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APPLICATION OF ECO-FRIENDLY ALTERNATIVE METHODS FOR CONTROLLING PSEUDOMONAS SPP. IN DAIRY PRODUCTS

Letícia Elisa Rossi
Doctor Scientiae

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LETÍCIA ELISA ROSSI

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

Adviser: Solimar Goncalves Machado

Co-advisers: Maria C Dantas Vanetti
Mariana da C N Pimenta Brandao

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Letícia Elisa Rossi
Author

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ABSTRACT

ROSSI, Letícia Elisa, D.Sc., Universidade Federal de Viçosa, September, 2024. **APPLICATION OF ECO-FRIENDLY ALTERNATIVE METHODS FOR CONTROLLING Pseudomonas spp. IN DAIRY PRODUCTS**. Adviser: Solimar Goncalves Machado. Co-advisers: Maria Cristina Dantas Vanetti and Mariana da Costa Novo Pimenta Brandao.

The spoilage of dairy products by psychrotrophic microorganisms, such as some species of bacteria from the *Pseudomonas* genus, represents a significant challenge for the food industry. These bacteria can produce thermoresistant hydrolytic enzymes, pigments, and biofilms, which directly affect the quality of dairy products and reduce their shelf life. Although conventional methods, such as heat treatments and the addition of preservatives, are widely used, they have limitations, such as the loss of sensory quality and the need to meet the growing demand for clean-label foods without synthetic additives. In light of this, the search for natural and effective preservation methods becomes essential. This study provides a comprehensive review of alternative methods to replace heat treatments and synthetic preservatives in dairy products. Among the methods evaluated are the use of enzymes, lactic acid bacteria, bacteriophages, essential oils, and technologies such as electromagnetic waves and pulsed electric fields. The study explores the application of these eco-friendly methods against *Pseudomonas* bacteria, highlighting the need to address the limitations and disadvantages of each method to optimize their use. Optimization is crucial to ensure that these methods can be effectively applied in food matrices, preserving both microbiological safety and sensory quality. The experimental phase of this study aimed to evaluate the inhibition of multiplication and blue pigmentation caused by *Pseudomonas paracarnis* A006 *in vitro* and *in situ* by free and nanoemulsified essential oils. Twenty essential oils were investigated as potential antibacterial agents against *P. paracarnis* A006. The study demonstrated significant inhibitory activity of cinnamon essential oil, standing out with the lowest values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), both at 0.625 µL/mL. The composition of cinnamon essential oil was determined by gas chromatography-mass spectrometry (GC-MS) and gas chromatography with flame ionization detection (GC-FID). The major compound present in cinnamon essential oil was identified as cinnamaldehyde. The formulation

of the cinnamon essential oil nanoemulsion was optimized using response surface methodology associated with central composite design (CCD). Based on the design of two independent variables (sonication time and lecithin percentage) and model construction, the stationary point (optimal point) corresponded to 4.26 min of sonication and 2.79% soy lecithin. The level of reactive oxygen species (ROS) in *P. paracarnis* A006 cells treated with free cinnamon essential oil, its nanoemulsion, and cinnamaldehyde was determined by fluorescence assay using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a probe. The results indicated low levels of intracellular ROS promoted by the action of the treatments, possibly associated with the antioxidant activity of cinnamon essential oil and cinnamaldehyde. The three treatments were applied *in situ* using a matrix simulating fresh cheese conditions (model cheese). Despite the *in vitro* efficacy, the nanoemulsion showed reduced performance compared to the free cinnamon essential oil when applied to model cheeses. The highest efficacy in inhibiting *Pseudomonas in situ* was observed in the treatment with cinnamaldehyde at four times the MIC concentration (4xMIC), which reduced *Pseudomonas* spp. counts by 5.58 ± 0.92 log CFU/g and 2.24 ± 0.54 log CFU/g compared to the control without added oil immediately after cheese production (T0) and after 7 days of refrigerated storage (T7), respectively. The free essential oil treatment at 4xMIC reduced the target microbial group's count by 5.21 ± 1.63 log CFU/g at T0 and 1.55 ± 0.68 log CFU/g at T7, while the nanoemulsion treatment at 4xMIC reduced *Pseudomonas* spp. population by 4.81 ± 0.66 log CFU/g at T0 and 1.46 ± 0.55 log CFU/g at T7. This reduction in the *Pseudomonas* population controlled blue pigment production in model cheeses. In the untreated control, pigmentation began to appear from the fourth day of storage, while in cheeses with added cinnamaldehyde, pigmentation was detected only on the seventh day. Cheeses with added free essential oil showed no blue pigmentation until the seventh day of storage, while cheeses with added nanoemulsion showed pigmentation on the fifth day. Cheeses from the three treatments were subjected to volatile compound analysis by headspace GC-MS. Cheeses with nanoemulsion presented a lower concentration of volatile compounds, indicating controlled release. However, this gradual release may be associated with reduced antimicrobial efficacy in the model cheese matrix. Based on these results, this work demonstrates that alternative methods, such as the use of free or nanoemulsified essential oils, have promising potential in controlling *Pseudomonas* in dairy products. However, adjustments are

