ANDRESSA FALQUETO

SPOILAGE POTENTIAL, BIOFILM FORMATION AND BLUE PIGMENT PRODUCTION BY *Pseudomonas paracarnis*

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements of the degree of *Magister Scientiae*.

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VIÇOSA - MINAS GERAIS
2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
F196b
2023

Falqueto, Andressa, 1997-
Spoilage potential, biofilm formation and blue pigment production by *Pseudomonas paracarnis* / Andressa Falqueto. – Viçosa, MG, 2023.
1 dissertação eletrônica (76 f.): il. (algumas color.).

Texto em inglês.
Orientador: Solimar Gonçalves Machado.
Dissertação (mestrado) - Universidade Federal de Viçosa, Departamento de Microbiologia, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.487
Modo de acesso: World Wide Web.

1. *Pseudomonas*. 2. Queijo - Deterioração. 3. Pigmentos. 4. Biofilmes. I. Machado, Solimar Gonçalves, 1986-. II. Universidade Federal de Viçosa. Departamento de Microbiologia. Mestrado em Microbiologia Agrícola. III. Título.

CDD 22. ed. 579.332

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

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APPROVED: February 16, 2023.

Assent:

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ACKNOWLEDGMENTS

Primeiramente a Deus, por permitir que todo o processo fosse possível, por permitir que cada passo da minha caminhada fosse cuidado por Ele, e por me fazer ter certeza que esses dois anos seriam como ele planejou para mim.

À Universidade Federal de Viçosa e ao Departamento de Microbiologia, pelo ensino, oportunidades e estrutura necessários para realização deste trabalho.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro. À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo financiamento do projeto, código APQ 00088-21.

À Profa. Solimar Gonçalves Machado, pela orientação, pelas conversas, pela confiança e pela autonomia que me fizeram amadurecer mesmo que em pouco tempo de contato. As minhas coorientadoras Maria Cristina Dantas Vanetti e Marisa Alves Nogueira Dias, pela disponibilidade, todo carinho e atenção que me foram dados, tranquilizando e apoiando todo o tempo, pela participação direta na execução dos experimentos e pelos cafezinhos compartilhados as 7 h da manhã no Departamento de Bioquímica. Ao professor Antônio Fernandes de Carvalho, pelos recursos, pela utilização do laboratório, por me agregar a família INOVALEITE, por todas as confraternizações, carinho e por toda a colaboração durante todo o período de trabalho. A todos os demais professores do departamento de Microbiologia que contribuíram para o meu crescimento e conhecimento.

A minha família e Willian, pelo amor, carinho e por cada reencontro que eram os mais especiais. Em especial, à minha Nonna Maria Breda, pelo abraço mais apertado, o beijinho estalado na minha bochecha e o cheirinho mais gostoso desse mundo inteiro, a Nica vai estar sempre por você!

Aos meus amigos do Laboratório Inovaleite, Isabella, Sidney, Frida, Camila, Gustavo, Raiane, Jaqueline, Fabrizio, Davide e demais por todas as festas, encontros, dificuldades passadas com experimentos, choros e risadas (não nessa ordem, nem separados) lamúrias e contribuições deixadas no nosso quadro Inova, sem vocês esse ano teria sido triste. Ainda bem que tenho vocês. A Rafaela Rodrigues, que foi essencial para minha construção profissional, nunca conseguirei agradecer o suficiente por todo apoio, suporte, dicas e puxões de orelha. Aos demais colegas de trabalho que sempre passam e deixam sua contribuição na minha vida. Aos meus

irmãos de orientação do LAMPOAH, Tatiane, Nathália, Letícia e Juniel pela convivência e experiências trocadas.

Aos meus amigos de forró, que se tornou minha terapia durante os momentos em que a cabeça apenas precisa parar de pensar. Meu muito obrigada por serem tanto.

As minhas amigas de república, Jaciara, Emuriela e Bruna, pelos cafés no sábado de manhã, pelas conversas no almoço, as risadas, conversas necessárias, caminhadas, por serem as melhores integrantes da República Irritadíssimas e serem a minha família em Viçosa.

A cada pessoa que direta e indiretamente encontrou comigo nesse período contribuindo com o que pode e com o que eu precisava no momento, na pesquisa ou na vida de modo geral.

"It's the degree of commitment that determines success, not the number of followers."

(Remo Lupin)

ABSTRACT

FALQUETO, Andressa, M.Sc., Universidade Federal de Viçosa, February, 2023. **Spoilage potential, biofilm formation and blue pigment production by Pseudomonas paracarnis**. Adviser: Solimar Gonçalves Machado. Co-advisers: Maria Cristina Dantas Vanetti and Marisa Alves Nogueira Dias.

Dairy product contamination with psychrotrophic microorganisms is a concern for the dairy industry. *Pseudomonas* spp. have been frequently associated with blue pigmentation on the surface of fresh cheeses in recent years, but the structure of this pigment has not yet been elucidated. Furthermore, the production of lipase and protease by the *Pseudomonas* genus has been studied for many years due to the importance of these enzymatic activities in food spoilage. In addition to the production of hydrolytic enzymes and pigments, this genus is also recognized for its capability of biofilm formation, which represents a great risk for the permanence of *Pseudomonas* in the industrial environment. The main goal of this work was to evaluate the spoilage potential, biofilm formation capacity, and blue pigment production of *Pseudomonas* isolated from spoiled cheese. The production of blue pigment by strains belonging to *Pseudomonas carnis*, *Pseudomonas paracarnis*, and *Pseudomonas fluorescens* species was screened in an *in vitro* approach. Metabolites produced by *P. paracarnis* A006 were identified using gas chromatography followed by mass spectrometry (GC-MS) after its solubilization and extraction. The influence of different cheese manufacturing parameters on the production of pigments in a cheese-mimicking matrix (mini-cheese) was assessed using Response Surface Methodology (RSM) for Box-Behnken design (BBD). The colorimetric analyses of mini-cheese were carried out to obtain color variations and validation of the RSM approach. The deteriorating potential of the pigmented (*P. paracarnis* - A006) and non-pigmented (*P. fluorescens* ATCC 13525) strains was evaluated *in vitro* and *in situ* (mini-cheese). Proteolytic and lipolytic activities were quantified using azocasein and *p*-nitrophenyl palmitate, respectively, as substrates. Its ability of biofilm formation was assessed by applying the crystal violet method. *P. paracarnis* A006 was selected as the best producer of blue pigment among the evaluated strains, but it was not possible to identify its pigment chemical structure using the GC-MS approach. However, another 114 metabolites were identified. RSM highlighted the use of starter culture containing *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*, in the cheese-

making process, inhibits the multiplication of *Pseudomonas.* The inoculation of these lactic acid bacteria led to the inhibition of *Pseudomonas* growth, as well as the acidification of the cheeses reduced the production of blue pigment. The mathematical model defined by RSM determines that the absence of salt, a pH of 6.28 and an inoculum of 1.2 % of starter culture minimize the blue pigment production. The availability of nutrients, time and temperature of incubation interfere with proteolytic and lipolytic activity. *P. paracarnis* A006 and *P. fluorescens* ATCC 13525 showed proteolytic above 2.0 ΔA/mL.h, which demonstrate that both strains have a high proteolytic potential. Lipolytic activity of tested strains, like its proteolytic activity, is a strain-dependent characteristic and strongly affected by temperature and incubation time. The results of this work also revealed lower biofilm formation capacity over time at 25°C in both nutritional conditions, except for *P. paracarnis* A006 cultured in MMP. Eight out of nine genes located in the *aprX-lipA* operon, which encode genes related to the proteolytic and lipolytic activity, were identified in the genome of *P. paracarnis* A006 and *P. fluorescens* ATCC 13525. Therefore, defining cheese-making parameters is an interesting strategy to minimize the technological problems caused by Pseudomonas spp. Regarding this context, the RSM approach proved to be efficient. However, the chemical structure of the blue pigment produced by *Pseudomonas* spp. must be elucidated to have more information about the factors that can be controlled to minimize its production.

Keywords: Response surface methodology. Mini-cheeses. Hydrolytic activity.

RESUMO

FALQUETO, Andressa, M.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Potencial de deterioração, formação de biofilme e produção de pigmento azul por *Pseudomonas paracarnis*.** Orientadora: Solimar Gonçalves Machado. Coorientadoras: Maria Cristina Dantas Vanetti and Marisa Alves Nogueira Dias.

A contaminação de produtos lácteos com microrganismos psicrotróficos é uma preocupação para a indústria de laticínios. *Pseudomonas* spp. têm sido frequentemente associada à pigmentação azul na superfície de queijos frescos nos últimos anos. Porém, a estrutura deste pigmento ainda não foi elucidada. Além disso, a produção de lipase e protease pelo gênero *Pseudomonas* tem sido estudada há muitos anos devido à importância destas atividades enzimáticas na deterioração de alimentos. Além da produção de enzimas hidrolíticas e pigmentos, este gênero também é reconhecido pela sua capacidade de formação de biofilme, o que representa um grande risco para a sua permanência no ambiente industrial. O principal objetivo deste trabalho foi avaliar o potencial de deterioração, a capacidade de formação de biofilme e a produção de pigmento azul de *Pseudomonas* isoladas de queijo deteriorados. A produção de pigmento azul por cepas pertencentes às espécies *Pseudomonas paracarnis* e *Pseudomonas fluorescens* foi testada em uma abordagem *in vitro*. Os metabólitos produzidos por *P. paracarnis* A006 foram identificados por cromatografia gasosa seguida de espectrometria de massa (GC-MS) após sua solubilização e extração. A influência de diferentes parâmetros de fabricação de queijos na produção de pigmentos em uma matriz que simula queijo (mini-queijo) foi avaliada usando Metodologia de Superfície de Resposta (RSM) para design Box-Behnken (BBD). As análises colorimétricas dos mini-queijos foram realizadas para obtenção de variações de cor e validação da abordagem RSM. O potencial deteriorante das cepas pigmentadas (*P. paracarnis* - A006) e não pigmentadas (*P. fluorescens* ATCC 13525) foi avaliado *in vitro* e *in situ* (mini-queijo). As atividades proteolítica e lipolítica foram quantificadas utilizando azocaseína e paranitrofenil palmitato, respectivamente, como substratos. Sua capacidade de formação de biofilme foi avaliada pela aplicação do método do cristal violeta. *P. paracarnis* A006 foi selecionado como o melhor produtor de pigmento azul entre as cepas avaliadas, mas não foi possível identificar a estrutura química do pigmento pela abordagem GC-MS. No entanto, outros 114 metabólitos foram identificados. A aplicação de RSM

destacou que o uso de cultura iniciadora contendo *Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis* e *Streptococcus thermophilus*, no processo de fabricação do queijo, inibe a multiplicação de *Pseudomonas*. A inoculação destas bactérias lácticas levou à inibição do crescimento de *Pseudomonas*, bem como a acidificação dos queijos reduziu a produção de pigmento azul. O modelo matemático defino por RSM determinou que a ausência de sal, pH de 6,28 e inóculo de 1,2 % de cultura starter minimizam a produção de pigmento azul. A disponibilidade de nutrientes, o tempo e a temperatura de incubação interferem na atividade proteolítica e lipolítica. *P. paracarnis* A006 e *P. fluorescens* ATCC 13525 apresentaram atividade proteolítica acima de 2,0 ∆A/mL.h, o que demonstra que ambas as estirpes possuem alto potencial deteriorador. A atividade lipolítica das estirpes testadas, tal como a sua atividade proteolítica, é uma característica estirpe-dependente e fortemente afetada pela temperatura e pelo tempo de incubação. Os resultados deste trabalho também revelaram menor capacidade de formação de biofilme das estirpes testadas ao longo do tempo quando incubadas a 25°C, exceto para *P. paracarnis* A006 cultivado em escassez nutricional. Oito dos nove genes localizados no operon *aprX-lipA*, que codifica genes relacionadas à atividade proteolítica e lipolítica, foram identificados nos genomas de *P. paracarnis* A006 e *P. fluorescens* ATCC 13525. Portanto, definir parâmetros de fabricação de queijos é uma estratégia interessante para minimizar os problemas tecnológicos causados por *Pseudomonas* spp. Neste contexto, a abordagem da RSM mostrou-se eficiente. Porém, a estrutura química do pigmento azul produzido por *Pseudomonas* spp. deve ser elucidado para obtenção de informações adicionais a respeito dos fatores que podem ser controlados para minimizar sua produção.

Palavras-chave: Superfície de resposta. Mini-queijos. Atividade hidrolítica.

SUMMARY

GENERAL INTRODUCTION

The *Pseudomonas* genus comprises ubiquitous species that can be pathogenic, such as the clinically relevant species *Pseudomonas aeruginosa*, or deteriorative, such as *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas lundensis*, among others. In the food industry, the spoilage species belonging to the *Pseudomonas* genus are abundantly found in refrigerated dairy products and are often associated with defects in dairy products that are not visible, such as protein and lipid hydrolysis leading to unpleasant odors and off-flavors, or visible defects such as pigmentation, loss of structure and changes in the rheological characteristics of the product (QUINTIERI et al., 2020).

In the last decade, *Pseudomonas* species were isolated and identified as causing blue pigmentation in dairy products (CHIESA et al., 2014; CARRASCOSA et al., 2015; CHIERICI, et al., 2016; DEL OLMO, CALZADA and NUÑEZ, 2018; CARMINATI, et al., 2019; RODRIGUES, et al., 2021). The production of blue pigment is a visual alteration that reduces consumers' purchase intention, causing a great economic and environmental impact due to the disposal of deteriorating products. The defect gained prominence in the European media in 2010, when more than 70,000 units of Mozzarella with blue pigmentation had to be recalled from the market (CENCI-GOGA et al., 2014). *Pseudomonas fluorescens* subsp. *libanensis and Pseudomonas tolaasii* were associated with the contamination of cheeses that showed blue spots. During the investigation of the cause, these species were identified as coming from the industrial water used in the cheese processing (RASFF, 2010). This problem has aroused the interest of the scientific community. *Pseudomonas* is known as producer of various types of pigments such as pyoverdins, pioquelins, pyocyanins, pyomelanins (Figure 1) and pyorubins (DEL OLMO et al., 2018). However, none of these pigments have been associated with the technological problem of blue spots in cheese. Some authors suggest that blue pigmentation problems in dairy products may be associated with the production of syncytianin (BAUTISTA, 2014), indigoidin (CAPUTO et al., 2015) or a hydrophobic indigo analogue (ANDREANI et al., 2015).

