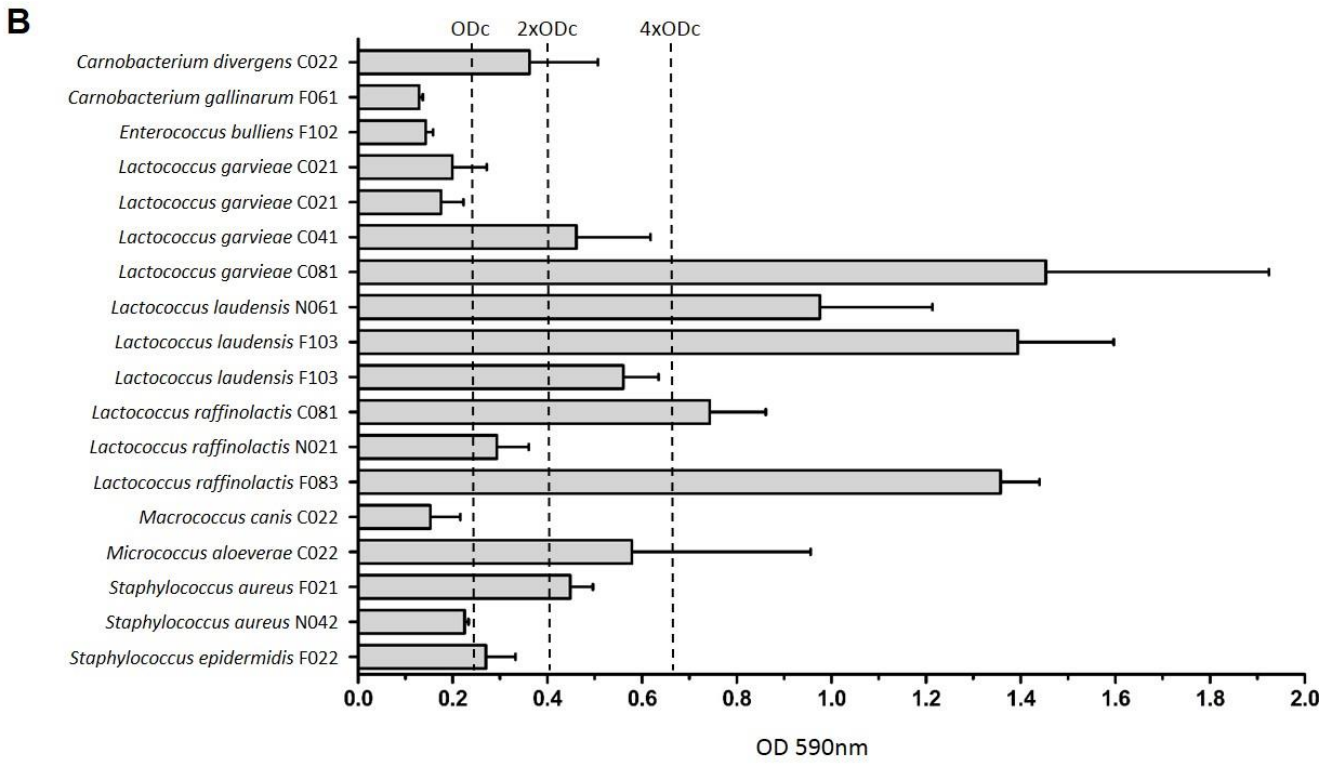


Figure 2- Biofilm formation in microplate of individual isolates formed at 25 ºC during 48 h. The X-axis represents the numerical values of the absorbance of each isolate and the Y-axis represents the identified isolates. (A) Gram-negative isolates. (B) Gram-positive isolates. Absorbance values represent the mean OD of each isolate. ODc= Optical Density of control. OD < ODc = non biofilm producer; ODc < OD ≤ 2xODc = weak biofilm producer; 2xODc < OD ≤ 4xODc = moderate biofilm producer; 4xODc < OD = strong biofilm producer.

A total of 11 isolates (30.5%) was identified as non-formers of individual biofilms on a polystyrene microplate. Although they are isolated from a multispecies biofilm, it is worth noting that in multispecies biofilm, different interactions can occur between species. These interactions can be crucial to allow the co-aggregation or biofilm formation of many species, which alone cannot adhere and develop a biofilm (Elias & Banin 2012; Yuan et al. 2019). In multispecies biofilms, interactions can be classified as cooperative, neutral, or competitive, depending on the species involved (Liu et al. 2016). Good biofilm formers, such as *Pseudomonas*, can interact by cooperating with the fixation of other microorganisms on the surface due to their high EPS production (Ibusquiza et al. 2012). Also, the culture medium, the surface analyzed and the temperature for biofilm formation differed from the one they

were isolated. These factors may have influenced the formation of biofilm in different isolates. The growth substrate and the availability of nutrients in the medium can be critical factors affecting the attachment of microorganisms to the surface (Sadiq et al. 2017). Yuan et al. (2018a) observed greater biofilm formation in stainless steel by psychrotrophic milk isolates when in the presence of TSB than in skimmed milk. For some bacteria, milk proteins can restrict bacterial binding to stainless steel, causing a blocking effect (Barnes et al. 1999; Sadiq et al. 2017). However, other studies have shown that milk components increased biofilm production by *S. aureus* and *Streptococcus uberis* in polystyrene microplate (Varhimo et al. 2011; Fabres-Klein et al. 2015).

Surface characteristics are of great importance for bacterial adhesion, so surfaces made of different materials can affect the ability to form a biofilm (Simões et al. 2010). *S. aureus* isolated from dairy products had more pronounced biofilm formation on polypropylene than on stainless steel (Pagedar et al. 2010). Different milk isolates were able to form biofilm on the microplate and stainless steel, such as *Pseudomonas* spp. species, while other species showed differences between the two surfaces, such as *Acinetobacter harbinensis*, which adhered preferentially to the polystyrene surface of a microplate, and *Acinetobacter guillouiae*, which adhered preferentially to the stainless steel surface (Yuan et al. 2018a).

Temperature can also be a determining factor in biofilm formation. Some psychrotrophic microorganisms have their biofilm formation favored at low temperatures (Tribelli & Lopez 2011; Aswathanarayan & Vittal 2014; Yuan et al. 2018a). *Pseudomonas extremoustralis* showed a higher EPS production at 10 °C compared to 28 °C (Tribelli & Lopez 2011). Rossi et al. (2016) also showed that different *P. fluorescens* isolates from dairy products that the ability to form biofilm was temperature and strain-dependent and that the ability of various isolates to form biofilm was associated with low temperature.