necessary to optimize the application of nanoemulsions in complex food matrices, ensuring their antimicrobial efficacy without compromising the sensory characteristics and rheology of the food.

Keywords: dairy product deterioration; blue pigment; alternative methods; essential oil; nanoemulsion

RESUMO

ROSSI, Letícia Elisa, D.Sc., Universidade Federal de Viçosa, setembro de 2024. **Métodos de controle de Pseudomonas spp. produtoras de pigmento azul responsáveis pela deterioração de produtos lácteos.**. Orientadora: Solimar Goncalves Machado. Coorientadores: Maria Cristina Dantas Vanetti e Mariana da Costa Novo Pimenta Brandao.

A deterioração de produtos lácteos por microrganismos psicrotróficos, como algumas espécies de bactérias do gênero *Pseudomonas*, representa um grande desafio para a indústria alimentícia. Essas bactérias podem produzir enzimas hidrolíticas termorresistentes, pigmentos e biofilmes, que afetam diretamente a qualidade dos produtos lácteos e reduzem sua vida útil. Embora métodos convencionais, como tratamentos térmicos e a adição de conservantes sejam amplamente utilizados, esses têm limitações, como a perda de qualidade sensorial e a necessidade de atender à crescente demanda por alimentos com rótulos limpos, sem aditivos sintéticos. Diante disso, torna-se essencial a busca por métodos de conservação naturais e eficazes. Este estudo faz uma revisão abrangente dos métodos alternativos para substituir tratamentos térmicos e conservantes sintéticos em produtos lácteos. Entre os métodos avaliados estão o uso de enzimas, bactérias láticas, bacteriófagos, óleos essenciais e tecnologias como ondas eletromagnéticas e campos elétricos pulsados. O trabalho explora a aplicação desses métodos ecologicamente corretos contra as bactérias do gênero *Pseudomonas*, destacando a necessidade de abordar as limitações e desvantagens de cada método para otimizar seu uso. A otimização é crucial para garantir que esses métodos possam ser aplicados de forma eficaz em matrizes alimentares, preservando tanto a segurança microbiológica quanto a qualidade sensorial. A fase experimental deste estudo teve como objetivo avaliar a inibição da multiplicação e da pigmentação azul causada por *Pseudomonas paracarnis* A006 *in vitro* e *in situ* por óleos essenciais livres e em nanoemulsão. Foram investigados 20 óleos essenciais como potenciais agentes antibacterianos contra *P. paracarnis* A006. O estudo demonstrou uma atividade inibitória significativa do óleo essencial de canela, se destacando com os menores valores de concentração mínima inibitória (MIC) e concentração mínima bactericida (MBC), ambos de 0,625 µL/mL. A composição do óleo essencial de canela foi determinada por cromatografia gasosa acoplada a espectrometria de massas (GC-MS) e por