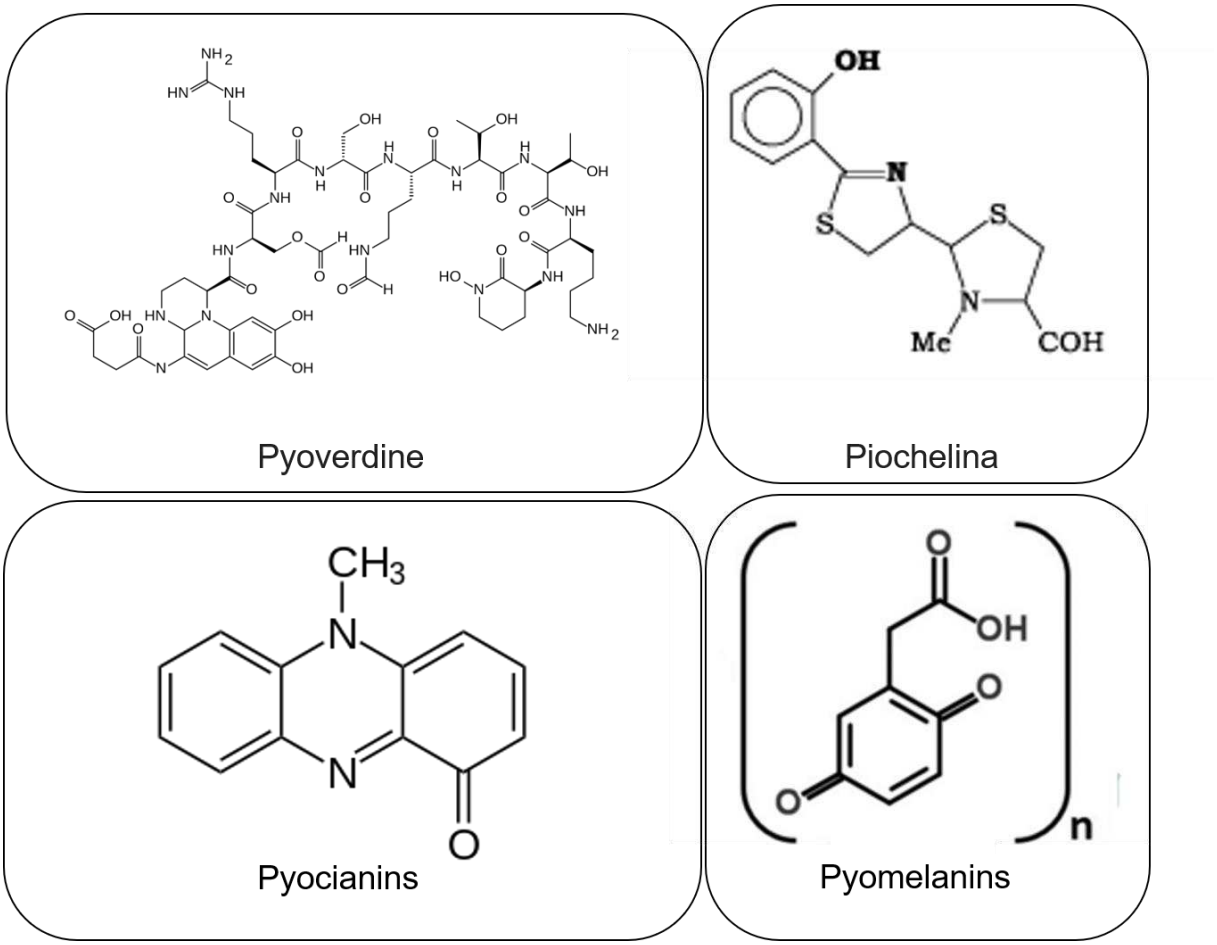

Figure 1 - Pigments produced by different species of *Pseudomonas* and their chemical structures.

Despite numerous studies published in recent years involving the production of blue pigment by *Pseudomonas* spp., the chemical structure, chemical and biological properties and metabolic pathways involved in the production of this molecule have not yet been elucidated. This hamper advances in studies related to the expression of the blue phenotype by spoilage bacteria in dairy products. Furthermore, blue pigments are still rarely found in nature (CIRCELLA, et al., 2020) and, therefore, new sources of blue compounds are welcome, especially if the molecule has a natural origin due to beneficial and more attractive social marketing.

Refrigeration temperatures, commonly used in the dairy sector, can favor the multiplication of psychrotrophic species belonging to the *Pseudomonas* genus that cause great damage to the industry. Although they are inactivated by heat treatments commonly used in the dairy industry, it is possible that *Pseudomonas* spp. spoil dairy

products due to the production of heat-resistant hydrolytic enzymes or post-processing contamination (STANBOROUGH, et al., 2018).

The production of lipase and protease by psychrotrophic strains has been studied for many years due to the importance of these enzymatic activities in the foods stored at low temperatures. Microorganisms that produce lipolytic enzymes, such as *P. fluorescens*, are important in the dairy industry because they can produce rancid flavors and odors in milk and dairy products that make these foods unfit for consumption (COUSIN 1982).

In milk, lipase activity leads to the hydrolysis of triglycerides, with preferential release of medium and short chain fatty acids. Hydrolysis of just 1% of milk triglycerides can lead to rancid flavors. Dogan and Boor (2003) reported that among 338 *Pseudomonas* spp. isolated from raw and pasteurized milk, 67% were positive for lipase production, imparting rancid flavors to the products. As lipases can cause spoilage of milk and dairy products even in low concentrations, the production of such enzymes by *Pseudomonas* affects the quality of milk and dairy products (CORRÊA et al., 2011).

Thermostable proteases of microbial origin are not present in fresh raw milk, but are produced during storage. Protease production by *Pseudomonas* in milk begins during the initial exponential/steady growth phase of the bacteria at cell counts of 10^7 –10^8 cfu/mL and increases during storage. Thus, the occurrence of thermostable *Pseudomonas* proteases in milk may be related to prolonged milk storage, even at low temperatures (PALUDETTI et al. 2020). Among the bacterial proteases involved in spoilage processes a predominant role seems to be played by the thermostable metallo-protease AprX, which is produced by various strains of the species *Pseudomonas fluorescens*. The AprX, produced and released by bacteria in milk, is resistant to heat and is able to maintain its activity even after heat treatments that milk may undergo during its processing such as pasteurization, UHT treatment, and cheese-making (ANDREANI et al. 2016).

Pseudomonas is also known to be able to colonize equipment and facilities for long periods, due to its ability to form persistent biofilms (TIRLONI, BERNARDI and STELLA, 2021). Microbial biofilms are three-dimensional bacterial structures that adhere to surfaces and differentiate into complex communities embedded in

extracellular polymeric substances (EPSs). The biofilms can be pure or mixed (MAGGIO, et al. 2021). The microbial biofilm has been prescribed in different areas, including the food industry, where they are responsible for potential food contamination and resistance. Particularly in the dairy industry, many bacterial species adhere and form biofilms on surfaces and equipment and, among them, the different species of *Pseudomonas* stand out (OLIVEIRA, et al., 2019). Recently, a relationship was made between the pigmentation ability and biofilm formation of *P. fluorescens*. Even though both are promoted at low incubation temperatures, suggesting their possible involvement in the dissemination and persistence of these strains in the dairy environment (ROSSI et al., 2018).

This work consists of three chapters. The first chapter aimed the identification of the blue pigment produced by *P. paracarnis* A006 isolated from Minas Frescal cheese (RODRIGUES, et al., 2021) as well as the identification of other metabolites produced by this bacterium. The second chapter aimed to optimize cheese manufacturing parameters (starter culture inoculum, pH and NaCl concentration) using cheese mimicking matrix and response surface methodology (RSM) for reduce pigment production by *P. paracarnis* A006. The third chapter addressed the spoilage potential of *Pseudomonas*, evaluating their hydrolytic enzymes activities *in situ* and *in vitro* and their biofilm formation capability.

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CHAPTER 1 – EXTRACTION AND IDENTIFICATION OF METABOLITES PRODUCED BY PIGMENTED *Pseudomonas paracarnis*

ABSTRACT

Pseudomonas spp. is widely associated with contamination of refrigerated dairy products. The genus is well known for its production of pigments of different shades. One of the most prominent is the blue pigment, whose structure has not yet been elucidated, and is associated with the blue pigmentation of fresh cheeses. It is already known that this pigment is produced under thermal and nutritional stress, difficult to solubilize, and often associated with the production of other metabolites. The objective of this study was to evaluate the production of blue pigment by *Pseudomonas* strains, select the best producer and carry out a screening of solvents for extraction in order to identify the metabolites produced by blue-pigment producing strain. Pigment production was carried out in Minimal Medium for *Pseudomonas* at 10 °C for 96 h by five different strains of *Pseudomonas* (four blue pigmented and one non-pigmented). Cells from the best blue-pigment producing strain (*Pseudomonas paracarnis* A006) were subjected to extraction with solvents of different polarities. The extract was purified by thin-layer chromatography and the fractions obtained were identified by gas chromatography followed by mass spectrometry. The purification was possible by mixing eluents (in TFA 99%: pure ethyl acetate; in HCl 37%: Ethanol + ethyl acetate 8:2 v/v), which allowed obtaining three promising fractions regarding the purity of their compounds. Concerning the metabolites, 115 compounds were identified (54 from pigment soluble in TFA and 60 from pigment soluble in HCl). This work highlighted the wide variety of metabolites produced by *Pseudomonas* including pigments. However, the blue pigment was not identified using the chosen approach. Despite the spoilage issues caused by *Pseudomonas*, this genus, mainly blue-pigment producing strains, constitute a group that can be exploited for the production of compounds of great industrial interest.

Keywords: Secondary metabolites, bacterial pigments, psychrotrophic.

1. INTRODUCTION

Among all the technological problems caused by *Pseudomonas* in dairy products, the production of blue pigment has gained prominence due to the increased incidence of the defect in different types of dairy products worldwide (CHIESA et al. 2014; CHIERICI et al. 2016; DEL OLMO, CALZADA, NUÑEZ, 2018; CARRASCOSA et al. 2021; RODRIGUES et al. 2021), soymilk, tofu (DE LEÓN et al. 2021) and meet (CIRCELLA et al. 2020). This defect, detected mainly in fresh cheese, has caused alarm among producers and consumers of dairy products, although no pathogenic bacteria have been found in the defective products (DEL OLMO, CALZADA and NUNEZ, 2018).

In the United States, in 2011, strains of *Pseudomonas fluorescens* were identified as causing blue pigmentation in fresh cheeses (MARTIN et al., 2011). In 2014, the same problem was reported in Italy, however the agents causing the defect were identified as *P. fluorescens* and *Pseudomonas koreensis*, isolated from “Blue Mozzarella” (CHIESA et al, 2014). That same year, the blue pigmentation affected Mozzarella production again, however, in Germany and England. Also in 2014, the problem was reported in fresh cheeses made in Gran Canaria, Spain, from a mixture of raw bovine and goat milk, without the addition of starter culture. The blue color in these cheeses persisted even after replacing raw milk with pasteurized milk in the cheese making process. In 2021, Brazil entered the list of countries affected by the unwanted pigmentation of fresh cheeses contaminated with *Pseudomonas carnis* and *Pseudomonas paracarnis* (RODRIGUES et al., 2021).

Fresh cheeses stand out as the most consumed cheese in Brazil (AGUIAR, 2022). The withdrawal of blue fresh cheeses from the market has an economic, financial, environmental, and social impact. Therefore, the study of the phenomenon of blue pigmentation becomes increasingly relevant. To minimize the damage caused by this type of deterioration, it is essential to identify the structure of the pigment and the metabolic pathway used for its synthesis in the microbial cell.

The hypothesis that the blue pigment causing spoilage in dairy products is a derivative of indigo is supported by the fact that *Pseudomonas* pigment-producing strains have multiple copies of the *trp* genes, which encode enzymes that participate in the Shikimate pathway (REICHLER et al., 2019). Indole is produced in this pathway

during the synthesis of tryptophan and can be converted to indigo or some derivative in reactions dependent on the presence of oxygen (ANDREANI et al. 2015).

Despite this hypothesis, the chemical structure of the pigment remains unknown. The present work aimed to extract, characterize and identify the blue pigment produced *in vitro* by *P. paracarnis* isolated from Brazilian fresh cheese (RODRIGUES et al. 2021) containing blue spots.

2. MATERIAL AND METHODS

2.1 Bacterial strains and culture conditions

Four blue pigment-producing bacterial strains belonging to the species *P. carnis* (B157 and C020) and *P. paracarnis* (A006 and RQ057) were used in this work. These strains were isolated from Minas fresh cheese (A006, B157, C020) and processed cheese (RQ057) (RODRIGUES et al., 2021). The *P. fluorescens* ATCC 13525 was used as negative control for blue-pigment production. Bacterial strains were kept at -80°C as pure stock culture in Brain Heart Infusion (BHI) broth supplemented with glycerol 20% (v/v). All strains were grown overnight before each experiment, using, 1% (v/v) of the pure stock culture inoculated in BHI broth followed by incubation at 25 °C for 24 h.

2.2 Standardization of cell cultures

Overnight cultures of tested bacterial strains (A006, B157, C020, RQ057 and ATCC 13525) were standardized by optical density (OD) after centrifugation for 15 min at 12,000 *g*. The supernatant was discarded, and the pellet was resuspended in 9 mL of saline solution (NaCl 0.85% w/v). This washing step was done twice. The final cell suspensions were diluted in saline solution to reach OD_{600nm} from 0.200 to 0.280 corresponding to a concentration of about 10^8 CFU/mL. The OD measurements were performed using the spectrophotometer UV-Vis UV-5100 (Global Trade Technology, China). The bacterial population in the standardized cell suspension was confirmed by spread plating on BHI agar followed by incubation at 25 °C for 24 h. The standardized cell suspensions were ten-fold serial diluted in saline solution (NaCl 0.85%) to reach

the concentration of about 10^5 CFU/mL before using in the subsequent experiments as initial inoculum.

2.3 *In vitro* pigment production

As blue pigment production was detected in minimal medium for *Pseudomonas* (MMP) broth (KIRNER, et al., 1996; CHIERICI et al., 2016), *Pseudomonas* species (A006, B157, C020, RQ057 and ATCC 13525) were evaluated for *in vitro* pigment production using this culture medium. Cell suspensions were standardized as described in section 2.2 to reach 10^5 CFU/mL. One hundred milliliters of standardized cell suspension in MMP were placed in a 250 mL flask in a shaker (Shaker Solab SL221 incubator, Brazil) and incubated at 10±2 °C rotated at 250 rpm for 96 h (ANDREANI et al., 2019). After 96h incubation, a volume of 1 mL from each culture was centrifuged for 15 min at 10,000 *g* to retain the bacterial cells. The UV-visible spectra (wavelengths from 300 nm to 800 nm) of the supernatants were recorded in a microplate spectrophotometer (Multiskan Go, Thermo Scientific, Finland). Each strain was evaluated mainly by the formation of peaks at wavelengths from 580 to 595 nm (SKOOG, 2006) and also by photographic recording in 96-well polystyrene plates. The strain that showed the highest production of blue pigment, i.e., the highest absorption from 580 to 595 nm (blue absorption zone), was selected for the extraction and characterization of the pigment.

2.4 Screening of solvents for pigment extraction

The strain with the highest production of blue pigment under the evaluated conditions was selected and cultivated in 100 mL of MMP broth with an initial inoculum of 10^5 CFU/mL. The incubation was performed in a shaker (Shaker incubator Solab SL221, Brazil) at 10 °C under stirring (250 rpm) for 96h to increase pigment production (ANDREANI et al., 2019). Then, the cells were pelleted by centrifugation at 12,000 *g* for 15 min. The supernatant was recovered, and the pigment extraction was performed from the supernatant and the pellet. The solubility of the pigment in solutions with different polarities was evaluated using the following solvents: NaOH 2 M, dimethyl sulfoxide (DMSO), Tween 80, ethanol, acetone, trichloroacetic acid (TCA) 37%, trifluoroacetic acid (TFA) 99%, HCl 37%, ethyl acetate, ethyl ether, chloroform and

hexane (ANDREANI et al., 2014). Solvents were added separately in the ratio 4:1 (solvent:supernantant or solvent:pellet).

To extract the pigment associated with the pellet, the mixture of pellet and solvent remained under vortex agitation for 2 min followed by centrifugation at 10,000 *g* for 5 min. After centrifugation, the supernatant was recovered and the same ratio of solvent was added, repeating the agitation and centrifugation process until the pellet was colorless. The recovered solvent was collected and stored for later use.

For the extraction of the blue pigment from the recovered supernatant, four parts of solvent were added to one part of the supernatant, followed by vortexing for 1 min and phase separation. The solvent with the highest extraction efficiency was selected for the following steps, this was measured based on the number of washes required for pellet discoloration or complete phase separation, carrying the highest possible proportion of the blue color of the culture supernatant.

2.5 Purification of metabolites

To identify the most efficient mobile phase for the purification process, the extract obtained from the pellet, as described in section 2.4, was spotted on a TLC plate of silica gel 60 with UV 254 fluorescence indicator (DC-Fertigfolien ALUGRAM Xtra SIL G/UV254) used for thin layer chromatography. The spots of pigment extract were applied by the capillary tube at 1 cm from the base and the sheet was added to a glass chromatographic tank containing the solvents used as mobile phase.