Isolates of *B. agrestis* showed a strong ability for microplate biofilm formation, in addition to *R. inusitata*, *P. fluorescens* and isolates belonging to the genus *Lactococcus*. Interestingly, isolates from the same species of *B. agrestis* and *R. inusitata* showed different biofilm formation abilities, but isolates of *B. agrestis* had medium or strong capacity. In species of the genus *Lactococcus*, this variation between strains was also observed. Thus, biofilm formation was considered a strain-dependent feature. Variation in biofilm formation between strains has also been reported among diverse bacteria as *Listeria monocytogenes*, *P. fluorescens*, *Aeromonas hydrophila* and *S. aureus* (Harvey et al. 2007; Nagar et al. 2017; Piechota et al. 2018; Thiran et al. 2018; Yuan et al. 2018a).

B. agrestis and *R. inusitata*, isolated from a milk sample, showed a weak ability for biofilm formation at 7 ºC (Yuan et al. 2018a). Isolates of *Lactococcus* spp. from refrigerated raw milk were able to form biofilm at temperatures of 10 ºC and 30 ºC (Hahne et al. 2019). *Micrococcus aloeverae* was also shown to be weak to moderate biofilm former on microplate at 37 ºC in a study performed by Saha et al. (2020). On the other hand, other species are well known for their ability to form a biofilm, such as *P. fluorescens* and *S. aureus*. *P. fluorescens* is often isolated from raw milk and surfaces in the dairy industry and has a high ability for biofilm formation on different surfaces, such as polystyrene and stainless steel (Teh et al. 2011; Rossi et al. 2018; Zarei et al. 2020). The foodborne pathogen *S. aureus* is also of great concern to the dairy industry, as its presence in milk and surfaces is recurrent and has a moderate ability for biofilm formation (Teh et al. 2011; Gutiérrez et al. 2012; Filipello et al. 2019).

3.3. Proteolytic activity

The proteolytic activity was evaluated in PCA-milk, and 23 (63.8%) of the 36 isolates were considered proteolytic (Table 1). Among the Gram-negative isolates, 11 (61%) showed proteolytic activity. Most of the isolates identified as *B. agrestis* and *R. inusitata* were

positive for the proteolytic enzymes, although there was no consensus among the isolates of these species. Trmčić et al. (2015) did not observe proteolytic activity in *R. inusitata*, isolated from pasteurized milk, but verified the β-galactosidase activity at 21 ºC, capable of causing a ropy milk defect. Yuan et al. (2018b) also reported no proteolytic activity by isolates of *R. inusitata* and *B. agrestis*, nor lipolytic activity by *B. agrestis*. Another proteolytic isolate was *Chryseobacterium haifense*, and this genus has been reported to have high proteolytic and lipolytic activity (Hantsis-Zacharov & Halpern 2007; Yuan et al. 2018b). *Pseudomonas* has been widely recognized for having a proteolytic activity (Marchand et al. 2009; Machado et al. 2015), and the isolate in our study reinforce this phenotype (Table 1). In addition to the ability of microorganisms to produce deteriorating enzymes, the great concern for dairy industries is the heat resistance shown by some of these enzymes to heat treatments commonly used in milk processing (Marchand et al. 2009; Baglinière et al. 2013; Machado et al. 2016).

Among the Gram-positive isolates *C. divergens*, *C. gallinarum*, *M. aloeverae*, *Staphylococcus* spp. and the vast majority of *Lactococcus* spp. showed proteolytic activity. *Staphylococcus* in general have been described with great spoilage potential with the production of proteolytic and lipolytic enzymes (Teh et al. 2011; Vidanarachchi et al. 2015; Hahne et al. 2019). Species of *Lactococcus* have been reported to have proteolytic activity (Tidona et al. 2018; Hahne et al. 2019), and although this phenotype has technological value and is explored in the production of different dairy products (Tidona et al. 2018; Fusieger et al. 2020), it is detrimental to raw milk quality.

Table 1- Identification of the 36 isolates from multispecies biofilm, classification of biofilm formation ability, classification with or without proteolytic activity, and ability to induce or not the production of violacein by *C. violaceum* CV026.

	Identification	16S rDNA	Biofilm formation	Proteolytic activity	Induction of violacein production	Accession number
Gram-negative	**C061**	*Acinetobacter albensis*	non	-	-	MZ905496
	C022	*Buttiauxella agrestis*	strong	+	-	MZ905643
	C042	*Buttiauxella agrestis*	strong	-	-	MZ908685
	C081	*Buttiauxella agrestis*	strong	+	-	MZ905501
	C081	*Buttiauxella agrestis*	moderate	+	-	MZ905502
	N062	*Buttiauxella agrestis*	moderate	-	-	MZ912700
	N081	*Buttiauxella agrestis*	moderate	+	-	MZ912493
	F021	*Buttiauxella agrestis*	moderate	-	-	MZ905642
	F063	*Buttiauxella agrestis*	strong	+	-	MZ913015
	F082	*Buttiauxella agrestis*	strong	-	-	MZ911747
	C023	*Chryseobacterium haifense*	non	+	-	MZ912998
	N102	*Pseudomonas fluorescens*	strong	+	-	MZ908777
	C082	*Rahnella inusitata*	strong	+	+	MZ908686
	N023	*Rahnella inusitata*	strong	-	+	MZ911749
	N043	*Rahnella inusitata*	non	-	+	MZ911847
	N102	*Rahnella inusitata*	non	+	+	MZ910713
	N103	*Rahnella inusitata*	non	+	+	MZ911848
	F083	*Rahnella inusitata*	strong	+	-	MZ913022
Gram-positive	**C022**	*Carnobacterium divergens*	weak	+	N	MZ905644
	F061	*Carnobacterium gallinarum*	non	+	N	MZ912701
	F102	*Enterococcus bulliens*	non	-	N	SUB10260360
	C021	*Lactococcus garvieae*	non	+	N	MZ901914
	C021	*Lactococcus garvieae*	non	+	N	MZ902026
	C041	*Lactococcus garvieae*	moderate	-	N	MZ911870
	C081	*Lactococcus garvieae*	strong	+	N	MZ911897
	N061	*Lactococcus laudensis*	strong	+	N	MZ912494
	F103	*Lactococcus laudensis*	strong	-	N	SUB10266551
	F103	*Lactococcus laudensis*	moderate	-	N	MZ911853
	C081	*Lactococcus raffinolactis*	strong	+	N	MZ911898
	N021	*Lactococcus raffinolactis*	weak	-	N	MZ905518
	F083	*Lactococcus raffinolactis*	strong	+	N	MZ911850
	C022	*Macrococcus canis*	non	-	N	MZ905640
	C022	*Micrococcus aloeverae*	moderate	+	N	MZ907678
	N042	*Staphylococcus aureus*	moderate	+	N	MZ913019
	F021	*Staphylococcus aureus*	non	+	N	MZ912699
	F022	*Staphylococcus epidermidis*	weak	+	N	MZ911739

(N) no tests were performed to detect AHL molecule.