cromatografia gasosa com detecção por ionização em chama (GC-FID). O composto majoritário presente no óleo essencial de canela foi identificado como cinamaldeído. A formulação da nanoemulsão do óleo essencial de canela foi otimizada utilizando a metodologia de superfície de resposta associada ao design de composto central (CCD). Com base no delineamento de duas variáveis independentes (tempo de sonicação e porcentagem de surfactante lecitina) e na construção do modelo, foi determinado o ponto estacionário (ponto ótimo) que correspondeu a 4,26 min de sonicação e 2,79% de lecitina de soja. O nível de espécies reativas de oxigênio (ROS) em células de *P. paracarnis* A006 tratadas com óleo essencial de canela livre, com nanoemulsão do mesmo óleo e cinamaldeído foi determinado por ensaio de fluorescência utilizando 2',7'-diclorodihidrofluoresceína diacetato (DCFH-DA) como sonda. Os resultados indicaram baixos níveis de ROS intracelular promovidos pela ação dos tratamentos, possivelmente associados à atividade antioxidante do óleo essencial de canela e do cinamaldeído. Os três tratamentos foram aplicados *in situ* utilizando uma matriz que simula as condições de queijos frescos (queijo modelo). Apesar da eficácia *in vitro*, a nanoemulsão apresentou desempenho reduzido em comparação ao óleo essencial de canela na forma livre ao ser aplicada em queijos modelo. A maior eficácia de inibição de *Pseudomonas in situ* foi observada no tratamento com o cinamaldeído em concentração quatro vezes maior que o MIC (4xMIC), que reduziu 5,58 ± 0,92 log UFC/g e 2,24 ± 0,54 log UFC/g da contagem de *Pseudomonas* spp. em relação ao controle sem adição de óleo logo após a fabricação dos queijos (T0) e após 7 dias de armazenamento sob refrigeração (T7), respectivamente. O tratamento com o óleo essencial livre 4xMIC reduziu 5,21 ± 1,63 log UFC/g e 1,55 ± 0,68 log UFC/g da contagem do grupo microbiano alvo em T0 e T7, respectivamente, enquanto o tratamento com a nanoemulsão 4xMIC reduziu 4,81 ± 0,66 log UFC/g da população de *Pseudomonas* spp. em T0 e 1,46 ± 0,55 log UFC/g em T7. Essa redução da população de *Pseudomonas* controlou a produção de pigmento azul nos queijos modelo. No controle sem tratamento, a pigmentação começou a aparecer a partir do quarto dia de estocagem, enquanto nos queijos com adição de cinamaldeído, a pigmentação foi detectada apenas no sétimo dia. Já os queijos com adição de óleo essencial livre não apresentaram pigmentação azul até o sétimo dia de armazenamento, enquanto nos queijos com adição de nanoemulsão, a pigmentação foi detectada no quinto dia. Queijos dos três tratamentos foram submetidos à análise de compostos voláteis por GC-MS de

headspace. O queijo adicionado da nanoemulsão apresentou menor concentração de compostos voláteis, indicando liberação controlada. No entanto, esta liberação gradual pode estar associada à menor eficácia do efeito antimicrobiano na matriz do queijo modelo. Com base nesses resultados, este trabalho demonstra que os métodos alternativos, como o uso de óleos essenciais na forma livre ou em nanoemulsões têm potencial promissor no controle de *Pseudomonas* em produtos lácteos. No entanto, ajustes são necessários para otimizar a aplicação de nanoemulsões em matrizes alimentares complexas, assegurando sua eficácia antimicrobiana sem comprometer as características sensoriais e a reologia dos alimentos.

Palavras-chave: deterioração de produtos lácteos; pigmento azul; métodos alternativos; óleo essencial; nanoemulsão

SUMMARY

1 INTRODUCTION

Microbial contamination of food is a constant challenge for the food industry, especially for perishable products like dairy. Among the microorganisms responsible for the spoilage of these foods, some species of *Pseudomonas* have stood out due to their ability to produce heat-resistant hydrolytic enzymes, pigments, and their capacity to form biofilms. These factors significantly contribute to the alteration of the texture, flavor, and aroma of food, leading to considerable economic losses and a reduction in the shelf life of products. Additionally, these bacteria can grow at low temperatures, favoring their proliferation in refrigerated foods, which presents an additional challenge for preserving dairy products.

Raw milk and its derivatives are particularly vulnerable to *Pseudomonas* contamination due to their characteristics, such as high water and nutrient content, neutral pH, and refrigerated storage practices. Although thermal treatments, such as pasteurization and ultra high temperature (UHT) processing, are widely used to combat microbiological contamination, these approaches can have adverse effects on product quality, including protein denaturation, vitamin loss, and sensory changes. Furthermore, some *Pseudomonas* species, such as *Pseudomonas paracarnis*, have demonstrated the ability to cause spoilage in fresh cheeses, including the production of blue pigments that impair the appearance and market acceptance of products.

In recent years, the increased demand for "clean label" foods has driven both research and the food industry to seek natural and sustainable alternatives for food preservation. Consumers are increasingly concerned about the presence of synthetic additives in their food, which drives the development of control methods that preserve the sensory quality of products without compromising health or the environment. In this context, alternative eco-friendly methods, such as the use of enzymes, lactic acid bacteria and their metabolites, bacteriophages, ozone, and electromagnetic waves, have emerged as promising alternatives.