For the pigment extracted with TFA, the following solvents were tested as mobile phase: ethyl acetate, hexane/ethyl acetate (95:5 v/v), acetone, isopropyl alcohol and dichloromethane. For the extracted with HCl 37%, the following solvents were tested as mobile phase: ethyl acetate, hexane, ethanol/ water (8:2 and 1:1 v/v), ethanol/ethyl acetate (8:2 v/v) methanol, ethanol and chloroform.

Once the most efficient mobile phase (solvent with an Rf value closer to 1.0) was identified, preparative thin layer chromatography (TLC) was performed to separate the blue pigment. The plates were analyzed at 254 and 365 nm, being these two excitation wavelengths of the evaluated molecules. The shorter wavelength with higher energy and the larger wavelength with lower energy, being able to cover larger chemical compositions and different bonds. The points identified in the length of 254

nm were obtained by scraping the silica, which proceeded to the extraction of the compounds obtained.

Each recovered spot was added to 150 mL of solvent used as mobile phase and kept at room temperature without stirring for 24 h to extract the compounds and separate the silica. After 24 h, the fractions were filtered through quantitative filter paper and concentrated once to a volume of 2 mL in a rotary evaporator IKA RV 3 (IKA Works / IKA Werke GmbH & Co. KG, Brazil), reapplied on the TLC plate. The silica extraction and concentration process were repeated. The concentrated extract obtained from silica fractions was subjected to TLC to verify the purity and the purified extract was stored in glass tubes in the dark.

2.6 Identification of metabolites

The purified fractions were analyzed by gas chromatography followed by mass spectroscopy (GC-MS) at the Analytical Center of the Chemistry Institute – University of Sao Paulo, using the GC-MS QP2010 Ultra (SHIMADZU, Japan). The samples were introduced with a split injector at 280 °C. The initial temperature of the column was 50ºC, being increased to 280ºC and maintained at this temperature for 3 min. The MS interface temperature was 280 °C. The mass spectrometer was operated in scan mode (electron impact at 70 eV, 1000 V).

3. RESULTS AND DISCUSSION

3.1 Assessment of *in vitro* pigment production

The pigment production potential of each strain was evaluated in MMP. The absorbance spectrum of cell-free crude extract from *Pseudomonas* strains was monitored from 300 nm to 800 nm using UV-Vis spectrophotometer (Figure 1). The crude extracts exhibited maximum absorption at 382 and 598 nm. A slightly bluish coloration was visually observed in the culture of *P. paracarnis* A006 after 72 h of incubation under stirring. *P. paracarnis* A006 showed the highest production of unknown blue pigment visually (Figure 1B) and in spectral absorption at wavelengths from 560 to 595 nm (Figure 1A), a possible zone of pigment absorption encompassing blue and violet colors (SKOOG, 2006). Then, *P. paracarnis* A006 was selected for the

next steps that comprise extraction and identification of blue pigment and other metabolites. At excitation wavelengths from 345 to 405 nm, a peak of absorbance was identified (Figure 1A). The highest peak observed at this range of wavelength is characteristic of the presence of pyocyanin, as described by Debritto et al. (2020).

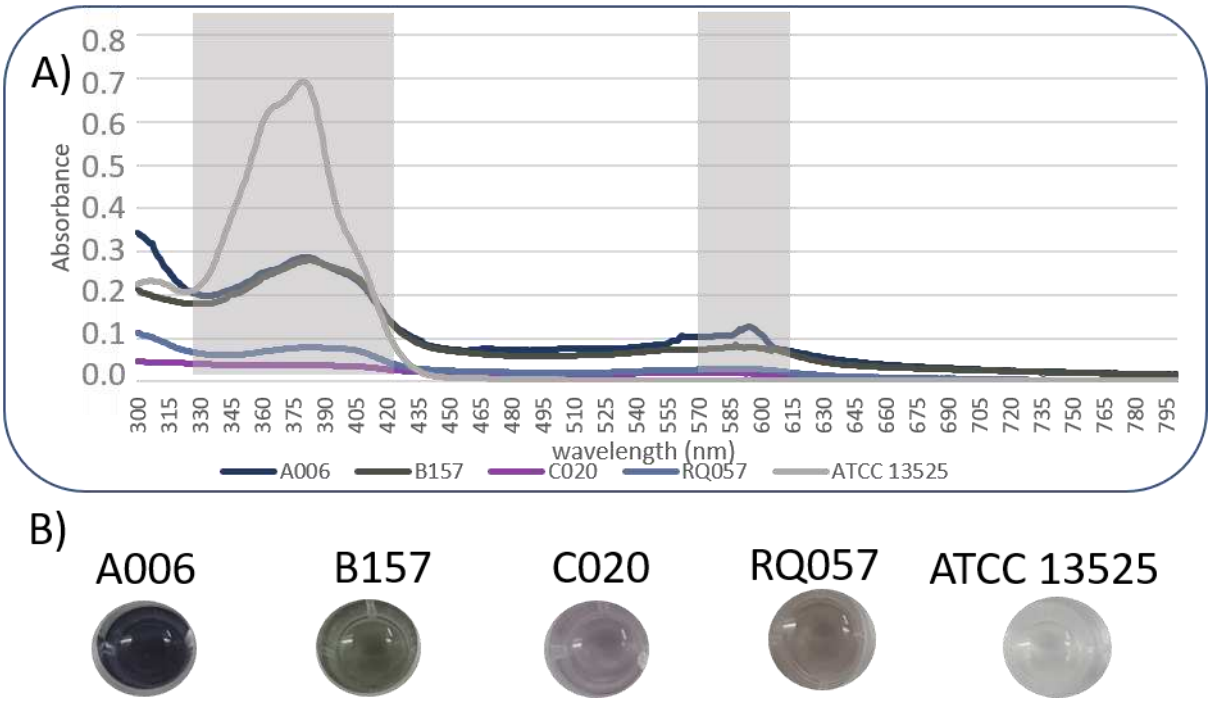

Figure 1 – UV-Visible absorption spectrum (A) and visual record (B) of cell-free crude extract of *Pseudomonas* strains cultured in Minimal Medium for *Pseudomonas* **(**MMP) after 96 h of incubation at 10 °C under stirring. *Pseudomonas carnis*: B157 (brown) and C020 (lilac); *Pseudomonas paracarnis*: A006 (blue) and RQ057 (light blue) and *Pseudomonas fluorescens* ATCC13525 (grey). The areas shaded in gray represent: the absorption peak between 330 and 420 nm for identification of pyocyanin and the peak between 570 and 615 nm for the absorption of the blue color. In this case, the peak is represented in the constituent line of the strain *P. paracarnis* A006 with maximum peak at 595 nm.

3.2 Screening of solvents for extraction

After the pigment production by *P. paracarnis* A006 in MMP, the cell-free supernatant was subjected to solvent screening for metabolites extraction. Among the tested solvents (Table 1), the blue pigment from pellet and cell-free supernatant showed high solubility in TFA 99% and HCl 37%. In the pellet fraction, a higher

concentration of pigment associated with the cells was observed (Figure 2). Thus, when solubilized, the color intensity was greater than in the supernatant, which presented a slightly bluish color, based on this result, the fraction chosen for the rest of the extractions was the pigment in cell pellets. This can be explained by the polarity of the pigment, which is insoluble in water (ANDREANI, 2015), therefore, in the aqueous phase (supernatant), the concentration of pigment was smaller. For this reason, the subsequent steps were carried out only with the solubilized pellet. In addition, both solvents maintained the intense blue color of the pigment. In DMSO, the pigment became partially soluble, however, changing color from blue to brown.

Solvent	Pellet	Supernatant
Ethyl acetate	NS	NS
TCA 34%: ether (1:1 v/v)	NS	NS
TCA 34%	NS	NS
Ether	NS	NS
Tween 80: ether (1:3v/v)	NS	NS
Acetone	NS	NS
Ethanol 99.5%	NS	NS
Ethanol 50%	NS	NS
Acetone: Tween 80 (1:3 v/v)	NS	NS
DMSO	PS	PS
Hexane	NS	NS
Chloroform	NS	NS
NaOH 2 M: ethyl acetate	NS	NS
NaOH 2 M	NS	NS
Ethyl acetate : DMSO (1 :1 v/v)	NS	NS
Tween 80	NS	NS
HCl 3 M	NS	NS
Chloroform: acetone (1:1 v/v)	NS	NS
TFA 99%	S	S
HCl 37%	S	S

Table 1 – Solvent blends used for pigment solubilization in cell-free supernatant and pellet from *P. paracarnis* A006 cultured in MMP at 10 °C for 96 h. PS – Partially soluble; NS – Not soluble; S – Soluble; TCA - Trichloroacetic acid; DMSO: dimethyl sulfoxide; TFA: trifluoroacetic acid.

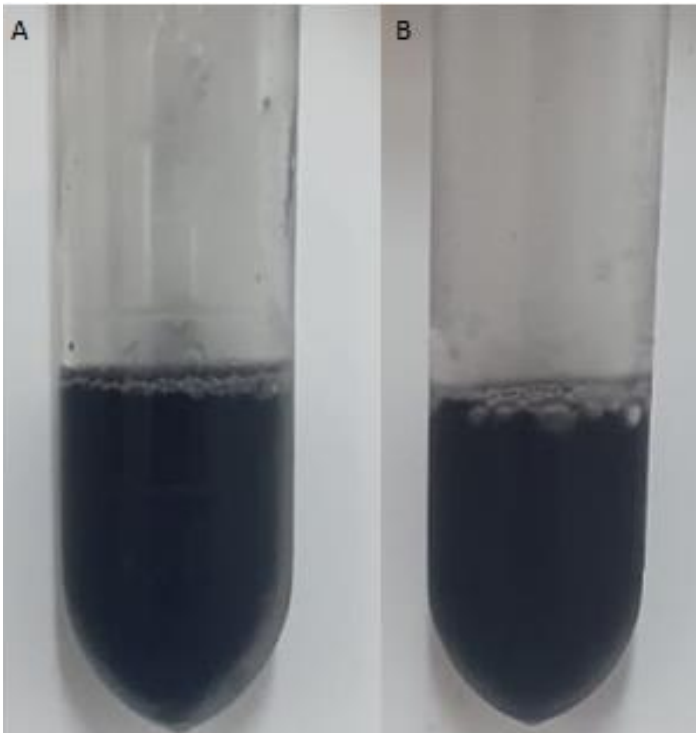

Figure 2 – Pellet of *P. paracarnis* A006 solubilized in TFA (A) and HCl (B).

To explain what triggers the color change in the pigment, it would be necessary to know its molecular structure. However, with anthocyanins for example, at pH above 7.0, it gradually changes the hue from blue to yellow, as an indirect result of chalcone formation, by breaking the anhydro base ring (HRAZDINA, 1977). In these structural modifications, increases in the number of hydroxyl groups tend to turn the color bluish. An increase in the number of methoxy groups increases the intensity of red (LOPES et al., 2007).

Cycles of solvent rinses were performed only on the cell pellet. When TFA and HCl were used, there was no cell pellet left after the first cycle, as the cells were ruptured by the action of the acid. In the third step of washing with DMSO, there was no visible pigment in the recovered solvent, but the pellet remained brownish, because the extraction was not complete due to pigment polarity and affinity with the solvent. In the other solvents there was no extraction due to the incompatible polarity of the pigment with the evaluated solvents. Finally, TFA (99%) and HCl (37%) solvents (Figure 2) were chosen for the other steps because they completely solubilized the pigment.

The results obtained in this research corroborate the results obtained by Andreani et al. (2015), where they also used the cell pellet obtained from the culture

for extraction and observed the low polarity of the molecule. Andreani et al. (2015) described that the pigmented material (cell pellet) physically extracted by centrifugation of *P. fluorescens* (Ps_20, Ps_22, Ps_40 and Ps_77) cultures was insoluble in water, NaOH 1 M, ethanol, methanol, acetonitrile and DMSO. However, other studies that evaluated the blue pigments associated with *P. fluorescens*, proved the molecule is freely soluble in water and DMSO, as is the case of indigo, indigoidin and leuco-indigoidin (CUDE et al., 2012; KURNIAWAN et al., 2014; CAPUTO et al., 2015).

3.4 The metabolites purification

For the purification of the blue pigment, five different solvents were tested as mobile phase in preparative thin layer chromatography. The solvent that presented the best separation of the compounds solubilized in TFA was pure ethyl acetate. It carried the compound a greater distance in the solvent run followed by pure acetone when observed at 254 nm. The other solvents showed little or no action in terms of affinity with the sample. When evaluating the purification of the pigment solution in HCl, only ethanol: ethyl acetate (8:2 v/v) and methanol were able to perform the separation of compounds. Then, ethyl acetate was used as mobile phase in TLC for purification of samples solubilized in TFA, while the blend ethanol: ethyl acetate (8:2 v/v) was used for samples solubilized in HCl.

TLC was performed and the elution of samples solubilized in TFA resulted in three spots (Figure 3). After being recovered from the silica and analyzed by TLC under the same conditions, the spots F1, F2 and F3 were subdivided in three new sub-fractions. These fractions were identified when subjected to ultraviolet light at a wavelength of 245 nm. After silica extraction, each fraction after drying the solvent resulted in a slightly golden colored extract that was subjected to GC-MS analysis (Figure 3). The fractions that maintained their color during the run and the fractions that were most separated in the silica were sent for analysis.

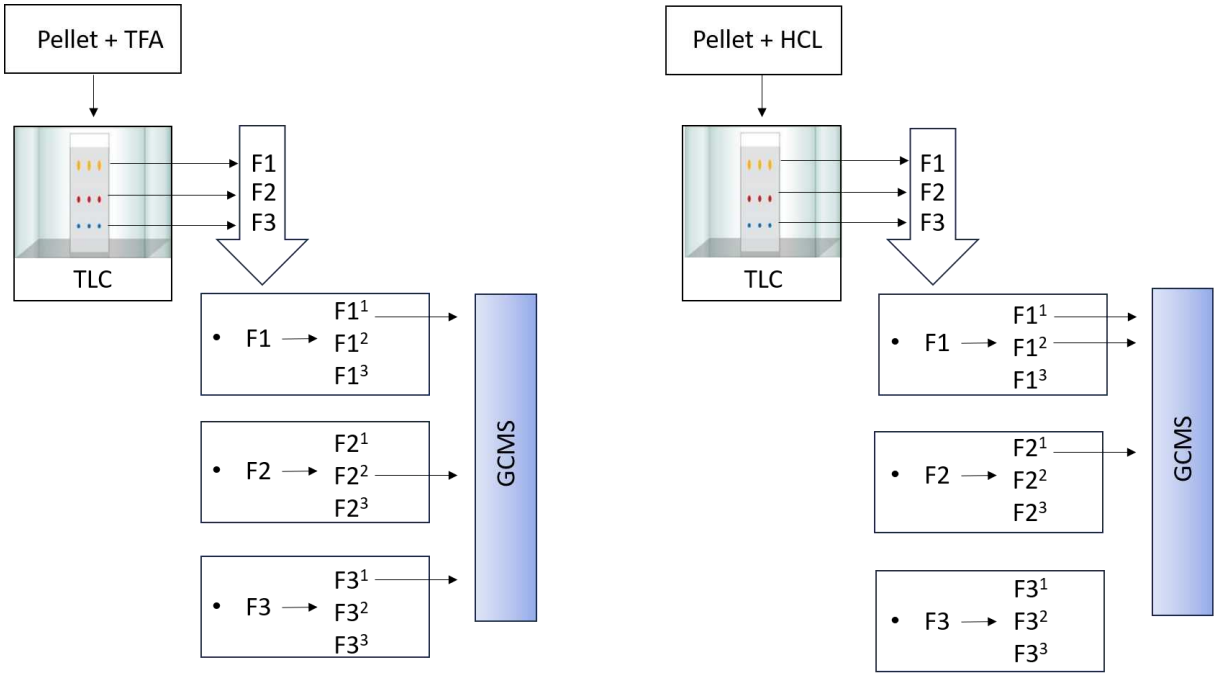

Figure 3 – Fractions obtained from the solubilization of cell pellets in TFA 99 % and HCl 37%. The mobile phases for separation are the same for fractions and sub-fractions. For those solubilized in TFA, the mobile phase was pure ethyl acetate. For those solubilized in HCl, the mobile phase was a ratio of ethyl acetate and ethanol (8:2 v/v). Only fractions indicated by black arrows were subjected to gas chromatography analysis.