In this work, the evaluation of proteolytic enzymes produced by the isolates was not performed with cells adhered to biofilms. However, Teh et al. (2014) observed that proteolysis may be greater in biofilm than in planktonic cultures. Therefore, these different bacterial species with biofilm production capacity and which are also capable of producing proteolytic enzymes is of concern in the dairy industry. The persistence of these bacteria in biofilm formed on dairy processing equipment could result in a constant contamination and promotes milk and dairy products spoilage.

3.4. AHL production

Among the 18 Gram-negative isolates tested for AHL production, only five (27.8%) were able to induce the monitor strain *C. violaceum* CV026 (Table 1). These five isolates, C082, N102, N023, N043 and N103, belong to the species *R. inusitata*. The genus *Rahnella* was recently reclassified as it originally consisted of only one species, *Rahnella aquatilis*, and so very little is known about *R. inusitata* (Brady et al. 2014). *R. aquatilis* has been described as a producer of AHL (Myszka et al. 2021). The presence of *R. inusitata* has been reported in raw milk (Yuan et al. 2017), but its ability to produce AHL has not yet been described.

Many Gram-negative isolates from raw milk samples produce different AHL (Pinto et al. 2007; Martins et al. 2018). AHL-dependent QS has been identified in a wide range of Gram-negative bacteria and is involved in the regulation of many functions, such as the production of proteolytic enzymes and biofilm formation of different species, thus becoming an important focus of study for food industry (Ponce et al. 2012; Bai A & Vittal 2014; Passos da Silva et al. 2017). As such, QS can play an important role in the deterioration of many products.

The production and response to AHLs may differ between bacterial species and even between isolates of the same species due to variations in the size of the carbon chain of this

molecule, as well as the presence or absence of modifications. The *C. violaceum* CV026 biomonitor strain is able to respond to AHL molecules with side chains ranging from four to eight carbons (McClean et al. 1997). This may have been a limitation for the identification of other AHL-producing isolates with longer carbon chains.

4. Conclusions

Many of the isolates identified in this study were able to form biofilms (69.5%), among them most were classified as moderate to strong biofilm former. Isolates of *R. inusitata*, *L. garvieae*, *L. laudensis* and *L. raffinolactis* showed the highest biofilm production values. Furthermore, most isolates showed proteolytic activity, but only five *R. inusitata* isolates were able to produce AHL. The formation of biofilm and production of deteriorating enzymes produced by different bacteria is still a major challenge for the dairy industries.

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CHAPTER 3

Application of response surface methodology (RSM) for inactivation of multispecies biofilm cells formed in milk by combining peracetic acid, time and temperature

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Application of response surface methodology (RSM) for inactivation of multispecies biofilm cells formed in milk by combining peracetic acid, time and temperature

Abstract

Biofilm can be formed on different surfaces and is generally associated with more than one microbial species. Particularly in the food industry, biofilm is often a contamination source and mitigation strategies are needed. In the present study, the response surface methodology (RSM) was used to optimize conditions for the inactivation of multispecies biofilm cells, using peracetic acid concentration (PAA) (0.05-0.5%), treatment time (5-30 min) and temperature (25-60 ºC) as three independent variables. The multispecies biofilm was formed by the Gram-positive and Gram-negative bacteria, previously isolates from biofilm formed in the presence of raw milk. The stainless steel (SS) coupons were immersed in reconstituted whole milk inoculated with *Pseudomonas fluorescens*, *Rahnella inusitata*, *Staphylococcus aureus* and *Micrococcus aloeverae*. Numbers of around 108 CFU/cm^2 of sessile cells were obtained after 10 days incubation at 4 ºC; with the exchange of milk and inoculum every 48 h. The response surface model showed a goodness fit with R^2 of 0.9489 and adjR^2 of 0.8832. All independent variables had a positive effect on the inactivation of biofilm cells. The maximum inactivation was approximately 6 log (CFU/cm^2), the highest values achieved when the three factors were used. Epifluorescence microscopy demonstrated that many cells in the biofilm were dead or injured after treatment corresponding to the central point. RSM is an interesting approach to accompany the inactivation of multispecies biofilm cells, and should be more explored.

Keywords: *Pseudomonas fluorescens*; *Rahnella inusitata*; *Staphylococcus aureus*; *Micrococcus aloeverae*; milk; stainless steel.

1. Introduction

The food industry is increasingly looking for effective ways to reduce the contamination of raw materials and products by spoilage and pathogenic microorganisms. Biofilm formed on food contact surfaces represents a significant well recognized concern in food safety as it can act as a reservoir of microbial contaminants, compromising the final product, and offering risks to the consumer's health if pathogens are present (Abdallah et al., 2014; Banda et al., 2020). The sessile cells present in biofilm are incorporated in an extracellular polymeric substance (EPS) composed of polysaccharides, proteins, nucleic acids and others, exhibiting greater resistance to removal and antimicrobial agents used in industries compared to planktonic cells (Singh et al., 2017; Barroso et al., 2019; Kamble & Pardesi, 2021).

Raw milk may contain an abundant and diverse contaminating microbiota, depending on the hygienic conditions in which it is obtained, and may contribute to the spread of contaminants in the dairy industry environments. Therefore, on the vast majority of real-case scenarios, the biofilm formed in the dairy environments are multispecies, characterized by great microbial diversity (Kostaki et al., 2012; Nozhevnikova et al., 2015; Oliveira et al., 2019). Multispecies biofilm with their species heterogeneity are more resilient to antimicrobial agents than their monospecies biofilm counterparts (Joshi et al., 2021) and could be more resistant to mitigation and persist more in the environment. Oxaran et al. (2018) demonstrated that the pathogens *Staphylococcus aureus* and *Listeria monocytogenes* when present in a mixed biofilm formed by five species were more resistant to peracetic acid (PAA) disinfectant compared to their monospecies biofilms. The higher EPS complexity of multispecies biofilm could contribute to this higher resistance (Lee et al., 2014a; Kim et al., 2021).

Due to the great resistance of biofilm, its persistence and the problems caused by it are constant in the food industries. Thus, its inactivation and prevention are necessary to achieve safe and quality products, and efforts to eliminate them from the industrial environment include effective cleaning and sanitizing process (Peng et al., 2002). One of the most widely used sanitizers is PAA, due to its broad spectrum of antimicrobial activity (Orth, 1998; Falsanisi et al., 2006). In addition to its efficiency against planktonic cells, PAA has shown great efficiency against monospecies and multispecies biofilms (da Silva Fernandes et al., 2015; Lee et al., 2016; Yuan, Wang, Sadiq, & He, 2020).

Investigating factors that may affect the inactivation of cells from biofilm is of great interest for the industry, in order to optimize cleaning and sanitization protocols. Mathematical modeling for developing predictive models is widely used to describe the growth or reduction of viable microorganisms (Song et al., 2014). Response surface methodology (RSM) is recognized as a powerful gathering tool of mathematical and statistical approaches which are helpful to analyze and model problems, where a response of interest is affected by multiple independent variables for response optimization (Montgomery, 2005; Chelladurai et al., 2021). The RSM has been used as a mathematical strategy to predict the inactivation of monospecies biofilm from different microorganisms (Kim & Rhee, 2018; Ranmadugala, Ebrahimnezhad, Manley-Harris, Ghasemi, & Berenjian, 2017; Zhang, Ye, Juneja, & Xu, 2017). Nevertheless, to our knowledge, no study has modeled the removal of multispecies biofilm, especially using the RSM.