Among the natural solutions, the use of essential oils (EO) has gained prominence for their antimicrobial properties. These plant-extracted compounds contain bioactive components that primarily act on the bacterial cell membrane, destabilizing its structure and leading to cell death. Additionally, the application of EO nanoemulsions has emerged as an interesting alternative, offering greater stability, controlled release of active compounds, and less impact on the flavor of foods while maintaining their antimicrobial properties. Recent studies indicate that these natural methods, when adequately formulated, can effectively inhibit spoilage bacteria, including *Pseudomonas* strains that produce blue pigment.

Given the relevance of the topic, this work aims to explore alternative and eco-friendly methods to combat *Pseudomonas* bacteria in dairy products, with a focus on the use of essential oils and their nanoemulsions. The search for preservation alternatives that meet the demand for clean label products, extend the shelf life of foods, and maintain their sensory qualities is crucial to addressing the challenges of the modern food industry. Throughout this study, both the advances and limitations of these approaches are discussed, with the objective of contributing to the development of effective and sustainable solutions for dairy product preservation.

Chapter 1: *Pseudomonas* in dairy plants and products: a review of challenges and control methods

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Pseudomonas in dairy plants and products: a review of challenges and control methods

Letícia Elisa Rossi[1] et al.

[1]Department of Microbiology, Federal University of Viçosa, Av. P.H. Rolfs, 365700-900, Viçosa- Brazil

ABSTRACT

Species of the genus *Pseudomonas* are among the main spoilage psychrotrophic bacteria of dairy products. Deterioration through the production of thermostable hydrolytic enzymes, the production of blue pigment and the ability to form a biofilm are challenges to be faced by industries that seek to extend the shelf life of foods. Eco-friendly methods are a new trend for food conservation due to the food industry's quest to please consumers who are increasingly looking for clean label products, with high nutritional value and closer to natural. Alternative methods are applied in order to replace the use of thermal methods and the addition of chemical additives. This review aims to present the damage caused by *Pseudomonas* in the dairy industry, explore the applications of conventional and environmentally friendly alternative methods in dairy products against spoilage bacteria of the genus *Pseudomonas*, and emphasize the need to address the disadvantages and limitations of these methods to optimize their use.

Keywords: *Pseudomonas*; food spoilage; dairy products; food preservation; eco-friendly methods; clean label.

1 Introduction

Bacteria of the genus *Pseudomonas* are known for their association with food spoilage, primarily due to their ability to produce pigments and heat-resistant hydrolytic enzymes. The hydrolysis caused by these enzymes, along with the production of volatile compounds and biosurfactants, can significantly alter the food texture, flavor, and odor (Carrascosa et al., 2021; Kumar et al., 2019). This deterioration not only compromises the sensory qualities of food products but also results in substantial economic losses due to reduced shelf-life and increased food loss and waste (Martin et al., 2021).

Pseudomonas genus is composed of Gram-negative bacteria, with some strictly aerobic and psychrotrophic species. Due to the ability to grow at temperatures below 7 °C, refrigerated food storage practices allow the growth of these microorganisms. Thus, they are often studied for their importance in the food industry and their ubiquity due to the ability to use a wide

variety of organic compounds as an energy source. They can be found in environments such as water, soil, animals, plants, and in foods that have high water activity, such as meat, vegetables, milk and dairy products (Meng et al., 2017; Hesse et al., 2018; Xiang et al., 2018).

Raw milk and dairy products are favorable habitats for bacteria due to their characteristics such as the availability of carbohydrates, lipids, proteins, vitamins, minerals and neutral pH. Its microbiota is directly influenced by the environmental conditions in which the dairy cattle is allocated, with contamination through water, feed, udder surface and milking equipment (von Neubeck et al., 2015). Although genera belonging to the lactic acid bacteria group are predominantly found in raw milk right after milking, the cooling process to which the milk is subjected before processing allows the inversion of the dominant microbiota. Psychrotrophic lactic acid bacteria species, despite being able to grow at low temperatures, become less abundant due to the competitive advantages of *Pseudomonas* species that have metabolic efficiency and the ability to degrade a wide range of nutrients. Consequently, psychrotrophic and spoilage species of *Pseudomonas* become abundant (Hoving-Bolink et al., 2023; Mörschbächer et al., 2018).