3.5 The metabolites identification

The fractions obtained from the pellet solubilized in TFA ($F1^1$, $F2^2$ and $F3^1$) were analyzed by GC-MS and 54 metabolites were identified (Figure 4). The metabolites that stand out in quantity are acetic acid butyl ester (63.17%), di-n-octyl phthalate (30.75%), acid ethyl ester (21.59%), 2-dimethyl sulfone (17.32%), tris(2,4-di-tert-butylphenyl) and phosphate (15.72%). From samples solubilized in HCl 37% (Figure 5), 60 metabolites were identified. The metabolites identified in greater amounts in these fractions were acid ethyl ester (24.85%), octadecanoic acid, (21.59%), di-n-octyl phthalate (15.72%), tris(2,4-di-tert-butylphenyl) acetamide (12.97%) and phosphate (11.53%).

In this study, the extraction and purification steps were not able to separate a fraction containing the blue pigment probably due to lack of affinity of the solvent used as mobile phase with the solubilized pigment. Considering the lack of compatibility of

the solvents used in the solubilization of the pellet with the chromatography column used, there was a limitation of the method for the extraction and the identification of the pigment of interest.

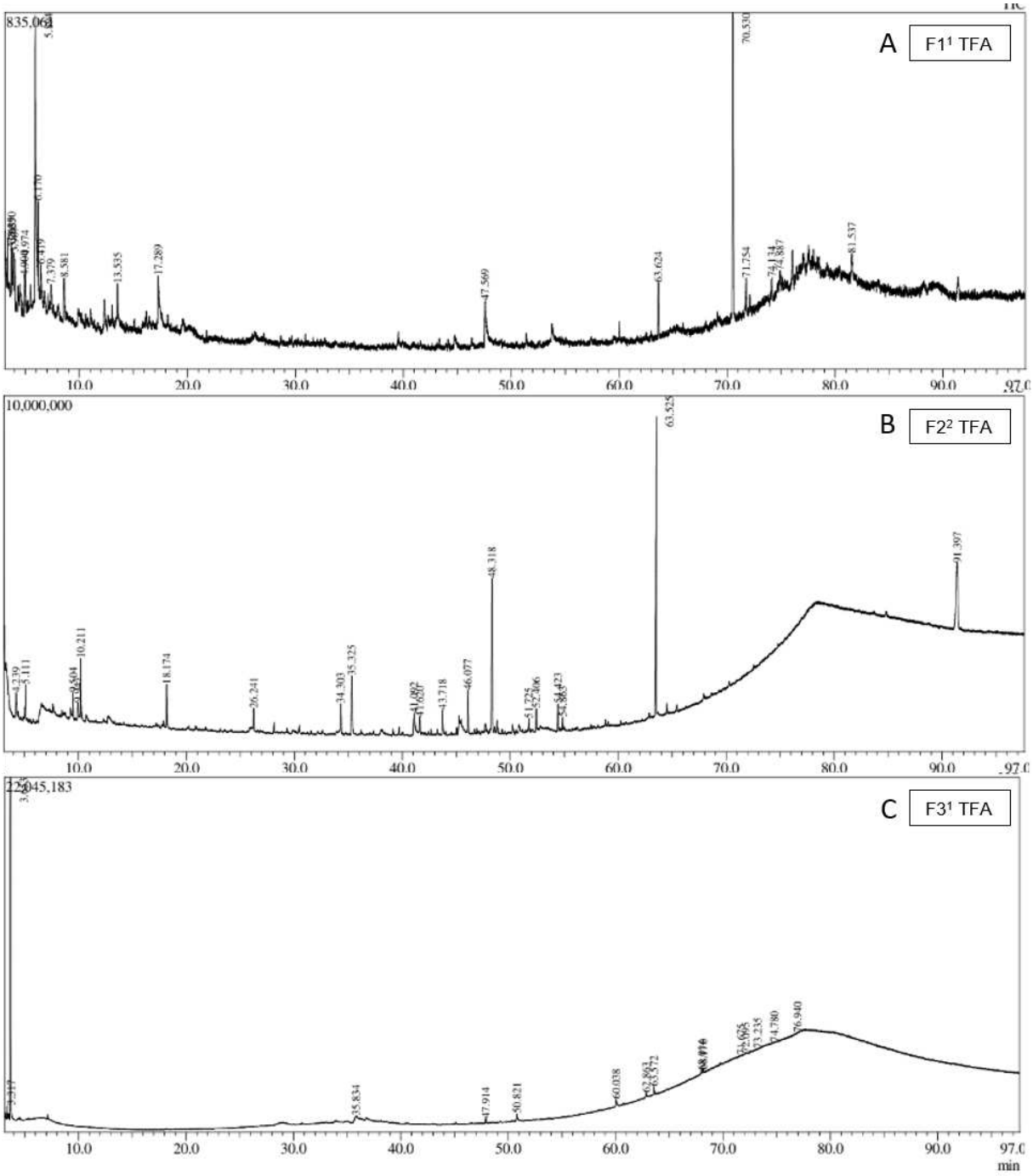

Figure 4 – Chromatograms obtained from fractions obtained from solubilization in solvent: Trifluoroacetic acid 99%. Images A, B and C correspond to the sub-fractions obtained by extractions in ethyl acetate F1[1], F2[2] and F3[1], respectively.

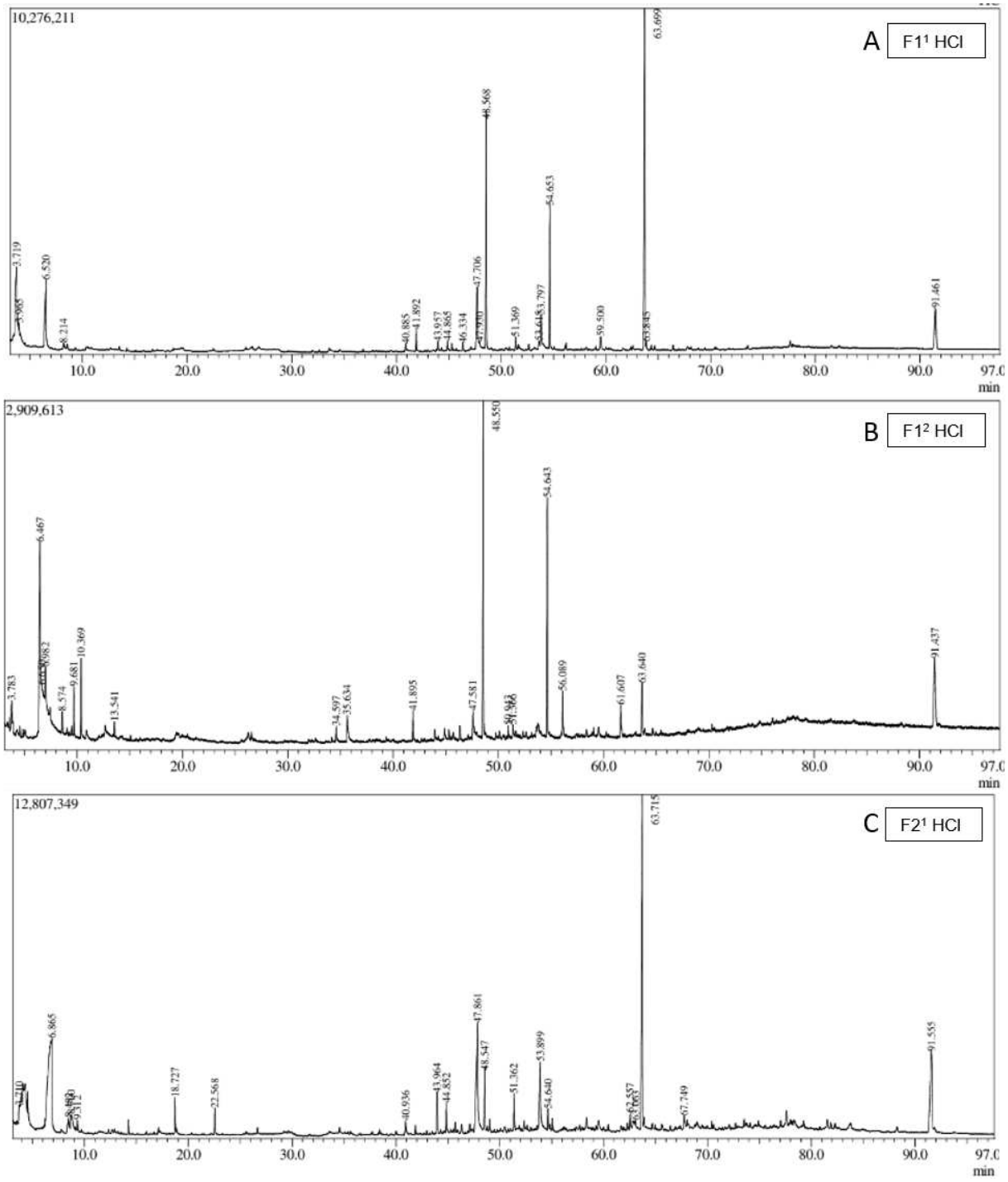

Figure 5 – Characterization of fractions obtained from *P. paracarnis* A006 cells solubilized in HCl 37%, purified by TLC using ethanol: ethyl acetate (8:2 v/v) and methanol as mobile phase followed by GS-MS analysis. A represents F1 fraction 1, B represents F1 fraction 2, and C represents F2 fraction 1.

Despite the large number of compounds identified by the approach used in this study, none of them are related to pigments. Therefore, the chemical structure of the blue pigment produced by *P. paracarnis* A006 remains unknown. However, it was possible to identify some metabolites of interest such as cyclotrisiloxane, hexamethyl: Antimicrobial and antioxidant (ISMAIL et al., 2019), cyclotetrasiloxane, octamethyl: Antimicrobial (KESKIN et al., 2012), 2-methylbutane-1,4-diol, 3-(1-ethoxyethoxy): antibacterial (NATH et al., 2019), benzophenone: chromophore (CUQUERELLA et al., 2012), hexadecenoic acid: antifungal, antitumor, antibacterial (TYAGI and AGARWAL, 2017), phthalic acid, ethyl 2-propylpentyl ester: Antimicrobial and antioxidant (JASIM et al., 2015), trichloroacetic acid, tetradecyl ester: Medicinal activities (JESSICA et al., 2016). Most compounds have not yet had their functions elucidated, however, among the groups found, most are antimicrobial and antioxidant compounds.

The chemical nature of the blue pigment has been investigated before, but no clear answer has yet been obtained. Some authors suggest that blue pigmentation outbreaks in dairy products may be associated with the production of sinvastatin (BAUTISTA, 2014). Caputo et al. (2015) detected leukoindigoidin, a colorless and reduced form of indigoidin, in mozzarellas with blue spots using the mass spectrometry approach. Based on a MALDI-TOF mass spectrometry analysis, Andreani et al. (2015) suggested a hydrophobic analogue of indigo.

Reichler et al. (2019) evaluated six isolates belonging to the *Pseudomonas* genus, related to cases of blue pigmentation in dairy products and compared them with three complete genomes of non-pigmented *Pseudomonas*. After this comparative analysis, the authors identified an accessory locus containing genes for tryptophan (*trp*) biosynthesis in all pigment-producing isolates and absent in the other evaluated isolates. These results reinforce what was described by Andreani et al. (2015) who used a genomic and transcriptomic approach. Based on these results, Reichler et al. (2019) proposed that pigment isolates produce an indigo-derived blue coloring pigment via the tryptophan biosynthesis pathway (Shikimate pathway) from anthranilate. Indole is produced by this pathway during the synthesis of tryptophan and can be converted to indigo or some derivative in reactions dependent on the presence of oxygen.

As previously reported, the nature of the blue pigment produced by the “blue branch” strains is unknown. In their study, Andreani et al. (2015) excluded that this pigment could be identical to that produced by *P. fluorescens* biovar IV or another pigment, such as pyomelanin. The hypothesis of Andreani et al. (2015) the pigment

could be pyomelanin was also refuted since the addition of lysine in the growth medium did not affect the production of the pigment of interest.

4. CONCLUSIONS

The findings from this study highlighted the blue pigment produced by *P. paracarnis* A006 is hydrophobic and insoluble in several organic solvents, which has made its extraction and identification difficult. The approach used in this work did not allow the elucidation of the blue pigment chemical structure. However, it was possible to describe two solvents (TFA and HCl) in which this pigment was completely soluble and visible blue. Investigations into the blue pigment interaction with cell structures and secretion systems are needed, to make possible the process of its extraction, purification and identification.

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CHAPTER 2 – OPTIMIZATION OF PROCESSING PARAMETERS FOR LOW PIGMENT PRODUCTION BY *Pseudomonas paracarnis* IN FRESH CHEESE USING RESPONSE SURFACE METHODOLOGY (RSM)

ABSTRACT

Fresh dairy products are often affected by the contamination of spoilage microorganisms. The *Pseudomonas* genus is well known for its food spoilage potencial, and lately it has been associated with product losses due to the occurrence of blue pigmentation on the surface of fresh cheeses. Different processing conditions can mitigate or favor the production of this pigment. It is already known that the addition of starter culture (SC), pH, temperature, bacterial population present in the food and several other growth conditions influence the color and intensity of the pigment. The objective of this work was to optimize the processing parameters for cheese making to reduce blue pigment production by *Pseudomonas paracarnis* A006 with the optimized experimental Box-Behnken design using response surface methodology. The cheese mimicking matrix (mini-cheese) was manufactured using ultra-filtered milk retentate with standardized fat content (2%). The experimental design provided 15 runs combining the three different levels of the three delimited parameters (SC, pH and NaCl content). The mini-cheeses were inoculated with a standardized cell suspension of *P. paracarnis* A006 (10^7 CFU/mL) and incubated at 10 °C for 144 h. After incubation, the color variation and *Pseudomonas* counting of mini-cheeses were assessed. The color variation was experimentally measured and predicted to carry out the validation of the Box-Behnken design and response surface methodology. The pH and the addition of SC modulate the blue pigment production, inhibiting or delaying its appearance depending on the parameters level. *Pseudomonas* was not detected in the mini-cheeses that contained SC after 24 h of incubation at 10 °C. On the other hand, the mini-cheeses that did not have SC addition presented *Pseudomonas* counts from 10^5 to 10^8 CFU/g. These cheeses presented higher values of color variation, which represents a greater difference between inoculated samples and negative controls (without *Pseudomonas* inoculation). The validation of RSM approach was successful, generating low differences between experimental and predicted color variations of maximized and minimized pigment production (1.1383 and 1.6033, respectively). In the case of this study, the parameters that led to the lowest color variation were 0% of NaCl, pH 6.28 and 1.1% of SC. RSM approach for optimization of processing parameters is of great importance for selecting their levels to mitigate the blue pigmentation problems in dairy industries.

Keywords: Statistical design, colorimetry, blue pigment.

1. INTRODUCTION

Microbial spoilage of fresh cheeses caused by *Pseudomonas* is a concern for the dairy industry, as changes in product characteristics reduce quality, damage cheesemakers' reputations, and can cause serious economic damage to dairy products (DEL OLMO, CALZADA and NUÑEZ, 2018). Defects and sensory changes in dairy products associated with the growth of psychrotrophic microorganisms correlate with the release of enzymes and pigments (CARRASCOSA et al., 2015 and TEIDER et al., 2019).