Thus, this study aimed to determine the effects of PAA concentration, exposure time and temperature on the inactivation of multispecies biofilm cells using the RSM.

2. Material and Methods

2.1 Bacteria

Pseudomonas fluorescens N102, *Rahnella inusitata* N023*, Staphylococcus aureus* F021 and *Micrococcus aloeverae* C022 were isolated from biofilm formed in stainless steel (SS) in the presence of refrigerated raw milk and identified by the 16S rDNA that was deposited in GenBank with accession number MZ908777, MZ911749, MZ912699 and MZ907678, respectively (Oliveira et al., Chapter 2). These isolates were selected based on previous results regarding their ability to form biofilm in a microplate assay, where they were moderate to strong biofilm formers at 25 ºC for 48 h (Oliveira et al., Chapter 2). The stock cultures of these microorganisms were kept at -20 °C in Tryptone Soy Broth (TSB), added with 20% glycerol. Before each experiment, each microorganism was grown in Brain Heart Infusion broth (BHI, Himedia, India) incubated at 30 °C for 24 h. Subsequently, the cultures were centrifuged at 13,000 *g* for 5 min at 4 °C and washed three times with Phosphate Buffer Saline (PBS) 10 mM pH 7.0. The inocula were standardized at an optical density (O.D.) of 0.1 using absorbance measurements at 600 nm in a spectrophotometer (Thermo Fisher Scientific, Finland).

2.2 Biofilm formation

The multispecies biofilm was formed in SS coupons AISI 304 (#4) with dimensions of 1 x 1 x 0.2 cm, sanitized as described by Oliveira et al. (2019). The coupons were hung in Erlenmeyer flasks containing 200 mL sterilized reconstituted whole milk at 10% (w/v), added 1% of standard inoculum from each of the four microorganisms and incubated at 4 ºC for 10 days. Every 48 h the coupons were washed with PBS, the reconstituted whole milk was exchanged for a fresh one inoculated with the respective microorganisms at the same

concentrations as described above. This procedure was carried out until completing 10 days of biofilm formation.

2.3 Sessile cell count

On the 10th day of biofilm formation, the biofilm cells were removed for counting. For this, the coupons were transferred to glass tubes containing 3 mL of PBS, exposed to ultrasound treatment (Vibra Cell, Newtown, USA) for 1 min continuous mode operating (130 W, 20 kHz) equipped with a 6 mm stainless steel probe and then, taken to the vortex for 30 s to remove cells from the biofilm (Bonkat et al., 2012). Then, serial dilution was performed from this cells suspension and plated by the drop plate method (Morton, 2001) on Plate Count Agar (PCA, Himedia, India). The plates were incubated at 30 °C for 8-24 h, and the colonies were counted and expressed as log CFU/cm^2.

2.4 Scanning Electron Microscopy (SEM)

The SEM was performed on the multispecies biofilm formed on the 10th day to observe the formed structure. The SS coupons were removed from the culture medium, rinsed with PBS, fixed as described by Campos-Galvão, Ribon, Araújo, & Vanetti (2016) and observed in a Scanning Electronic Microscope (JEOL, model JSM 6010LA, Japan).

2.5 Preparation of peracetic acid (PAA)

The PAA was prepared by diluting the commercial sanitizer peracetic acid 20% (Start, Brazil) in distilled water, in concentrations ranging of 0.05 to 0.5%. The solutions were prepared and used on the same day.

2.6 Response surface modelling and statistical analysis

The central composite face-centered (CCFC) experimental design was used to determine the response surface in the optimum region for inactivation multispecies biofilm cells. The independent variables used were PAA (0.05-0.5%) concentration, time (5-30 min)

and temperature (25-60 ºC) (Table 1). A total of 17 runs were employed, including three replicates at the central point to calculate the pure error of the regression analysis (Table 2). The runs were performed randomly according to a CCFC configuration for the three variables. Response value was expressed as logarithmic reductions by the difference between the logarithm of the viable cells number in multispecies biofilm at the initial and final of each treatment (log (CFU/cm^2 initial) – (log CFU/cm^2 final)).

Table 1- Experimental ranges and levels of independent variables for the central composite face-centered design used in optimization inactivation multispecies biofilm cells.

Independent variable	Range level		
	-1	0	+ 1
PAA (%)	0.05	0.275	0.50
Time (min)	5.0	17.5	30.0
Temperature (ºC)	25.0	42.5	60.0

Table 2- Central composite face-centered design matrix, actual and predicted response values for inactivation multispecies biofilm cells as a function of PAA concentration, time and temperature.

Run	Independent variables			Reduction log (CFU/cm^2)	
	PAA (%)	Time (min)	Temperature (ºC)	Actual Values	Predicted values
1	0.050	5.0	25.0	3.05941	3.19939
2	0.500	5.0	25.0	3.78850	3.92501
3	0.050	5.0	60.0	3.94997	4.02731
4	0.500	5.0	60.0	4.75838	4.82417
5	0.050	30.0	25.0	3.77340	3.81701
6	0.500	30.0	25.0	5.33350	5.36555
7	0.050	30.0	60.0	5.35698	5.32987
8	0.500	30.0	60.0	6.98023	6.94965
9	0.050	17.5	42.5	3.75838	3.52457
10	0.500	17.5	42.5	4.90105	4.69727
11	0.275	17.5	25.0	3.85529	3.50314
12	0.275	17.5	60.0	4.79459	4.70915
13	0.275	5.0	42.5	3.78850	3.36887
14	0.275	30.0	42.5	4.75838	4.74042
15	0.275	17.5	42.5	3.71699	3.79598

16	0.275	17.5	42.5	3.42939	3.79598
17	0.275	17.5	42.5	3.36639	3.79598

The Minitab 17.0 statistical software was used for data analysis using multiple linear regressions.

The data were examined by analysis of variance (ANOVA) for statistical significance and goodness of fit of the response surface (RS) model. To assess how much the variability of the response variable can be influenced by the independent variables, the determination coefficient (R^2) and the adjusted determination coefficient ($adjR^2$) were used. To verify the significance of individual terms and their interactions, the *p*-value was calculated and to verify the terms that most affect the response using the *t*-distribution values, the Pareto's diagram was constructed (Bevilacqua et al., 2013). Using Software Sigmaplot version 12.0, response surface plots were generated to show the effects between the independent variables (PAA concentration, time and temperature), in the inactivation of multispecies biofilm. The level of significance used was 5% for all statistical tests.