The ability to form a biofilm is also a strategy observed in deteriorating bacteria belonging to *Pseudomonas* genus, which favors survival in adverse environmental situations such as abiotic surfaces with sanitizer residues. Consequently, contamination of food products can occur not only in the initial production phase, but also through cross-contamination in processing equipment (Meliani and Bensoltane, 2015). Furthermore, it was reported that, under refrigeration at 4 °C, some species of *Pseudomonas* genus demonstrated an increase in biofilm structure and resistance to sanitizers such as sodium hypochlorite, with the accumulation of polysaccharides and extracellular proteins (Liu et al., 2023).

The shelf life of dairy products is directly influenced by the quality of the raw material, the control methods associated with processing and packaging, in addition to the storage conditions subjected to the products (Rauh and Xiao, 2022). Thus, microbial control methods are already commonly applied in the food industry to extend this shelf life. Heat treatments such as pasteurization and ultra high temperature (UHT) are conventional methods widely used in dairy products, despite their limitations. Thermal sensitivity can trigger protein denaturation, loss of amino acids and vitamins, in addition to calcium phosphate precipitation, browning by the Maillard reaction and flavor changes (van den Oever and Mayer, 2021).

Synthetic preservatives are also conventionally used methods to extend the shelf life of products such as cheese. However, the growing interest of consumers in eating products that are closer to natural, increases the tendency of researchers and industries to seek more eco-

friendly conservation methods for the production of clean label foods (Ritota and Manzi, 2020). Accordingly, there are already both biocontrol and physicochemical methods being research targets to test their abilities to eliminate deteriorating microorganisms such as bacteria belonging to the *Pseudomonas* genus.

Although there is significant interest in the search for new alternatives for preserving dairy products, further studies on new technologies are still needed to address the persistence of spoilage microorganisms. Such advancements would help to reduce food waste and promote the trend towards clean label food production. Therefore, the objective of this review is to present the damage caused by *Pseudomonas* in the dairy industry, to report the conventional and alternative methods already tested against these microorganisms in dairy matrices, and to disseminate knowledge about what can still be explored to face the challenges caused by contamination by *Pseudomonas* and its spoilage potential.

2 Methodology

The studies used in this review were selected from searches in scientific databases, such as Google Scholar. The keywords used in the searches were chosen according to the main theme of the review and included terms such as: “alternative methods”, “clean label”, “*Pseudomonas*”, “blue discoloration”, and “dairy products”. The search was directed to identify studies that specifically tested alternative methods in dairy products contaminated by bacteria of the *Pseudomonas* genus.

The review followed a qualitative approach, analyzing and synthesizing the results of the studies identified in the literature, aiming to provide an overview of alternative methods that have been applied to control *Pseudomonas* contamination in dairy products.

The following inclusion criteria were adopted for this review:

2.1 Type of study

Priority was given to studies that conducted tests on dairy products contaminated with *Pseudomonas*, excluding those that focused on *in vitro* analyses without using a real food matrix.

2.2 *Pseudomonas* species

Although *Pseudomonas aeruginosa* is an important contaminant, it was excluded from this review because it is more commonly associated with human health issues. Therefore, the focus was on *Pseudomonas* species more commonly related to food spoilage.

2.3 Publication years and language

Priority was given to studies published in the last 20 years and written in English.

3 Thermostable hydrolytic enzymes

Since the 1970s, there have been reports of spoilage of cheeses and sterilized milk caused by *P. fluorescens* due to thermostable hydrolytic enzymes (White and Marshall, 1973; Law et al., 1977). Even powdered milk, which has a low water content, which reduces the risk of microbial growth, can have its functionality and flavor compromised during storage by enzymes capable of withstanding high-temperature processing steps (Chen et al., 2003).