In June 2010, the Rapid Alert System for Food and Feed (RASFF) reported many cases referred to as "blue mozzarella cheese". The phenomenon was first observed with high-moisture mozzarella cheese made in Germany and later in other European countries. These cheeses, properly stored in a refrigerated chamber, turned blue after opening the packages. After investigations, German authorities concluded that tap water, containing *Pseudomonas* spp., was the source of product contamination (RASFF, 2010).

The production of metabolites, including pigments, and microbial growth are strongly affected by the composition of the medium, such as sources of carbon, nitrogen, salt content, in addition to cultivation conditions, such as aeration and agitation, pH, incubation time and temperature (MAHROUS et al., 2013). These parameters can be adjusted at points that maximize or minimize the level of pigment production (BREIG and LUTI, 2021).

The response surface methodology (RSM) is the procedure frequently used to analyze the best cultivation conditions (MADDOX and RICHERT, 1977). The RSM is a combination of statistical and mathematical techniques for building models, evaluating the effect of several independent variables in order to arrive at the optimal value of the variables to obtain desirable products (BEZERRA, et al., 2008). The RSM can be applied to determine the minimum and maximum production of one or more responses, investigating optimal nutrient composition and environmental conditions.

Therefore, it becomes necessary to determine process parameters used in the cheese making that minimize the damage caused by the production of blue pigment by *Pseudomonas*. Then, this study aimed to test different manufacturing parameters (starter culture, pH and NaCl content) for cheese making using mini-cheeses that

mimic the industrial processing and to optimize these parameters for lower pigment production through *Pseudomonas* contamination in fresh cheeses by RSM approach.

2. MATERIALS AND METHODS

2.1 Culture condition

The blue pigment-producing strain belonging to *Pseudomonas paracarnis* species (A006) were used in this work. *P. paracarnis* A006 was isolated from spoiled fresh cheese (RODRIGUES et al., 2021). Bacterial strains were kept at -80 °C as pure stock culture in Brain Heart Infusion (BHI) broth supplemented with glycerol 20% (v/v). All strains were grown overnight before each experiment inoculating 1% (v/v) of the pure stock culture into BHI broth followed by incubation at 25 °C for 24 h.

2.2. Production of cheese-mimicking matrix

In the present study, different bacterial growth conditions were analyzed to minimize the production of pigments produced by *P. paracarnis* A006 in a cheese-mimicking matrix (mini-cheeses). The mini cheeses were prepared in six-well plates using standardized ultrafiltration (UF) milk retentate according to Garnier et al. (2018) with some modifications. Briefly, skimmed milk was ultrafiltered (HFM - 180 - Koch Membrane Systems Inc., MWCO 80 kDa, 0,22 µm) at 45 °C and three times concentrated.

The fat content of UF milk retentate was standardized to 20 g/kg. Then, the NaCl concentration was adjusted following the Box-Behnken Design design (BBD). The salted retentate was thermally treated at 95 °C for 2 min under stirring in a Thermomix (Vorwerk, Brazil) and cooled down to 45 °C. The starter culture CT111 (*Lactococcus lactis* ssp. *cremoris, Lactococcus lactis* ssp. *lactis* and *Streptococcus thermophilus)* (DSM, Denmark) was added and pH adjusted according to BBD for subsequent addition of rennet (Maxiren XDS, France). The rennet was diluted five times more than manufacture's recommendation to avoid excessive contraction of the retentate proteins. The six-well plates (10 mL/well) were incubated at 32 °C for 2 h 30 min for milk coagulation. Then these plates were stored at 25 °C for three days followed by cheese draining and subsequent inoculation of *P. paracarnis* A006.

Each treatment was inoculated with 200 µL of *Pseudomonas* culture standardized (10^7 CFU/mL) by optical density (OD). Overnight culture was centrifuged for 15 min at 12,000 *g*. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 9 mL of saline solution 0.85% (w/v). The cell suspension was centrifuged at the same conditions as describe previously. This washing procedure was repeated twice. The cell suspensions were diluted to reach the OD in a range from 0.200 to 0.280 at 600 nm corresponding to 10^8 CFU/mL. The OD measurements were performed using the spectrophotometer (UV-Vis UV-5100, Global Trade Technology, China). The bacterial concentration in the standardized cell suspension was confirmed by plate counting on BHI agar. The standardized cell suspensions were diluted to the concentration of about 10^7 CFU/mL before using mini-cheeses) inoculation. The six-well plates containing mini-cheeses inoculated with *Pseudomonas* were incubated at 10 °C for 144h. The same was done with the control treatments (mini-cheeses without bacteria).

2.3 *P. paracarnis* count

P. paracarnis concentration in mini-cheese was assessed by plate counting in *Pseudomonas* agar base (peptone 16 g/L, tryptone 10 g/L, potassium sulphate 10 g/L, magnesium chloride hexahydrate 14 g/L, lactose 10 g/L agar 11 g/L, bromothymol blue 0.02 g/L, pH 7.2) (MACHADO et al., 2015) supplemented with cetrimide, fucidin and cephalosporin (CFC) supplement (Oxoid Limited, Thermo Fisher Scientific Inc., UK). Ten grams of each mini-cheese were homogenized in 90 mL of sterilized MiliQ water in a stomacher (BagMixer 400, Interscience, France) followed by ten-fold serial dilutions in saline solution (NaCl 0.85%). The plating was performed using the microdrop technique (MORTON, 2001). The plates were incubated for 24 h at 25 °C.

2.4. Assessment of blue pigment production

The surface color of the 15 mini-cheeses samples were determined with a colorimeter (Minolta CR 400, Minolta Corporation, Osaka, Japan). The coordinates L* (lightness), a* (green-red value), and b* (blue-yellow value) were obtained. Three readings from different random points were taken from each evaluated sample. The results were expressed in terms of color difference (DE, Equation 1).

$$DE = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \text{ (Equation 1)}$$

The DE values were used within the BBD to identify the best processing parameters for lower pigment production by the *P. paracarnis* A006 strain.

2.5. Optimization of cheese manufacturing conditions using RSM

A RSM using BBD was applied to optimize the effects of the three factors (NaCl, pH and SC) on the DE response. This design generates a second degree polynomial model, which in turn is used in the optimization of a process using a smaller number of experimental runs. With the values determined, it was possible to optimize the combinations of parameters according to equation 2:

$$N = k^2 + k + c_p \text{ (Equation 2)}$$

Within the equation, k represents the number of variables ([NaCl], [pH] and [SC]); c_p is the number of repetitions of the center point (3). The RSM package was used for experimental design (LENTH, 2009) in R software version 4.0.2. The factors and their coded and non-coded (actual) levels are shown in Table 1. A total of 15 experiments were performed, including three replicates at the center point, as shown in Table 2.

Variables	**Code**	**Encoded Level**		
		-1	0	1
		Real Values		
[NaCl] %	X_1	0	0.7	1.4
[pH]	X_2	5.4	6.3	7.2
[SC]%*	X_3	0	1.0	2.0

Table 1 – Coded levels and actual values for minimum, average, and maximum points for each of the variables in Box-Behnken Design for a response.

Trat.	X_1	X_2	X_3	[NaCl]%	pH	[SC]%	DE
1	-1	-1	0	0	5.4	1	0.52
2	1	-1	0	1.4	5.4	1	2.04
3	-1	1	0	0	7.2	1	1.55
4	1	1	0	1.4	7.2	1	0.99
5	-1	0	-1	0	6,3	0	2.92
6	1	0	-1	1.4	6.3	0	27.58
7	-1	0	1	0	6.3	2	0.57
8	1	0	1	1.4	6.3	2	1.07
9	0	-1	-1	0.7	5.4	0	23.71
10	0	1	-1	0.7	7.2	0	24.04
11	0	-1	1	0.7	5.4	2	1.89
12	0	1	1	0.7	7.2	2	2.58
13	0	0	0	0.7	6.3	1	1.28
13	0	0	0	0.7	6.3	1	0.77
13	0	0	0	0.7	6.3	1	1.48

Table 2 – Design matrix using BBD with experimental data for comparison between DE of mini-cheeses inoculated with *P. paracarnis* A006.

The central points were used to find the experimental error. The process performance was evaluated by analyzing the responses (Y), which are dependent on the input factors. The relationship between the response and the input process parameters is described by the equation 3.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + 2 \sum \beta_{ij}\, x_i x_j \quad \text{(Equation 3)}$$

Where Y is the DE response, β_0 the compensation term, β_i the first order main effect, β_{ii} the second order main effect and β_{ij} the interaction effect. X_1, X_2 e X_3 are the three experimental variables studied, that is, [NaCl%], pH and [SC%], respectively.

2.6 Experimental validation of statistical model

After all treatments were done, the response surface model needs to be validated testing the optimum conditions (maximum and minimum points for maximum and minimum pigment production, respectively). With the maximum and minimum points obtained for NaCl, pH and SC, mini-cheeses were made to validate the DE value obtained by the statistical model.

2.7 Statistical analysis of experimental data

The RSM package in R software version 4.0.2. was used to statistical analyses. The best model was selected automatically using the Akaike information criterion (AIC). A constrained nonlinear optimization method, based on Sequential Quadratic Programming (SQP) algorithm, was used to optimization performed using the R package NlcOptim (CHEN and YIN, 2019). The values of the coefficient of determination (R2), adjusted R^2 and response graphs were obtained to verify the quality of the polynomial model and the analysis of variance - ANOVA was performed.

3. RESULTS AND DISCUSSION

It is well known that processing parameters influence pigment production by *Pseudomonas* in dairy matrix. The RSM approach was applied to investigate the influence of NaCl content, pH and SC as well as to optimize these processing parameters to obtain the lowest pigment production. The effect of NaCl content, pH and SC on pigment production (DE) in mini-cheeses were evaluated and validated experimentally using BBD. Based on the BBD, the experimental levels of DE under each set of conditions were determined and compared with the corresponding predicted values (Table 2). The analysis of variance – ANOVA for DE is presented in Table 3. The coefficient of determination (R^2) and the adjusted-R^2 were calculated and used to indicate the adequacy of the regression coefficients, the fittingness, and the goodness of the model equations.

A quadratic polynomial model fitted the experimental data well and satisfactorily with high R^2 value of 0.9258 indicating the significant performance of the model (Table

3). The R^2 provides the proportion of the total variation of the response predicted by the model, indicating the ratio between the sum of squares due to the regression and the total sum of squares (AZIZ, MAT NOR and AROF, 2020). A high coefficient of R^2 (close to 1) describes an effective correlation between predicted values and experimental values (MARAN and MANIKANDAN, 2012). The adjusted-R^2 value corrects the R^2 value for the sample size and the number of terms in the model. High adjusted-R^2 value (0.8515) was obtained for DE responses indicating that the experimental values could be accurately predicted by the established model. R^2 values should be at least 0.80 to imply quadratic fits expressing the design space are satisfactory (VARANK, GUVENC and DEMIR, 2018).

Model terms	**Coefficient estimate**	**Standard error**	**t-value**	**Pr > |t|**
Intercept	1.177	2.190	0.537	0.607708
NaCl	3.265	1.341	2.435	0.045114*
pH	0.125	1.341	0.093	0.928349
SC	-9.018	1.341	-6.724	0.000271***
$NaCl^2$	-2.461	1.974	-1.247	0.252632
pH^2	2.559	1.974	1.296	0.235926
SC^2	9.319	1.974	4.721	0.002155**
NaCl×SC	-6.040	1.897	-3.185	0.015389*
R^2	*0.9258*			
adj R^2	*0.8515*			

Table 3 – ANOVA of the quadratic polynomial model describing the effect of the independent variables (NaCl content, pH and SC) of the response. '***' Significant at P<0.001; '**' Significant at P<0.01; '*' Significant at P<0.05.

The model is expressed according to equation 4, in terms of coded factors, and with the actual values in equation 5:

$$\textit{Coded}\quad Y = 1.177 + 3.265\ NaCl + 0.125\ pH - 9.018\ SCl - 2.461\ NaCl^2 + 2.559\ pH^2 + 9.319\ SCl^2 - 6.040\ NaCl{\times}SCl \qquad \text{(Equation 4)}$$

Real values $Y = 132.272 + 20.324\ NaCl - 39.670\ pH - 21.616\ SCl - 5.022\ NaCl^2 + 3.159\ pH^2 + 9.319\ SCl^2 - 8.629\ NaCl \times SCl$ (Equation 5)

The effect of each control variable on the response is illustrated in Figure 1.

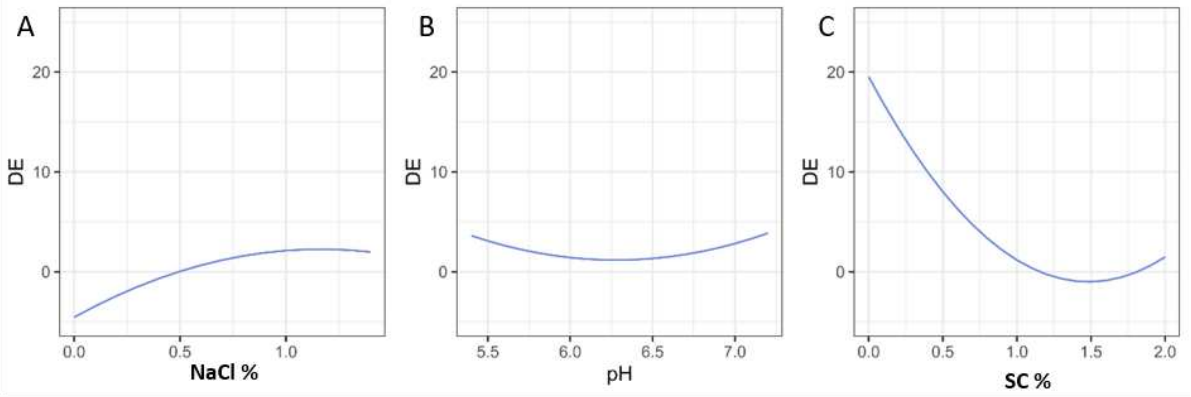

Figure 1 – Individual effects plots of variables at response level (DE). A) Influence of NaCl; B) Influence of pH and C) influence of added yeast on colorimetric variation

Low NaCl levels (showing a negative linear effect with ascending convexity) negatively control pigment production, but with less influence on the response variable (DE) (Figure 1A). Mean pH values (showing a positive linear effect with downward convexity) have no significant influence on the response when evaluated alone (Figure 1B). The change in the levels of SC produces a positive effect, i.e, it decreases the DE response, being a variable that, when absent, favors the pigment production. When evaluated individually, it presents a high level of significance in the change of the response value obtained (Figure 1C).

Higher DE values indicate high pigment production. The interactions between NaCl x SCl and pH x SCl values, were the ones that presented the highest response variable, that is, greater pigment production in relation to the control treatments (Figure 2).

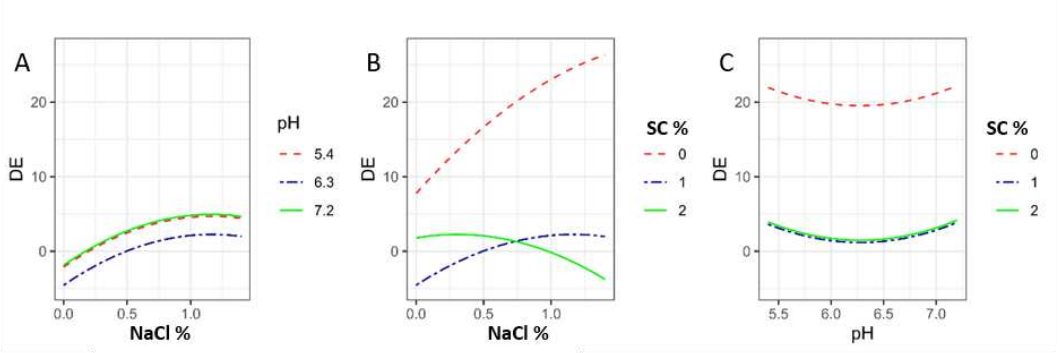

Figure 2 – Interaction plots between two variables as a function of DE response. A) Interaction between NaCl content and pH; B) Interaction between NaCl and SC content and C) interaction between pH and SC content in the response obtained for pigment production.