2.7 Inactivation of multispecies biofilm by combining PAA concentration, time and temperature

The multispecies biofilms formed in SS in reconstituted whole milk were rinsed with PBS, to remove non-adherent cells and placed in tubes with different concentrations of PAA, time and temperature according to CCDR (Table 2). The temperature treatment was conducted by incubating the tubes in a water bath. After each treatment, the coupons were immersed for 2 min in 3 mL of neutralizing solution (0.34 g/L KH_2PO_4; 5 g/L $Na_2S_2O_3$; 3 g/L lecithin from soybeans, 1 g/L L-histidine and 3% (v/v) Tween 80) (Luppens et al., 2002), at room temperature. Then, the cells were removed from the multispecies biofilm and counted colonies as described previously (section 2.3).

2.8 Model validation

The validation of the proposed model was performed by selecting nine random experimental conditions within the range of the experimental domain but different from the combinations used to build the model. According to the procedure described above, these nine selected conditions were submitted to treatment (section 2.6 and 2.7). The experimental results obtained were compared with the predicted values of the mathematical models in the nine proposed experimental conditions (Table 4). The experiments were carried out three times.

2.9 Epifluorescence microscopy

Epifluorescence microscopy was performed on the control biofilm and the biofilm exposed to the treatment of the central point (PAA = 0.275%; Time = 17.5 min; Temperature = 42.5 ºC). The SS coupons were removed from the culture medium after 10 days, rinsed with PBS and then subjected to the central point treatment according to section 2.7 or not for the control treatment. Subsequently, the coupons were stained with the Live/Dead kit (Invitrogen, Sweden) for 20 min in the absence of light and observed under an epifluorescence microscope (Thermo Fisher, model EVOS M5000, USA), using GFP and RFP filters with excitement wavelength of 470 nm and 531 nm, respectively.

3. Results and Discussion

3.1 Biofilm

The multispecies biofilm formed by *P. fluorescens* N102, *R. inusitata* N023, *S. aureus* F021 and *M. aloeverae* C022 in SS coupon reached a population of 10^8 CFU/cm^2, on the 10th day of incubation at 4 ºC in reconstituted whole milk. It was possible to observe by images obtained on SEM, the presence of a mat of cells forming the structure of biofilm (Figure 1). It is noteworthy that these isolates were obtained from biofilms formed by the microbiota of refrigerated raw milk (Oliveira et al., Chapter 2). These four bacterial species are also frequently isolated from raw milk and dairy surfaces (Porcellato et al., 2021; Ribeiro Júnior, Tamanini, de Oliveira, Alfieri, & Beloti, 2018; Sharma & Anand, 2002; Trmčić, Martin, Boor, & Wiedmann, 2015; Yuan, Burmølle, Sadiq, Wang, & He, 2018).

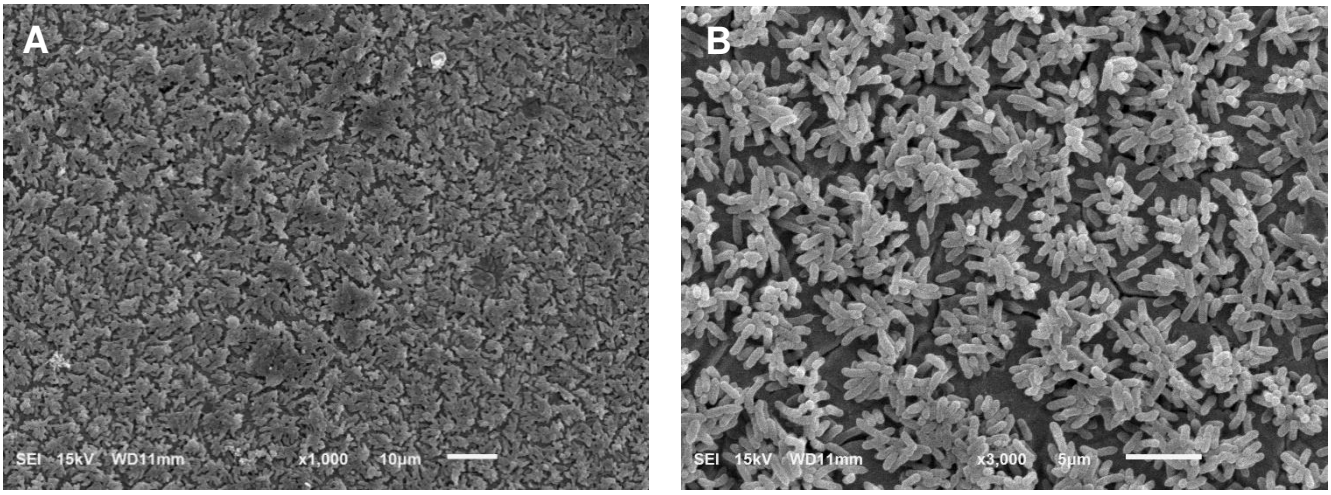

Figure 1- Scanning Electron Microscopy of the multispecies biofilm formed in SS coupon, immersed in reconstituted whole milk on the 10th day of incubation at 4 ºC. A- Magnification of 1,000 x; B- Magnification of 3,000 x.

Among the Gram-negative bacteria found commonly in milk, *P. fluorescens* is of great concern for the dairy industry due to its deteriorating potential with the production of proteolytic and lipolytic enzymes (Machado et al., 2015; 2017; Martins, Pinto, Riedel, &

Vanetti, 2015). It is also proven its ability to form biofilm at temperatures varying from 7 to 30 ºC (Aswathanarayan & Vittal, 2014; Rossi et al., 2016; Wang, Cai, Li, Xu, & Zhou, 2018). *P. fluorescens* has a high production of EPS that can facilitate fixation and provide protection to other microorganisms, including pathogenic bacteria (Puga, Dahdouh, SanJose, & Orgaz, 2018; Wang, Hong, Liu, Zhu, & Chen, 2020). The genus *Rahnella* is commonly found in milk (Lindberg, Ljungh, Ahrne, Löfdahl, & Molin, 1998; Vithanage, Yeager, Jadhav, Palombo, & Datta, 2014; Vithanage et al., 2016), but the species *R. inusitata* has only been reported recently (Trmčić et al. 2015; Yuan et al. 2017). *R. inusitata* has been isolated from pasteurized milk exhibiting a ropy defect at a temperature of 6 ºC (Trmčić et al. 2015). It grows at low temperatures and, according to Yuan et al. (2018), formed biofilm at 7 ºC in SS, both in TSB medium and skimmed milk.