Hydrolytic enzymes produced by contaminating psychrotrophic bacteria can reduce the shelf life of dairy products due to changes in sensory quality. Proteinases cause proteolysis of caseins in UHT milk, developing a bitter taste and increasing the viscosity of the product, causing gelation (Datta and Deeth, 2003). Zhang et al. (2018) highlighted that the rapid gelation of skimmed UHT milk is promoted by higher concentrations of proteases produced by *P. fluorescens*, with a predominant role in the hydrolysis of κ-casein. D'Incecco et al. (2019) corroborated these findings by observing that these proteases have an activity similar to chymosin, destabilizing casein micelles by cleaving Caseinomacropeptide (CMP) and pseudo-CMPs from κ-casein. Through mass spectrometry, CMP accumulation was observed well before the onset of UHT milk gelation, being presented in the study as an early indicator of this gelation. Other species of *Pseudomonas* genus, such as *Pseudomonas jessenii* and *Pseudomonas gessardii*, were also identified by Zarei et al. (2020) as producers of proteases, thus playing a notable role in the deterioration of Iranian commercial UHT milk.

The reduction in cheese yield can also occur due to casein destabilization. In the 1980s, Ellis and Marth (1984) analyzed the yield of cheddar cheese made from milk artificially contaminated with proteolytic species of *Pseudomonas* and *Flavobacterium*, stored at 7°C. *Pseudomonas* grew more rapidly and reached larger populations, resulting in an average reduction of 0.53% in cheese yield per day after 7 days of storage. Paludetti et al. (2020a) reinforced these observations, demonstrating that the rennet coagulation time and curd firmness were affected during milk storage at 4°C. This occurred because the protease primarily acted on caseins essential for coagulum formation and structure, such as κ-casein and β-casein. Additionally, Paludetti et al. (2020b) also observed the proteolytic activity of heat-resistant enzymes produced by *P. fluorescens* in cheddar cheese, using contaminated milk stored at 4°C for 48 hours. However, the average cheese yield and the recovery of fat and protein were not affected by protease activity, a result attributed to the low pH of the curd, which reached 5.35

during the milling stage, a value below the enzyme's optimal pH (7 to 9). Furthermore, Frohbieter et al. (2005) demonstrated that microbial proteolysis can trigger the activation of plasminogen under conditions similar to those in cheese, increasing processing costs due to delayed ripening and negatively affecting the flavor and texture of the cheese.

The taste of dairy products can also be threatened by lipolytic enzymes, which cause the hydrolysis of lipids into free fatty acids, which leads to an unpleasant, "rancid" taste (Deeth and Fitz-Gerald, 2009). There are reports that lipases have a more eminent activity at refrigeration temperatures (Rajmohan et al., 2002; Rick G. Woods et al., 2001). Hayes et al. (2002), with the help of 10 trained panelists, conducted a descriptive analysis of the aroma of skim and whole milk inoculated with *Pseudomonas fluorescens*, *Pseudomonas fragi*, and *Pseudomonas putida*, stored at 5°C. Over 3 weeks, aromas such as "rotten," "barn," "shrimpy," "veterinary office," "cheesy," "fruity," and "cooked" were described. The variations in responses demonstrated that the type and onset of spoilage depended on the *Pseudomonas* strain and the fat content of the milk. Bekker et al. (2016) also evaluated, through sensory analysis, aromas produced in UHT whole milk inoculated with *P. fluorescens*. In this study, high levels of lipolytic activity and production of volatile compounds such as 2-butanone, 2-pentanone, and 2-heptanone were identified, which were associated with "rancid," "fruity," and "blue cheese" odors, respectively. The production of volatile compounds was also identified in UHT milk inoculated with *P. fluorescens*, *P. fragi*, *Pseudomonas mosselii*, and *Pseudomonas rhodesiae* by Decimo et al. (2018). The compounds identified as potential indices of chemical spoilage of the milk were 3-methylbutan-1-ol, 2-methylpropan-1-ol, 3-hydroxybutan-2-one, butan-2,3-dione, butanoic and hexanoic acids.

The use of molecular biology technologies makes it possible to understand the coding and regulation of the production of thermoresistant enzymes. From a strain of *P. fluorescens*, Liao & Mccallus (1998) identified an extracellular protease AprX, named after the coding *aprX* gene. With advances in molecular studies, this gene has become the target of investigations in other members of the genus *Pseudomonas* (Maier et al., 2021, 2020; Zarei et al., 2020).