Regarding the effect of interactions between variables pH x NaCl, all pH values were influenced by the presence of the maximum concentration of NaCl. However, pH 6.3 inhibited the pigment production (Figure 2A). Chemical acidification (pH 5.4) and the pH closest to neutrality (pH 7.2) did not demonstrate a difference in terms of influence on pigment production. On the other hand, the absence of SC was directly linked to the production of pigments in the matrix used in this study (Figure 2B and C). The interaction between SC and NaCl (Figure 2B), with the contents of 0 and 1% of SC, shows a higher level of response when the NaCl content is increasing. In mini-cheeses with 2% of SC, the presence of salts decreases the blue pigment production. When only the interaction between pH and SC was evaluated, the pH 6.3 decreased the response when there was no addition of SC (Figure 2C). In the evaluated range of pH, the addition of 1 and 2% of starter culture did not impact the obtained response (Figure 2C).

Figure 3 presents the three-dimensional (3D) surface plots, obtained using the BBD, correlating the variables with the response (DE). The response against two experimental factors was plotted, while the other factors were held constant at their optimum conditions. By applying these 3D response surface plots, the simultaneous effects of independent parameters and their interaction on DE measurements can be analyzed.

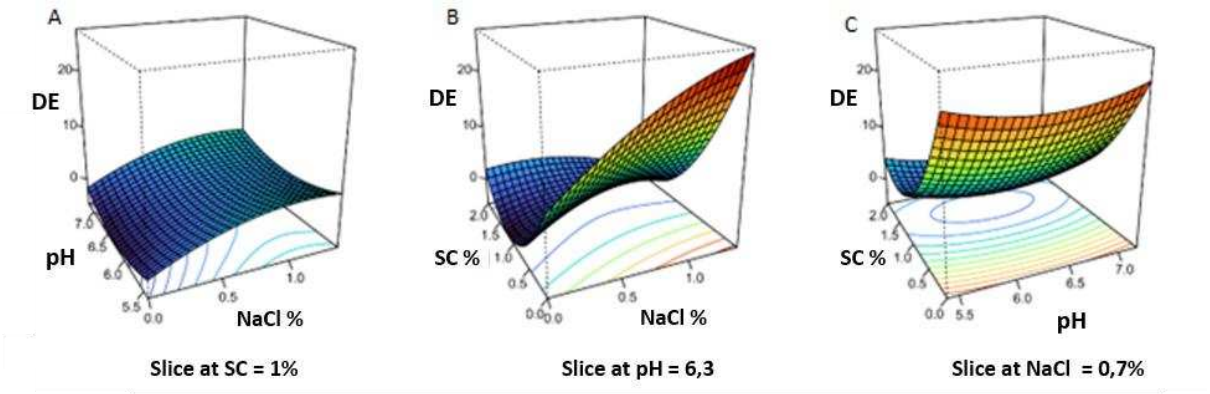

Figure 3 – Response surface plots (3D) with the midpoints of each of the fixed variables and the interactions between the minimum and maximum points in the obtained response. The response surface plots (3D) were made keeping one independent variable at the central level and changing the other two. A: Center point of SC; B: Center point of pH and C: Center point of: NaCl.

When the central point of the SC is fixed, it is noticed that there is little interaction between NaCl content and pH which influences little on the response. When the NaCl content increases (point +1), the maximum and minimum pH values (+1 and -1, respectively) favor the inhibition of pigment production (Figure 3A). When the central pH value is fixed (pH 6.3), the maximum pigment production, given by DE, is increased (Figure 3B). When the SC level is 0% and the salt content increases proportionally, a maximum point of DE with 0 % SCI and 1.4% salt, with coded values of -1 and -1, respectively, was observed (Figure 3B). When the NaCl content is fixed in the DE response plot as a function of SC and pH, the response varies little along the pH. However, the interaction of SC as a function of pH with average NaCl content is still very high, providing a large variation of DE (Figure 3C).

Based on RSM approach, the values obtained from the validation (Table 4) of the predicted values indicate good adequacy of the methodology to the design used. The residue generated (difference between predicted response and experimental response) was 1.1383 and 1.6033 for the maximum and minimum points, respectively. The smaller the residue generated by the experimental values, the better the adequacy of the data. The values obtained confirm that the different processing conditions can influence the appearance of pigment production as well as mitigate the problem of appearance of color through changes in the formulation of the matrix used through the proposed methodology.

Optimization direction	Coded			Uncoded			Predicted response	Experimental response
	NaCl	pH	SC	NaCl (%)	pH	SC (%)		
Maximization	0.9009	0.4339	-1.000	1.3307	6.6905	0.0000	26.4353	27.5736
Minimization	-1.0000	-0.0244	0.1597	0.0000	6.2780	1.1597	4.7885	3.1852

Table 4 – Variables in their coded and actual levels for predicted response values by the Box-Behnken design (BBD) and experimental response obtained from the validation of the proposed method. SC: starter culture.

The effect of SC on pigment production is highlighted in Figure 4. The treatments that showed the greatest visual and colorimetric difference were treatments 6, 9 and 10. These are the runs that did not contain SC. These treatments without SC reached *Pseudomonas* counts of 5.0×10^8 in T9, 7.6×10^8 in T10 and 1.7×10^7 CFU/mL in T6 after 144 h of incubation at 10 °C. These results indicate the strong influence of population density on pigment production as well as the influence of having a competing community when SC is present. In addition, in T5, it was noticed that even without the addition of SC, the visual difference of the control compared to the inoculated mini-cheese was much smaller, even if the color blue appeared slightly on the sixth day of incubation. The output pH is too low for bacteria to grow. This occurs because the percentage of NaCl in this formulation is the minimum used for the analysis and can be a limiting factor for pigment production.

The absence of microbial competition and the acidification promoted by CS do not prevent bacterial growth, which at the end of 144 hours of analysis had a population of 2.6x105 CFU/mL but prevented the production of the blue pigment under these conditions.

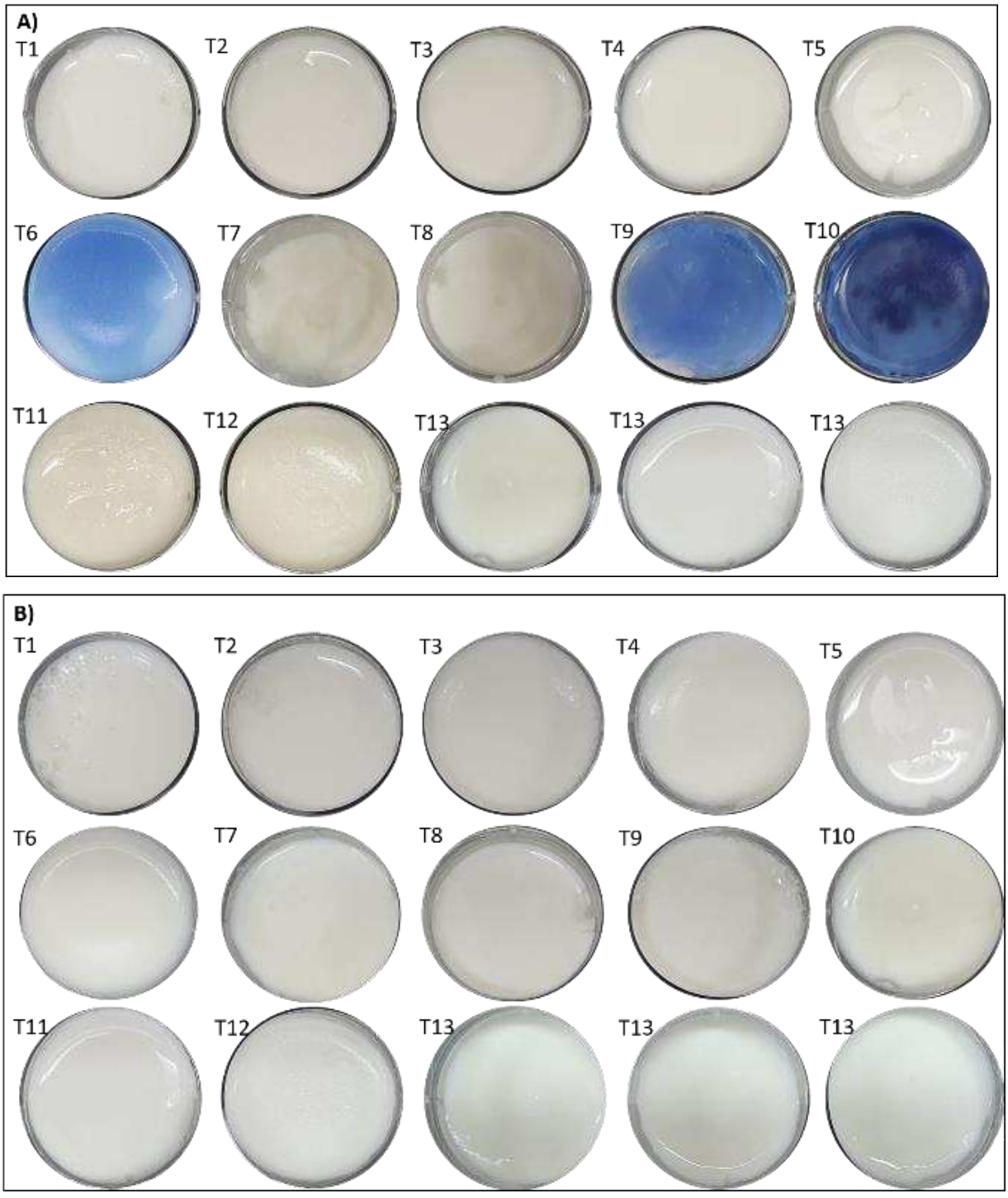

Figure 4- Pigment production in cheese mimicking matrix by *P.paracarnis* A006 (A) under different cheese processing conditions predicted by the statistical model and their respective controls (B) without spoilage bacteria inoculation.

Previous studies have shown that detection of the blue phenotype can be affected by growth conditions, substrate composition, as well as incubation temperature (ANDREANI et al., 2014 and CHIERICI et al., 2016). Carrascosa et al.

(2015) noticed that among cheeses produced inside a cheese factory, fresh cheeses without the addition of SC presented the blue pigmentation. However, cheeses with SC, produced in the same cheese factory, never showed the blue spots. Caputo et al. (2015) found that cheeses containing chemical or microbial acidification prevented pigment production when stored at 4 °C for 5 days. Samples acidified by microbial cultures showed a greater difference in color when compared to chemical acidification, demonstrating the positive influence of adding SC. The results of the scientific literature corroborate the findings of this study, where microbial acidification was more efficient even with high initial pH (7.2) than chemical acidification for mitigate the blue pigmentation in dairy matrix. The addition of SC concentrations in the manufacturing process inhibits the growth of *Pseudomonas*, possibly due to competition between cells and the acidification of cheese matrix (NELLI et al. 2023).

4. CONCLUSIONS

RSM through BBD proved to be a simple and practical approach to develop mathematical models for predicting the effects of SC, pH and NaCl content in reducing blue pigment production on dairy matrix by *Pseudomonas*. The predicted data and experimental measurements highlighted that the use of SC for cheese making mitigate the blue pigment production when samples are contaminated with pigment-producing *Pseudomonas*.

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CHAPTER 3 – SPOILAGE POTENTIAL AND BIOFILM FORMATION OF *Pseudomonas paracarnis* A006 UNDER DIFFERENT CULTURE CONDITIONS

ABSTRACT

The *Pseudomonas* genus is one of the most abundantly found in cold raw milk and often related to spoilage caused by the production of pigments or hydrolytic enzymes. These bacteria are inactivated during heat treatments used in milk processing. However, they produce thermos-resistant hydrolytic enzymes. Therefore, they have high resistance to cleaning and sanitation plans due to their high biofilm formation capacity, and their removal is a frequent problem. The aim of this study was to evaluate the enzymatic activity and biofilm formation potential of two species of *Pseudomonas*, a blue pigment producer *Pseudomonas paracarnis* A006 and a non-producer *Pseudomonas fluorescens* ATCC 13525, *in vitro* and *in situ,* using a cheese-mimicking matrix. Enzymatic activity measurements were performed by colorimetric assays. Biofilm formation was tested by crystal violet polystyrene microplate assay. The availability of nutrients interferes with proteolytic activity when the strains evaluated are subjected to cold stress. The pigment-producing strain, *P. paracarnis* A006, incubated at 10 °C and 25 °C showed the same lipolytic profile independeing on the nutritional availability. The non-pigmented strain, *P. fluorescens* ATCC13525, presented the opposite profile when incubated in BHI and MMP at 10 °C, therefore, lipolytic activity is also a strain-dependent characteristic and is strongly affected by temperature and incubation time. *P. fluorescens* ATCC 13525 showed a lower biofilm formation capacity over time at 25 °C, while *P. paracarnis* A006 showed the same profile, except when it was cultivated in nutrient definiency. Both strains showed statistically similar pattern of biofilm formation at 48 and 96 h of incubation. In addition to producing blue pigment, *P. paracarnis* A006 strain can persist in the industrial environment by forming biofilms in addition to hydrolyze proteins and milk fat molecules causing technological problems and economic loss.

Keywords: Protease, lipase, psychrotrophic, fresh cheese.

1. INTRODUCTION

Pseudomonas spp. are known to be psychrotrophs abundantly found in refrigerated dairy products, mainly refrigerated ready-to-eat products (STELLATO, 2017). This genus is often associated with deterioration caused by the activity of hydrolytic enzymes, in addition to the formation of highly resistant biofilms (CARRASCOSA et al., 2015; YUAN, et al., 2022). However, in the last decade, *Pseudomonas* species have been frequently and abundantly associated with problems related to blue pigmentation in dairy products (DEL OLMO, CALZADA and NUÑEZ, 2018; CARMINATI, et al., 2019; RODRIGUES, et al., 2021).

Pseudomonas spp. are one of the main contaminating bacteria responsible to produce thermostable proteases and lipases that hydrolyze caseins and lipids affecting negatively the sensory quality and shelf life of dairy products (AGUIAR et al., 2019; NARVHUS et al., 2021 and SARAIVA, et al., 2021). The spoilage potential of species belonging to this genus, associated with the ability to produce extracellular enzymes even at refrigeration temperatures, has been a great concern (ROSSI et al., 2018).

Until now, only one extracellular peptidase has been characterized in *Pseudomonas*, namely the AprX metallopeptidase. The caseinolytic endopeptidase AprX belongs to the serralysin family and contains a Zn^{2+} and Ca^{2+} ions for its stability and functionality (SCHOKKER and VAN BOEKEL, 1997). It has a size of 45–50 kDa (MARCHAND et al., 2009), shows the highest substrate turnover at 37–45°C, and is functional from slightly acidic to alkaline pH (DUFOUR et al., 2008; MARTINS et al., 2015; MATÉOS et al., 2015). The corresponding *aprX* gene is located in the *aprX-lipA2* operon, consisting of several genes controlled by a single promoter upstream of the *aprX* gene. So far, it is known that the *aprX-lipA2* gene cluster comprises up to nine different genes in total (MA et al., 2003). Strain-specific deviations in operon organization, for example, a missing *prtA* gene in *Pseudomonas brassicacearum* NFM421, loss of *prtAB* in *P. fluorescens* SIK W1, an additional lipase gene in *P. fluorescens* Pf0-1, and a completely different gene structure of the operon in *P. aeruginosa* PAO-1, were mentioned in the literature (DUONG et al., 2001; MA et al., 2003).

The issues caused by *Pseudomonas* in the dairy industry are compounded by the long persistence of this genus in the industrial environment thanks to its ability to form biofilms on surfaces. The formation of biofilm in the dairy industry causes many

damages, whether structural, due to the corrosion of pipes and equipment, or the post-processing contamination that may occur through the presence of adhered cells in the processing line. Post-processing contamination, mainly by *Pseudomonas*, leads to spoilage and economic losses that could be avoided. Furthermore, multispecies biofilms can be formed together with food pathogens (OLIVEIRA et al., 2019) raising, even more, the industry's concern about removal and control of this cellular adherence to the environment.