The Gram-positive pathogen *S. aureus* also presents a deteriorating potential, with the production of proteolytic and lipolytic enzymes (Vadehra & Harmon, 1965; Teh et al., 2011). Its biofilm formation ability has been observed at temperatures of 20 ºC to 37 ºC (Fabres-Klein et al., 2015; Thiran et al., 2018). However, when present in the milk, it can adhere to processing surfaces, where temperatures are generally low (Lee et al., 2014b; Teh et al., 2011). We used *S. aureus* isolate which proved to be frequent in biofilm formed from raw milk maintained at 4 °C. Although *S. aureus* is not recognized as a psychrotrophic bacteria, its maintenance in biofilm at low temperature is possible. Bacteria of the genus *Micrococcus* are part of the contaminating microbiota of raw milk (Aaku et al., 2004; Ribeiro-Júnior et al., 2020). *M. aloeverae* are proteolytic and lipolytic, in addition to forming biofilm on a polystyrene surface at 37 ºC (Ribeiro Júnior et al., 2018; Saha et al., 2020). No reports were found on biofilm formation by this species at a refrigeration temperature.

3.2 Modeling

The RSM was used for investigated the influence of three independent variables (PAA concentration, time and temperature) and for optimizing these parameters on the inactivation of cells of the multispecies biofilm. The current and predicted values are shown in Table 2 and were used to establish a model containing the three variables. Based on the actual values (Table 2), a second-order polynomial equation was established as described by Freitas, Prudêncio, Peña, & Vanetti (2019) and was presented as follows:

$$y = 4.45 - 2.29\ PAA - 0.0666\ Temp - 0.0565\ T + 6.22\ PAA^2 + 0.001013\ Temp^2 + 0{,}00166\ T^2 + 0.0045\ PAA*Temp + 0.0731\ PAA*T + 0.000783\ Temp*T$$

Equation (1)

where y is the log (CFU/cm^2) inactivation of multispecies biofilm cells; *PAA* is the concentration of peracetic acid (%); *Temp* is treatment temperature (ºC); T is treatment time (min).

ANOVA results for the RS model indicated that the p-value of the model is low (0.001) and all linear terms were statistically significant ($p < 0.05$) (Table 3). In addition, the results presented a R^2 of 0.9489 and adjR^2 of 0.8832. These obtained values indicate that the model has a goodness of fit and can explain about 90% of the variability in the response. R^2 values above 0.7 are strong indicators of the model's adequacy to describe the importance of independent variable's response (Granato & Masson, 2010). Lack of Fit test and F-test were also used to determine the statistical significance. Results showed that the model for predicting the effect of the PAA concentration, time and temperature on the inactivation of multispecies biofilm cells was considered statistically significant. The Pareto chart showed that the term linear time exhibited the greatest effect on the inactivation of multispecies biofilm cells, followed by temperature and concentration (Figure 2).

Table 3- ANOVA for the predictive model and estimated coefficients of the adjusted RS model for inactivation multispecies biofilm cells.

	Degrees of freedon	Sum of squares	Mean square	F Value	*p*-value
Model	9	14.531	1.614	14.45	0.001
PAA	1	3.438	3.438	30.77	0.001
Time	1	4.702	4.702	42.08	<0.001
Temperature	1	3.636	3.636	32.54	0.001
PAA^2	1	0.265	0.265	2.38	0.167
$Time^2$	1	0.179	0.179	1.60	0.246
$Temperature^2$	1	0.257	0.257	2.31	0.173
PAA*Temperature	1	0.0025	0.0025	0.02	0.884
PAA*Time	1	0.338	0.338	3.03	0.125
Time*Temperature	1	0.234	0.234	2.10	0.191
Residual error	7	0.782	0.111		
Lack of fit	5	0.712	0.142	4.08	0.209
Pure error	2	0.069	0.034		
Total	16	15.313			

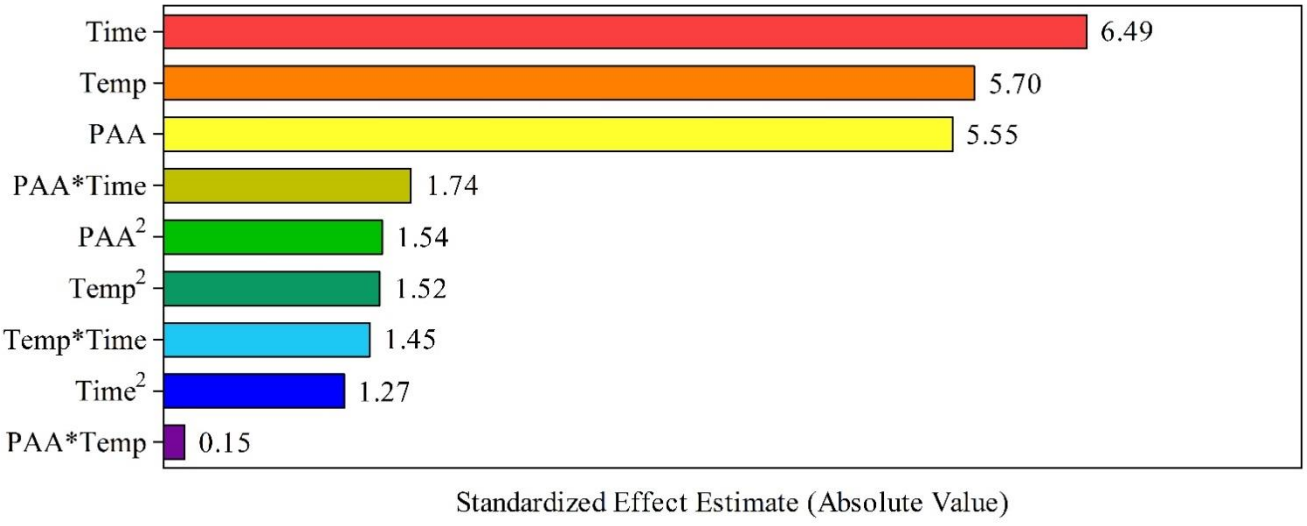

Figure 2- The standardized Pareto chart showing the effect of all factors on the inactivation multispecies biofilm cells. Time: treatment time; Temp: treatment temperature; PAA: peracetic acid concentration.

The effect of independent variables that can be adjusted to achieve a satisfactory result in the sanitization of equipment and food contact surfaces has been used to build statistical models to estimate the inactivation of the cell of the biofilm. Previous results indicated that RSM could be used to predict cell removal from the biofilm with good statistics adjustments. The RSM was used to optimize the removal of *Bacillus cereus* biofilm from SS coupons using the combination time (10-30 min), temperature (40-65ºC) and sodium hydroxide (1-2%) concentration (Kumari & Sarkar, 2014). Also, RSM was used to investigate the influence of the concentration of sodium chloride, calcium chloride, and urea in reducing biofilm formation by *B. cereus* (Ranmadugala et al., 2017). A predictive model for estimating the removal of *Salmonella* biofilm in SS coupons was obtained using various concentrations of chlorine (50-150 ppm), ethanol (10-30%), and lactic acid (1-3%) (Zhang et al., 2017). RSM was also used to build a model to predict reductions in uropathogenic *Escherichia coli* biofilms using the combination of cranberry extract (3–9%), caprylic acid (0.01-0.05%) and thymol (0.01-0.5%) (Kim & Rhee, 2018).