The *aprX* gene is located in a cluster of genes that constitute the *aprX-lipA* operon. In this operon there is the *aprX* gene that encodes the protease, in addition to having genes that encode a protease inhibitor (*aprI*), a secretion system (*aprDEF*), two autotransport proteins (*prtAB*) and a lipase (*lipA*) (Woods et al., 2001). By investigating the genetic organization of the *aprX-lipA2* operon in different *Pseudomonas* spp. isolates, Maier et al. (2020) identified some differences, such as the absence of the *prtA* gene or more than one lipase-coding gene, which has been shown to affect the bacterium's proteolytic potential. Aguilera-Toro et al. (2023)

also evaluated the correlation between proteolytic activity and genotypic characteristics of 24 strains of *Pseudomonas* spp. through whole genome sequencing. From this analysis, they observed that variations in amino acid sequences in biologically relevant motifs, such as the Zn^{2+}-binding motif in the catalytic domain and the type I secretion signaling mechanism in the C-terminal region, were highly conserved within alignment groups. Thus, the authors identified these genetic modifications as potential biomarkers to determine the spoilage potential of the strains. **Figure 1** shows the organization of the *aprX* operon. Operon A represents one of the most abundant configurations and was associated with the highest proteolytic potential, as reported by Maier et al. (2020). On the other hand, operons B, C, and D depict the different organizational types identified by Aguilera-Toro et al. (2023), in addition to operon A.

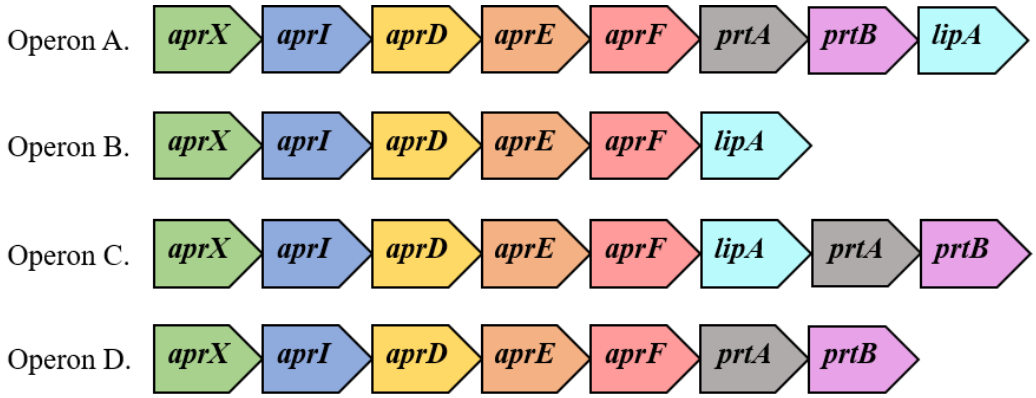

Figure 1. Different structures of the *aprX* operon. Schematic representation of the genes present in the operon with different coding activities: *aprX* – protease; *aprI* – protease inhibitor; *aprDEF* – secretion system; *prtAB* - autotransport proteins; *lipA* – lipase.

In order to evaluate the possibility of the *aprX* gene being a marker of proteolytic bacteria, Martins et al. (2005) evaluated the limit of detection of this gene by Polymerase Chain Reaction (PCR). The technique was performed using samples of reconstituted skimmed milk powder, sterilized and inoculated with *P. fluorescens* isolated from raw milk. The limit of detection was a population of 10^5 CFU/mL with the extracted DNA. However, inhibitory substances such as proteinases and Ca^{2+} used in DNA extraction can make it difficult to detect genes by PCR (Ramesh et al., 2002), leading the authors to the conclusion that new methods should be investigated to improve the sensitivity of the technique.

The fact that a bacterium has the *aprX* gene does not allow us to state that it is being expressed. Thus, in addition to improving gene detection techniques, Marchand et al. (2009) sought to understand the variability of proteases in different species of *Pseudomonas* (*P. lundensis*, *P. fragi* and members of the *P. fluorescens* group) at the level of nucleotides and amino acids. According to them, with the information acquired by these analyzes it becomes

possible to develop immunological detection methods for proteases. Of the 55 strains of *Pseudomonas* analyzed, 42 had the gene that encodes the protease. The technique presented possibilities for the *aprX* gene to be used as a taxonomic marker, however it has limitations due to the heterogeneity in the amino acid sequence level of different species of the genus.

The study by Von Neubeck et al. (2015) confirms the heterogeneity between species of the genus *Pseudomonas* regarding enzymatic activity. By analyzing the composition and patterns of this microbiota activity in refrigerated raw milk, they found the genus *Pseudomonas* to be among the most abundant and active in terms of extracellular enzymatic activity. The species *Pseudomonas proteolytica*, *Pseudomonas* sp. nov. (1), and *Pseudomonas meridiana* showed lipolytic activity for 99 of 107 isolates. *P. lundensis*, *P. fragi* and *Pseudomonas* sp. nov. (2) showed lipase activity less frequently, which may indicate genetic differences, such as the absence of the lipase gene, or differences in the regulation of these genes among members of the same genus.