The investigation of the potential for biofilm production, proteolytic and lipolytic activity had not yet been reported in the *P. paracarnis* A006, which is a blue pigment-producing strain. Thus, the aim of this study was to investigate the ability of *P. paracarnis* A006 to adhere and form biofilms on polystyrene surfaces and to produce hydrolytic enzymes *in vitro* and *in situ* using a cheese-mimicking matrix.

2. MATERIAL AND METHODS

2.1. Microorganisms and culture conditions

For the assessment of the deteriorating potential, bacterial strains belonging to the species *P. paracarnis* (A006) and *Pseudomonas fluorescens* (ATCC13525) were used in this work. The first was isolated from Minas fresh cheese (RODRIGUES et al., 2021). Bacterial strains were kept at -80 °C in Brain Heart Infusion (BHI) broth supplemented with 20% v/v glycerol. All strains were grown overnight before each experiment adding 1% (v/v) of the pure stock culture into BHI broth followed by incubation at 25 °C for 24 h.

2.2. Cell suspension standardization

The concentration of *Pseudomonas* in cell suspensions was standardized by optical density (OD). The overnight culture of each strain was centrifuged for 15 min at 12,000 *g*. The cell pellet was resuspended in 9 mL of saline solution 0.85% (w/v). This washing step was performed twice. The final cell suspensions were standardized for OD at 600 nm from 0.200 to 0.280 corresponding to a concentration of about 10^8 CFU/mL. OD measurements were performed using UV-Vis spectrophotometer (UV-5100, Global trade technology, China). The final population of the standardized cell

suspension was confirmed by microdroplet counting on BHI agar (MORTON, 2001). The standardized cell suspensions were ten-fold diluted in saline solution to reach the concentration of about 10^5 CFU/mL before each experiment.

2.3. Assessment of spoilage potential of *Pseudomonas*

The spoilage potential of *P. paracarnis* A006 and *P. fluorescens* ATCC 13525 were assessed using *in vitro* and *in situ* approaches. In both approaches, *P. paracarnis* A006 and *P. fluorescens* ATCC13525 were inoculated at an initial concentration of about 10^5 CFU/mL.

For *in vitro* evaluation, four different culture conditions were used: i) BHI at 10 °C; ii) BHI at 25 °C; iii) Minimal Medium for *Pseudomonas* (MMP) (KIRNER, et al., 1996; CHIERICI et al., 2016) at 10 °C and iv) MMP at 25 °C. The hydrolytic potential and biofilm formation capability were measured after 48 and 96 h of incubation.

For *in situ* analysis (mini-cheeses), the *Pseudomonas* cell suspension were inoculated in mini-cheeses, which were incubated at 10 °C for 96 h. After incubation, proteolytic and lipolytic activities were measured. In addition, the mini-cheeses were visually assessed for monitoring the blue coloration during the incubation time.

2.4. Production of mini-cheeses for *in situ* analyzes

The cheese-mimicking matrix, mini-cheeses, were made in six-well plates according to Garnier et al. (2018) with some modifications. Briefly, skimmed milk was ultrafiltered (HFM - 180 - Koch Membrane Systems Inc., MWCO 80 kDa, 0,22 µm) at 45 °C and 3 times concentrated. The fat content of UF milk retentate was standardized to 20 g/kg. Then, the NaCl (0.7% w/v) was added to standardized and concentrated UF milk. This mixture was thermally treated at 95 °C for 2 minutes under stirring in a Thermomix (Vorwerk, Brazil), cooled to 45 °C for subsequent addition of rennet (five times diluted) (Maxiren XDS, França), according to the manufacturer's recommendation. Then, the mixture was distributed in six-well microplates (10 mL/well). The plates were incubated at 32 °C for 2 h 30 min for milk coagulation. After first step of incubation, the plates were stored to 25 °C for 72 h followed by whey draining for subsequent inoculation of 200 µL of *P. paracarnis* A006 and *P. fluorescens* standardized cell suspension (10^5 CFU/mL). The plates kept incubated at 10 °C for 96

h. Mini-cheeses without inoculum were kept for the same time to control color and enzymatic activity.

2.5. *P. paracarnis* plate counting

For *P. paracarnis* counting in mini-cheese, *Pseudomonas* agar base (peptone 16 g/L, tryptone 10 g/L, potassium sulphate 10 g/L, magnesium chloride hexahydrate 14 g/L, lactose 10 g/L agar 11 g/L, bromothymol blue 0.02 g/L, pH 7.2) (MACHADO et al., 2015) supplemented with cetrimide, fucidin and cephalosporin (CFC) supplement (Oxoid Limited, Thermo Fisher Scientific Inc., UK). Ten grams of each mini-cheese were homogenized in 90 mL of sterilized MiliQ water in a stomacher (BagMixer 400, Interscience, France) followed by ten-fold serial dilutions in saline solution (NaCl 0.85%). The plating was performed using the microdrop technique (MORTON, 2001). The plates were incubated for 24 h at 25 °C.

2.6. Proteolytic activity

One mL of cell cultures was centrifuged at 12,000 *g* for 10 min. The supernatant was used as crude extract for *in vitro* analyzes. For *in situ* enzymatic activity measurements, the mini-cheeses were manually homogenized in plastic bags in the absence of diluent. One mL of homogenized cheese was centrifuged at 12,000 *g* for 10 min. The supernatant obtained after centrifugation was named crude extract.

The quantification of proteolytic activity in crude extracts was performed according to the technique described by Dufour et al. (2008) with modifications. Briefly, 100 µL of crude extract were added in 1.5 mL eppendorfs followed by the addition of 100 µL of azocasein solution 3% (w/v) (Sigma-Aldrich, St. Louis, MO, USA) and 300 µL of disodium phosphate (Na_2HPO_4, 50 mM, pH 7.5). The mixture was shaken and incubated in a water bath at 37 °C for 1 h. After incubation, 500 µL of TCA 20% (w/v) were added and the reaction was incubated in the dark for 15 min. The mixture was centrifuged at 12,000 *g* for 10 min followed by measuring the absorbance in a spectrophotometer (UV-Vis UV-5100, Global trade technology, China) at 366 nm. Proteolytic activity was calculated by subtracting the absorbance of the non-incubated sample from the value for the incubated sample and expressed as $\Delta A \times h^{-1} \times mL^{-1}$.

2.7. Lipolytic activity

The same crude extracts used for proteolytic activity quantification were submitted to lipolytic activity measurements. The last was performed as described by Salgado et al. (2021). Using p-nitrophenyl palmitate (p-NPP) as a substrate obtained from Sigma-Aldrich, St. Louis, MO, USA, the assay procedure was as follows: Crude extract aliquots of 15 µL were dispensed into a 96-well microplate, followed by the addition of 150 µL of the substrate solution. The microplate was then incubated at 30 °C for 1 hour. Subsequently, the absorbance at 410 nm was measured using a spectrophotometer from Thermo Fisher Scientific, Finland. The substrate solution was prepared by mixing Solution A (comprising 0.3% (w/v) p-NPP from Sigma Aldrich, USA, dissolved in 2-propanol) with Solution B (consisting of nine volumes of 0.2% (w/v) sodium deoxycholate and 0.1% (w/v) arabic gum in a 50 mM sodium phosphate buffer at pH 8.0). It's important to note that the blank sample did not contain any enzyme. For *in situ* lipolytic activity quantification, a negative control was performed. The negative control consisted of the cheese without bacteria inoculated which went through the same analysis steps.

To obtain lipolytic activity in U/mL, the absorbance of the control and blank was subtracted from the absorbance of each sample. The activity in U/mL of each sample was calculated using a standard curve. The standard curve of the hydrolysis product of *p*-NPP, *p*-NP, was established at concentrations ranging from 300 to 3000 µM. One unit of lipase (U) was defined as the amount of enzyme that releases 1.0 µM of p-NP per min at pH 8.0 and 30 °C (CHRISTENSEN et al. 2003; SALGADO et al. 2020).

2.7. Biofilm formation

The *Pseudomonas* ability to form biofilm was evaluated in 96-well polystyrene microplates using the methodology adapted from Quintieri et al. (2020). Standardized cell suspensions with a population density of about 10^5 CFU/mL in MMP and BHI were distributed in microplates (200 µL/well). The microplates were incubated in BOD at 10 °C and 25 °C for 96 h without stirring. Sessile cells were quantified at 48 and 96 h using the crystal violet approach. At the end of each incubation time, the planktonic cells were removed, and the wells were washed three times with 200 µL sterile distilled

water followed by drying at 37 °C for 20 min. The sessile cells forming the biofilm were stained with 200 µL crystal violet (1% w/v) for 15 min, and then washed with 200 µL of sterile distilled water three times. The crystal violet was solubilized with 200 µL of ethanol and left at room temperature for 15 min. The aliquots were transferred to a clean 96-well microplate to proceed with the absorbance measurements at 590 nm using a spectrophotometer (MultiskanGo, Thermo Fisher Scientific Inc., Finland). The appearance of blue color on the microplates was visually monitored every 24 hours of analysis.

2.8. Comparison of the *aprX-lipA* operon sequences

The sequences of *aprX-lipA* operon from strains *P. paracarnis* A006 (NCBI accession number: JAEFBF000000000) and *P. fluorescens* ATCC13525 (NCBI accession number: LT907842.1) were compared. To build the alignment figure, it was necessary to find the region of the genes of interest. Then, the genomes were annotated using Prokka v.1.14.5 (SEEMANN, 2014). The files with the *AprX-LipA* operon sequences were aligned using BLASTn comparison using Easyfig v.2.2.6 (SULLIVAN, PETTY, & BEATSON, 2011) against the predicted proteins genome file to find out the protein identification. Genetic templates were determined. Clustering and orientation deduced for closely linked genes were done by the Easyfig software.

2.8 Statistical analysis

Each experimental analysis had three repetitions performed in triplicate. The homogeneity of variances and normality were verified, respectively, by the Bartlett and Shapiro-Wilk tests. The Kruskal-Wallis' test with Conover's test (using Bonferroni correction), ANOVA and Tukey's multiple comparisons was used for post-hoc analysis of multiple group comparisons. Comparisons between two groups were performed by LSD t-test. Statistical analyzes were performed using R, version 4.0.2, and the statistical significance considered was $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Assessment of spoilage potential and biofilm formation of *Pseudomonas in vitro*

3.1.1 Proteolytic activity

Proteolytic potential of *P. paracarnis* A006 e *P. fluorescens* ATCC13525 was measured by the azocasein assay (Figure 1). At 10 °C, both evaluated strains showed a higher proteolytic activity in BHI after 48 h of incubation comparing to MMP. Despite the enzymatic activities are correlated with the population present in the medium, the increase in proteolytic activity cannot be associated with variation in the population which remained higher than 7.5 Log CFU/mL. These results show that nutrient availability can interfere with enzymatic activity when the evaluated strains are subjected to cold stress. Wang et al. (2022) highlighted nutrients availability, mainly whey protein, influences the production and the activity of AprX from *P. fluorescens* W3 cultured at 4°C.

Regardless the difference in proteolytic activity in BHI and MMP in the first 48 hours of incubation, after 96 hours of incubation, the proteolytic potential was similar in two culture media at 10 °C. At 25 °C, the proteolytic activity of *P. paracarnis* A006 became greater after 96 h of incubation in both BHI and MMP. However, this pattern was not repeated for *P. fluorescens* ATCC13525, which showed lower proteolytic activity after 96h of incubation in MMP.

Zhang et al. (2019) and Woo et al. (2023) had already demonstrated that the relative expression ratios of *aprX* gene is higher when *Pseudomonas* is cultivated under cold storage. Although these studies have demonstrated the influence of low temperatures on the proteolytic potential of *Pseudomonas*, the variation in *aprX* expression and AprX activity within the same genus and, even, within the same species must be highlighted. Aguilera-Toro et al. (2023) observed that proteolytic potencial of *Pseudomonas* strains are greatly related to genotypic characteristics at *aprX-lipA* operon level. These authors also classified *Pseudomonas* with proteolytic activity greater than 2.0 A/mL.h as highly spoilage, which reinforces the spoilage potential of the *P. paracarnis* A006 (Figure 1). Besides the blue pigment production, this strain can also hydrolyze milk proteins and dairy products, causing technological problems.

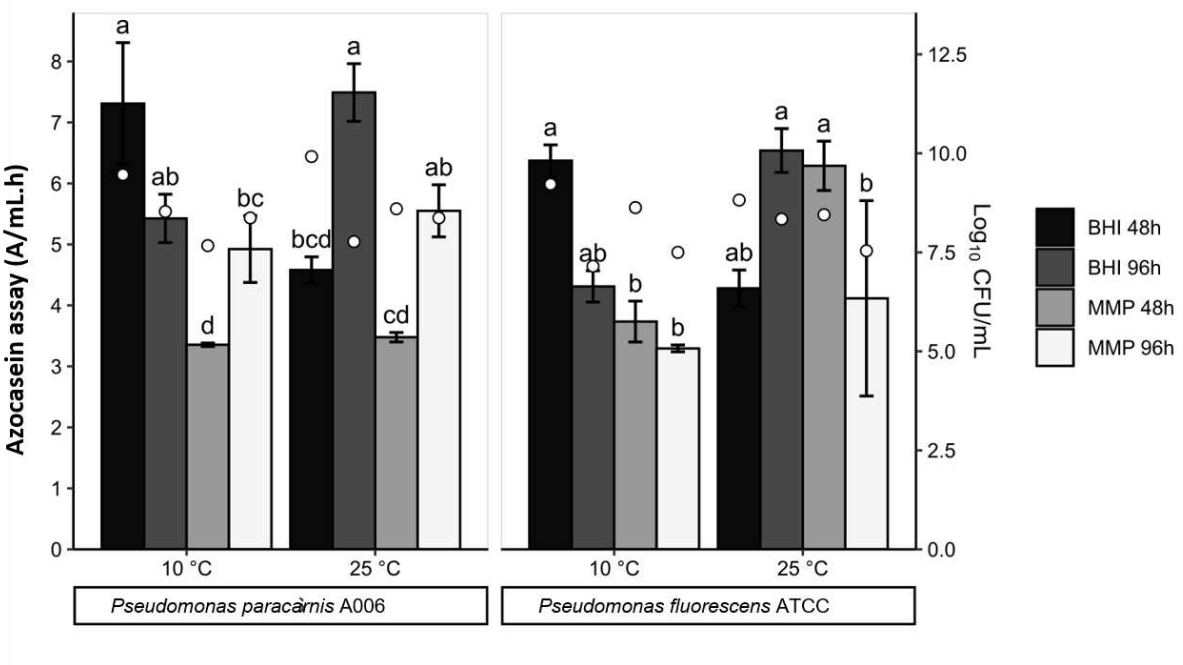

Figure 1 – Proteolytic activity (expressed in activity/mL/h) and cell concentration of *P. paracarnis* A006 and *P. fluorescens* ATCC13525 under different culture conditions: 10 °C and 25 °C for 48 h and 96 h in BHI and MMP. Error lines represent standard deviation of the mean. Different letters above the bars indicate significant difference between groups for each strain (P<0.05). ANOVA and Tukey's multiple comparisons post hoc test were performed.

3.1.2 Lipolytic Activity

The pigment-producing strain, *P. paracarnis* A006, incubated at 10 °C and 25 °C had the same lipolytic profile in both culture media (Figure 2), with a large increase in enzymatic activity in the last 48 h of analysis. The non-pigmented strain, *P. fluorescens* ATCC13525, had opposite profile when incubated in BHI and MMP at 10 °C. The lipolytic activity decreased in the last 48 h of growth, as well as the number of viable cells found in the culture, although the populations remained above 7.5 Log CFU/mL, indicating that the culture was in a stationary growth phase. When *P. fluorescens* ATCC 13525 was cultured at 25 °C in rich medium (BHI), an increase in lipolytic activity was observed, while, in MMP, there was a decrease.