3.3 Inactivation of multispecies biofilm

Fixing the values of the central points (PAA concentration = 0.275%, time = 17.5 min and temperature = 42.5 ºC) the response surface graphs were generated according to the model (Eq. 1). The values obtained for cells inactivation of multispecies biofilm ranged from approximately 3 to 6 log CFU/cm^2 cycles depending on the intensity of the treatment applied (Figure 3). The results indicate that the increase in temperature and treatment time causes an increase in the inactivation of biofilm cells (Figure 3A). Similarly, the high temperature (above 50 ºC) and a higher concentration of PAA (above 0.4%) caused an inactivation greater than 4.5 log CFU/cm^2 cycles (Figure 3B). The same can be observed with the increase in the time and concentration of PAA, which results in inactivation of more than 6 log cycles in the

sessile cells number (Figure 3C). Thus, according to Figure 3, all the independent variables tested positively affected the efficient inactivation of cells present in the biofilm.

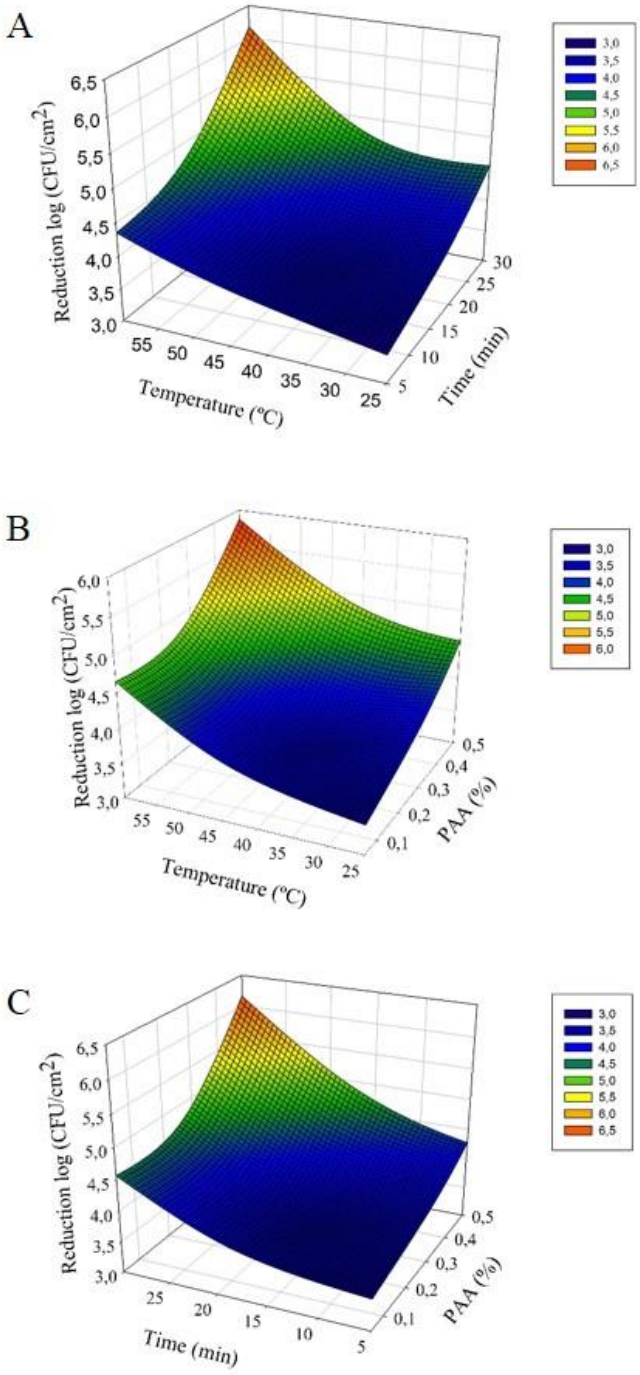

Figure 3- 3D response surface plots for inactivation multispecies biofilm cells using interaction between three different variables: (A) temperature *vs*. time and fixed PAA concentration of 0.275%; (B) temperature *vs*. PAA concentration and fixed time of 17.5 min; (C) time *vs*. PAA concentration and fixed temperature of 42.5 ºC.

The efficiency of PAA against monospecies biofilms of several microorganisms has been demonstrated (Rasimus et al., 2011; Akinbobola et al., 2017; Iñiguez-Moreno et al., 2018). Marques et al. (2007) obtained reductions of up to 5.26 and 4.5 log CFU/cm^2 of *S. aureus* from the biofilm formed on SS and glass surfaces, respectively, using a concentration of 0.3% PAA for 30 s at room temperature. On the other hand, *S. aureus* biofilm formed in SS was almost completely inhibited when treated with PAA at a concentration of 0.5% for 1 min (Lee et al., 2016). Castro, da Silva Fernandes, Kabuki, & Kuaye (2021) observed only a 2.28 log reduction in the *P. fluorescens* monospecies biofilm after treatment with PAA at 200 ppm for 10 min. Biofilm formed by more than one species becomes of great concern for the food industry, as they generally exhibit greater resistance to different antimicrobials, when compared to monospecies biofilms. The increased survival of *L. monocytogenes* to the sanitizer benzalkonium chloride was correlated with the presence of *P. fluorescens* growing in a two-species biofilm (Haddad et al., 2021).

3.4 Model validation and epifluorescence microscopy

Validation is a critical step in the development of predictive models. Therefore, in this study, the model obtained was validated using additional random nine conditions and the results are shown in Table 4.

The values obtained experimentally agree with the prediction of the model (Table 4). These data confirm that the model has good adjustments and can be considered validated to predict the inactivation of multispecies biofilm cells. Obtaining a model for the inactivation of multispecies biofilms can assist in the optimization of hygiene protocols adopted in the industries, aiming at a better result. Whereas, in the food industries, microorganisms can interact and form these multispecies biofilms on food contact surfaces, and deteriorating and pathogenic microorganisms can coexist (Sterniša et al., 2019)..

Table 4- Validation of the RS model to inactivation of multispecies biofilm cells and the results of the observed and predictive values of the nine conditions.

Test	Independent variables			Reduction log (CFU/cm^2)	
	PAA (%)	Time (min)	Temperature (ºC)	Actual value[a]	Predicted value
1	0.20	25	25	3.33 (0.12)	3.70
2	0.40	20	55	5.45 (0.35)	5.00
3	0.05	8	30	3.06 (0.03)	3.14
4	0.30	15	50	3.90 (0.02)	4.03
5	0.30	20	55	4.37 (0.37)	4.63
6	0.40	25	60	5.99 (0.43)	5.81
7	0.50	10	40	3.95 (0.03)	4.18
8	0.05	30	45	3.96 (0.03)	4.37
9	0.10	15	30	3.37 (0.25)	3.19

[a] Mean (standard deviation, n=3).

Epifluorescence microscopy was performed to visualize the effect of the treatment (PAA = 0.275%; Time = 17.5 min; Temperature = 42.5 ºC) on the viability of the multispecies biofilm cells. As seen in Figure 4, the untreated biofilm showed viable cells (green) in greater numbers, while the treated biofilm showed dead or injured cells (orange) in greater proportion. The antimicrobial action of PAA occurs due to the release of reactive oxygen species (ROS), which cause damage to DNA and lipids, in addition to causing denaturation of proteins and enzymes and increasing the permeability of the cell wall, which can lead to cell death or a viable but non-culturable state (VBNC) (Baldry, 1983; Salive et al., 2020; Small, Chang, Toghrol, & Bentley, 2007; Zhang, Brown, & Hu, 2018). In the VBNC state the bacterial cell shows an inability to grow in routine culture media, although they are still metabolically active (Li et al., 2017). The VBNC state can be induced in response to stress conditions, such as in the presence of the PAA sanitizer (Salive et al., 2020). Therefore, using more than one technique in the study of biofilms subjected to certain sanitizers becomes

essential, as the bacterial cells present may be in metabolically active or metabolically inactive states (Huang, Yu, McFeters, & Stewart, 1995).

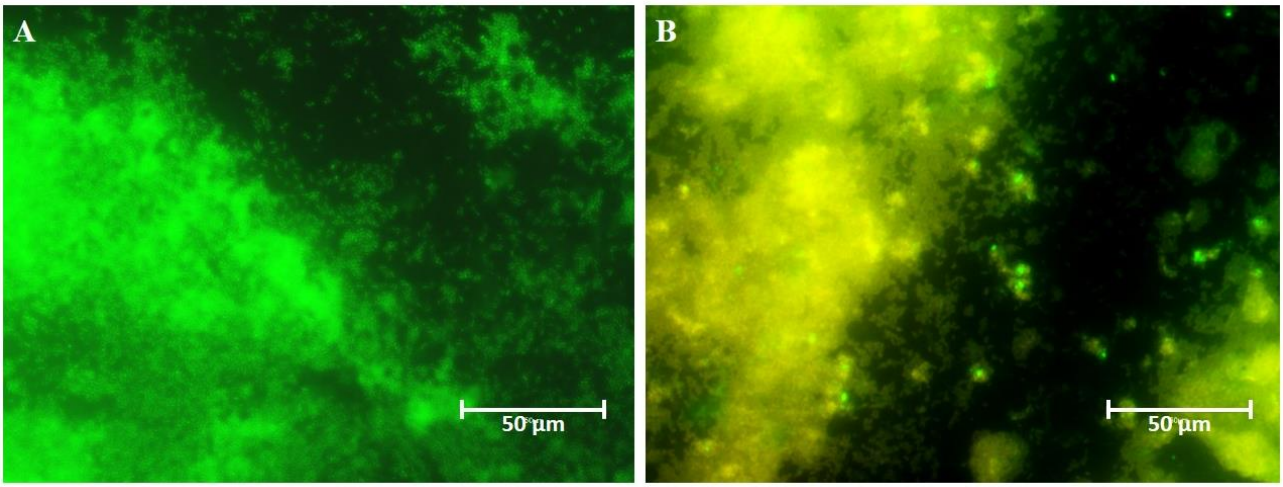

Figure 4- Epifluorescence microscopy of the control (A) and central point (PAA = 0.275%; Time = 17.5 min; Temperature = 42.5 ºC) (B). The images represent the overlap of the GFP and RFP filtersum.

The difficulty of inactivating all biofilm cells is recognized and explained by the protective effect of the EPS layer and adhered cells that protect the microorganisms against the action of sanitizers, in addition to decreasing their efficiency (Yuan et al., 2020; Zhang et al., 2019). Although we have achieved an inactivation of the order of 6 log CFU/cm^2, it is necessary to emphasize that the sanitization was done in just one step. The previous cleaning step before applying sanitizer should obtain good results in the inactivation of biofilms (da Silva Fernandes et al., 2015).

4. Conclusions

According to the results of the RSM, the independent variables, PAA concentration, time and temperature, were important factors for the inactivation of cells of the multispecies biofilm formed by *P. fluorescens*, *R. inusitata*, *S. aureus* and *M. aloeverae* in milk. The increase in the intensity of these three factors was accompanied by increased inactivation of biofilm cells. In this work, the mathematical model for the inactivation of multispecies biofilm cells was developed with only four microorganisms. Thus, new studies can be carried out trying to cover other microorganisms frequently isolated in the dairy industries to evaluate the potential of this model.

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CONCLUSÃO E PERSPECTIVAS

Os resultados do presente estudo mostraram que biofilmes multiespécies podem ser formados nas superfícies de aço inoxidável a partir da microbioata contaminante do leite cru. Nisina e furanonas não diminuíram a formação de biofilme multiespécie, mas influenciaram na sua composição. Os principais gêneros encontrados nesses biofilmes são de grande importância para a indústria de laticínios, pois muitos são microrganismos deteriorantes e patogênicos. O aprofundamento do conhecimento das comunidades bacterianas formadoras de biofilmes multiespécies pode auxiliar na busca de estratégias eficientes de prevenção e controle dessas estruturas para a indústria de laticínios.

Estudos futuros ainda são necessários para entender o efeito, causado pelos compostos nisina e furanonas, na alteração da composição e interações microbianas, e se essas alterações interferem na resistência desses biofilmes aos tratamentos com sanitizantes.

Os 36 isolados bacterianos obtidos a partir dos biofilmes multiespécies, foram afiliados a 15 diferentes espécies. Dentre eles, 69,5% foram capazes de formar biofilmes, sendo a maioria classificada como moderado a forte formador de biofilme. Isolados de *R. inusitata*, *L. garvieae*, *L. laudensis* e *L. raffinolactis* apresentaram os maiores valores de produção de biofilme. Além disso, a maioria dos isolados apresentou atividade proteolítica, mas apenas cinco isolados de *R. inusitata* foram capazes de produzir AHL. A formação de biofilme e produção de enzimas deteriorantes produzidas por diferentes bactérias ainda é um grande desafio para as indústrias de laticínios.

Os resultados da MSR demonstraram que as variáveis independentes, concentração de PAA, tempo e temperatura, foram fatores importantes para a inativação de células do biofilme multiespécies formado por *P. fluorescens* N102, *R. inusitata* N023, *S. aureus* F021 e *M. aloeverae* C022 no leite. O aumento desses três fatores foi acompanhado pelo aumento da inativação das células do biofilme. O modelo matemático para inativação de células do biofilme multiespécie foi desenvolvido com apenas quatro microrganismos, dessa forma, novos estudos podem ser realizados tentando abrangir outros microrganismos frequentemente isolados em indústrias de laticínios para avaliar o potencial deste modelo.