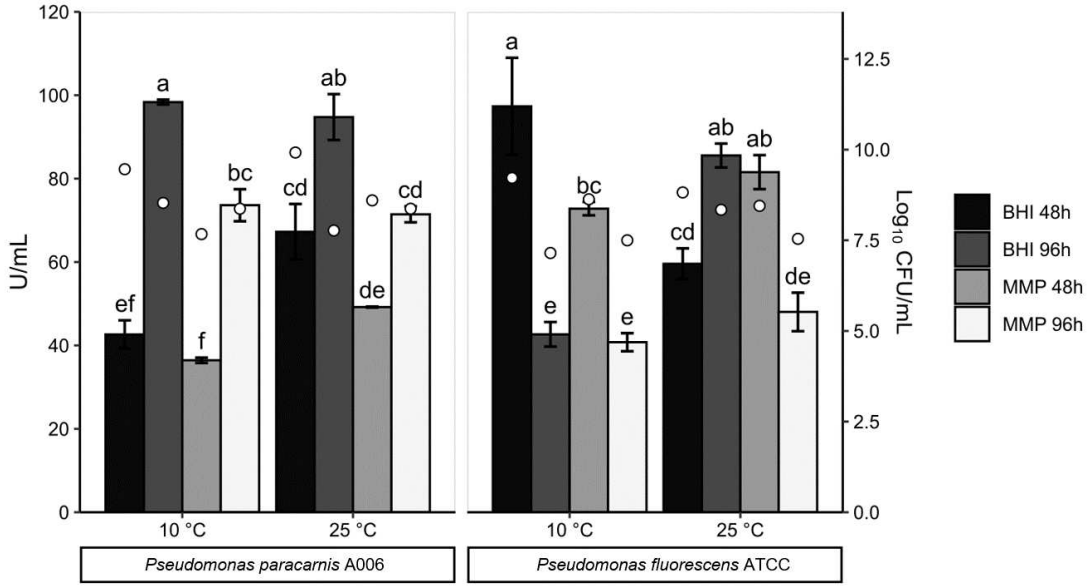

Figure 2 - Lipolytic activity of *P. paracarnis* A006 and *P. fluorescens* ATCC13525 (expressed as optical density at 410nm) under different culture conditions: 10 °C and 25 °C for 48 h and 96 h in BHI and MMP. Error lines represent standard deviation of the mean. Different letters above the bars indicate significant difference between groups for each strain ($P<0.05$). ANOVA and Tukey's multiple comparisons post hoc test were performed.

Psychrotrophic bacteria can impair milk quality at levels around 7 log CFU/mL. However, the number of psychrotrophic bacteria in raw milk required to impart off-taste pasteurized milk varies according to the strain of the bacteria, the length of the latency phase for their growth, the rate of growth at the particular storage temperature, the activity of proteolytic and lipolytic enzymes and the thermal stability of these enzymes (CHAMPAGNE, et al., 1994). For *Pseudomonas*, an association was found between extracellular enzyme production and cell density in the late log phase and early stationary phase of growth. According to McKellar (1989), such a relationship occurs when the bacterial count reaches 8 log CFU/mL while Dunstall et al. (2005) found such a correlation in bacterial counts greater than 6 log CFU/mL. Although these factors interfere with the lipolytic potential of *Pseudomonas*, lipolytic activity is also a strain-dependent characteristic and strongly affected by temperature and incubation time (NARVHUS et al., 2021).

3.1.3 Biofilm formation

P. fluorescens ATCC 13525 showed smaller capability of biofilm formation over time in both media (BHI and MMP) at 25 °C (Figure 3). *P. paracarnis* A006 presented the same profile, except when it was cultivated in MMP, which showed statistically similar biofilm formation capacity at 48 and 96 h of incubation.

The lower capacity for biofilm formation over time may be due to the detachment of cells in their last stage of biofilm formation (maturation), when the adhered cells begin to detach and become planktonic cells again (HINSA et al, 2003.).

Pigment production of *P. paracarnis* A006 was visually monitored during the analysis. At 10 °C, the pigment appeared first in MMP, with a slightly bluish color after 48h, while at 25 °C the pigment appeared first in BHI after 72 h of incubation. However, the predominant color was brown/black, different from the blue color characteristic of the defect described in cheeses. The same was observed by Rossi et al. (2018) who described after 72 h of incubation and biofilm formation, the change of substrate color was observed both in *P. fluorescens* single culture and in mixed culture with *L. monocytogenes* LM5.

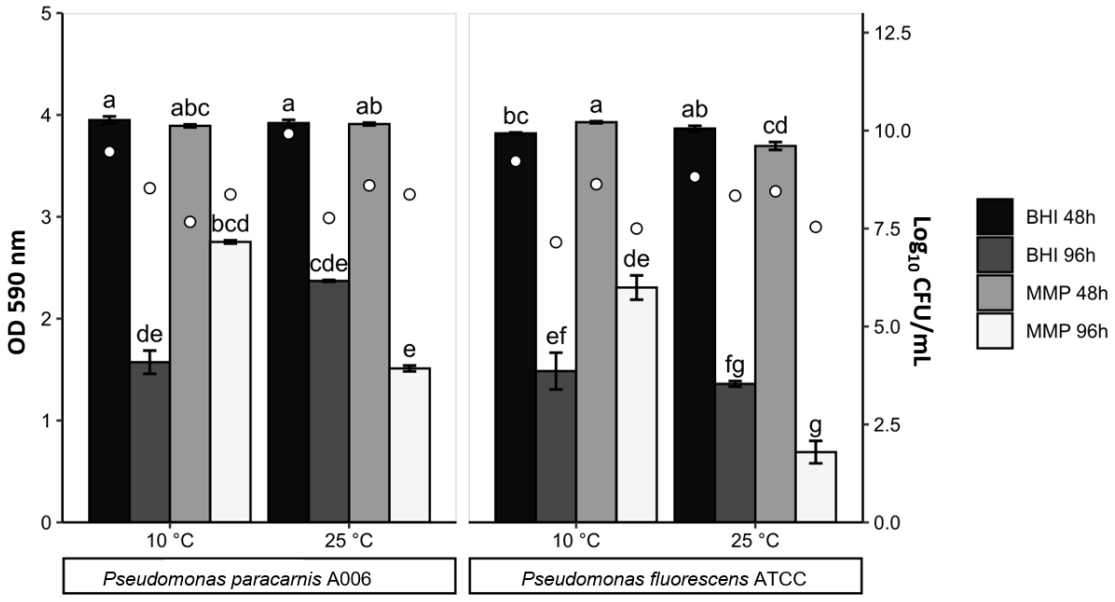

Figure 3- Biofilm formation by *P. paracarnis* A006 and *P. fluorescens* ATCC 13525 on polystyrene plates, incubated at 10 °C and 25 °C for 48 h and 96 h in BHI and MMP. Error lines represent standard deviation of the mean. Different letters above the bars indicate significant difference between groups for each strain (P<0.05). Kruskal-Wallis' test with Conover's multiple comparisons post hoc test were performed.

3.2. Assessment of spoilage potential of *Pseudomonas in situ*

In this study, the two tested strains showed proteolytic and lipolytic potential when inoculated into mini-cheese (Table 1). The cheese matrix was stored at 10 °C for 96 h and the color change was monitored at the end of the experimental period (Figure 4). On the third day, it was possible to observe the beginning of the appearance of the blue/greenish coloration, being evidenced on the fourth day of analysis (Figure 4). The enzymatic activity of both evaluated strains differed statistically *in situ*. *P. paracarnis* A006 has higher lipolytic potential and lower proteolytic potential than *P. fluorescens* ATCC 13525.

The relationship between the appearance of the blue color and the arrival of the bacterial count at 10^6 CFU/g was easier to observe, corroborating to similar studies (CANTONI, STELLA, RIPAMONTI and MARCHESE, 2001; ANDREANI et al., 2014; CHIERICI et al., 2016).

Strain	**Proteolytic activity ($\Delta A*h^{-1}*mL^{-1}$)**	**Lipolytic activity (U/mL)**	***Pseudomonas* couting (Log_{10} CFU/g**
A006	5.919 ± 0.0196*	3.287± 0.0226*	10.9
ATCC13525	7.226 ± 0.0583*	2.7347± 0.0223*	9.7

Table 1 – Proteolytic and lipolytic activity measured in mini-cheese after inoculation of *Pseudomonas* spp. followed by incubation at 10 °C for 96 h. Asterisk indicates that means differed significantly by least significance difference (LSD) t-test ($P<0.05$). Data are expressed as mean ± standard deviation.

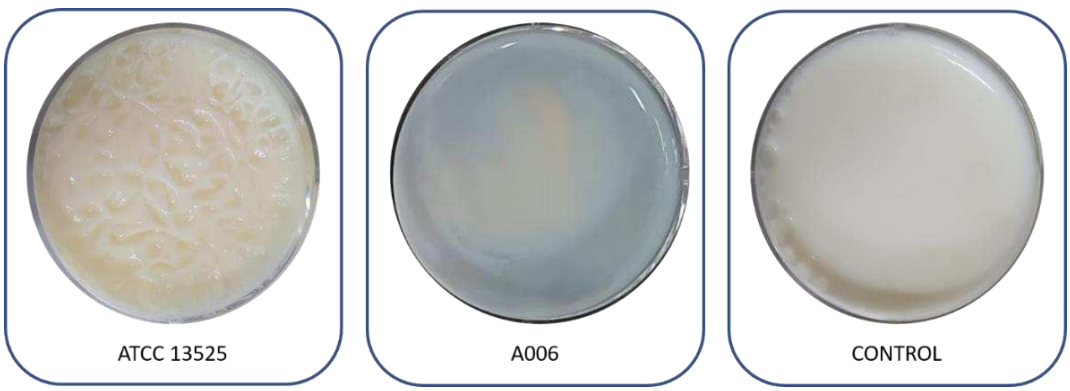

Figure 4 – Mini cheeses inoculated with *P. fluorescens* ATCC 13525, *P. paracarnis* A006 and control (without inoculation) after 96h of incubation at 10 °C.

Reichler et al. (2019) showed the correlation of the appearance of a gray pigment in pasteurized milk after 3 days of *P. fluorescens* ATCC 13525 inoculum incubated at 21 °C with extensive proteolysis evidenced by coagulation and separation of the inoculated samples that initially appeared after 3 days.

Del Olmo, Calzada and Nuñez (2018) also correlated the strong proteolytic activity to the production of the blue pigment by strains of *P. fluorescens* ATCC 13525 in fresh cheese from Burgos, followed by the increase in the expression of such enzymes with the appearance of the blue color in these products.

The above studies bring a strong correlation between the production of pigmentation and the deteriorating potential. This correlation has not yet been elucidated. However, some authors evidenced there is some type of regulation of virulence and deterioration factors by the production of the pigment, which is a response to stress, conditioned to the presence of oxygen (CAPUTO et al., 2015; ROSSI et al., 2018).

3.3 Genetic organization

The behavior of the *P. paracarnis* A006 and its deterioration properties have still been poorly studied. An analysis of its genome which encodes proteins, related to dairy spoilage, was performed. After identifying the operon in *P. paracarnis* A006, it was compared with the non-pigmenting model strain used in this work (ATCC13525) (Figure 5). Both bacteria have the operon that explain the high proteolytic and lipolytic enzymatic activity. Species that contain the complete *aprX-lipA* operon have high proteolytic and lipolytic activity (MAIER, et al., 2020) which corroborates the results found *in vitro* and *in situ*. Figure 5 also indicates that among the nine possible genes (Table 2) to be found in the *aprX-lipA* operon, the two tested strains have eight of them, with a high degree of similarity, which explains the similarity of both in the lipase and protease activity assays.

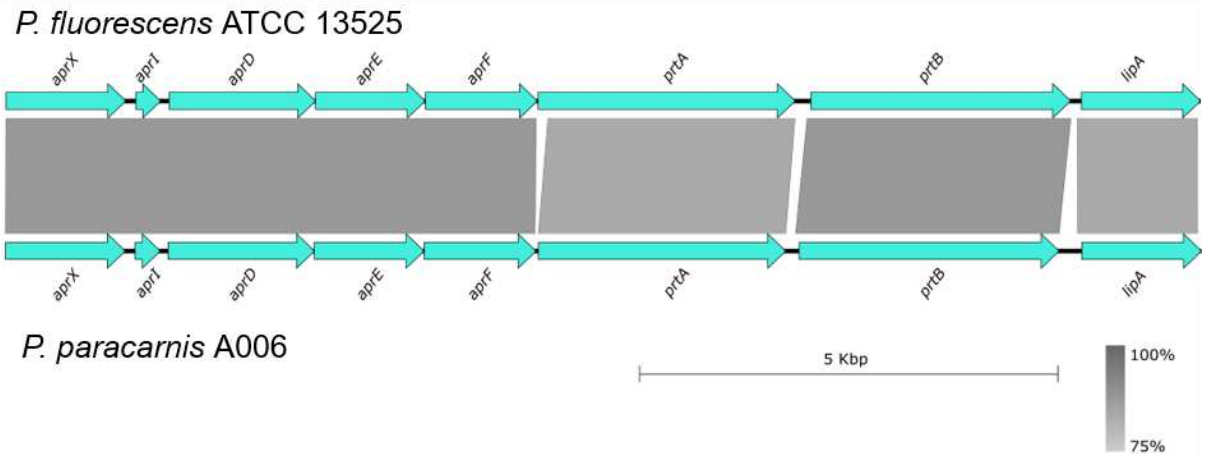

Figure 5 – Comparison between *aprX-lipA* genetic organizations of *P. fluorescens* ATCC13515 and *P. paracarnis* A006.

Proteins Original Annotation of Proteins (PGAP)	
AprX M10	Family metallopeptidase
AprI AprI/Inh	Family metalloprotease inhibitor
AprD type I	Secretion system permease/atpase
AprE HlyD family type I	Secretion periplasmic adaptor subunit
AprF TolC	Family outer membrane protein
PrtA	Autotransporter domain-containing protein
PrtB	Autotransporter domain-containing protein
LipA	Lipase

Table 2 – Genes of the *AprX-lipA* operon present in *Pseudomonas paracarnis*; PGAP: NCBI Prokaryotic Genome Annotation Pipeline.

3. CONCLUSION

P. paracarnis A006 and *P. fluorescens* ATCC showed proteolytic and lipolytic potential in culture media and cheese matrix although the extent of proteolysis and lipolysis was strain dependent and affected by nutritional factors, time, and temperature of incubation. Both strains demonstrated the capability of biofilm formation regardless the nutrient availability. It may contribute to the permanence of these microorganisms in the industrial environment. The results of this study further demonstrated that *P. paracarnis* A006 can cause spoilage of dairy products that goes beyond the production of blue pigment.

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GENERAL CONCLUSION

P. paracarnis A006 produces blue pigment insoluble in aqueous solutions and soluble in acids. The production of blue pigment by *P. paracarnis* A006 *in situ* is highly influenced by the product formulation and storage conditions. The use of starter culture inhibits the appearance of blue pigment by preventing bacterial growth, which is closely linked to the production of the pigment itself. Optimization using the RSM enabled the validation of parameters to mitigate the problem of the appearance of blue pigmentation, as well as validating pH values, NaCl and starter culture content for greater pigment production. The genus *Pseudomonas* presents variability in its enzymatic activity. However, the pigment-producing strain is strongly proteolytic and lipolytic, *in situ* and *in vitro*. Besides hydrolytic potential, *P. paracarnis* A006 can persist in industrial environment forming biofilms.

This work highlighted the processing parameters used in cheese production to mitigate the damage caused by strains that produce blue pigmentation in dairy industries. It is also expected that this work will be continued on the elucidation of the molecule that causes the blue pigmentation, since knowing its molecule, structure and production pathways, the compound can be explored.