It is important to highlight that the regulation of the *aprX-lipA* operon is controlled by a complex system of environmental and genetic factors. The study by Liu et al. (2007) aimed to examine the regulation of the *aprX* operon promoter in *P. fluorescens*. They identified a quorum sensing system based on *N*-acyl homoserine lactone (AHL), which regulates protease production at the transcriptional level. Furthermore, they observed that promoter activity significantly increased during the late exponential phase of growth, indicating growth phase-dependent regulation. The regulatory role of AHL-based quorum sensing was also confirmed by Yuan et al. (2020), who demonstrated that exogenous AHLs increased both lipolytic and proteolytic activities. This study further highlighted the involvement of quorum sensing in enzyme production, bacterial growth regulation, metabolism, and stress response. Additionally, Liu et al. (2018) identified that *RpoS*, a regulator associated with stress survival and virulence in various pathogens, also positively regulated AHL-based quorum sensing in *P. fluorescens*.

Environmental factors such as temperature, iron content, and the presence of calcium have also been previously described as factors influencing the regulation of the *aprX-lipA* operon. Marchand et al. (2009) evaluated the production of heat-stable proteases in different strains of *P. lundensis*, *P. fragi*, and *P. fluorescens*. The variation among species showed that while a certain temperature, such as 22 ºC, may be optimal for one species, it can be limiting for another. Additionally, while no protease production by *P. fragi* was observed at 30 ºC, all strains of the other species exhibited proteolytic activity. Martins et al. (2015) reinforced the influence of temperature by demonstrating maximum protease activity of *P. fluorescens* at 37 ºC and in the presence of Ca^{+2}. Besides calcium, Maunsell et al. (2006) demonstrated that iron

interferes with the activation of proteolytic activity in *P. fluorescens* by repressing the transcription of the AprA metalloprotease under high iron conditions, a regulation mediated by the Fur (Ferric Uptake Regulator) factor.

4 Blue pigmentation

The genus *Pseudomonas* is known for producing various types of pigments. Cantoni et al. (2006) evaluated the discoloration of mozzarella cheese, finding orange tones produced by *Pseudomonas aureofaciens* and *P. putida*, orange-red-brown tones by *P. gessardii*, greenish by *P. fluorescens*, and fluorescent tones by *P. fluorescens*, *P. putida*, and *Pseudomonas palleroni*. In 2010, the case of "blue mozzarella" gained public attention when it was reported by the RASFF (Rapid Alert System for Food and Feed) due to the formation of blue pigmentation in mozzarella imported from Germany. This event occurred in Italy between June and September 2010, when consumers reported the appearance of blue pigmentation in mozzarella cheese after opening the packaging, leading to the waste of around 70,000 mozzarella balls. The case became the subject of studies to identify the cause of this spoilage.

In order to better understand the origin of the "blue mozzarella" contamination, Nogarol et al. (2013) collected 181 samples of dairy products, 123 from Italian factories and 58 from products sold in Italy imported from German factories. The aim of this study was to identify molecular correlations of isolates using Pulsed Field Gel Electrophoresis (PFGE) to assess whether contamination of products with blue discoloration came from the same source. The results showed that the processing of dairy products can involve different sources of contamination, from raw milk to the milking environment. In this way, the hypothesis that only one strain of *P. fluorescens* could have been massively spread was overturned, further increasing the interest in analyzing dairy products and finding ways to combat and avoid this type of contamination.

The PFGE technique was also used by Martin et al. (2011) for specific detection and tracking of the origin of the *P. fluorescens* strain that produces blue pigment in fresh cheese. To investigate the persistence of contamination over a period of nine months in a commercial factory, samples were collected from environments associated with different stages of cheese processing. With the use of the restriction enzyme *Xba* I, the researchers were able, through PFGE, to distinguish and identify that the contaminating strains came from an agitator system that was part of the processing.

P. fluorescens had already been reported as predominant among the spoilage species that make up the microbiota of raw and pasteurized milk and the industrial environment of dairy

processing (Dogan and Boor, 2003). However, since the discovery of this species as the cause of blue discoloration in fresh cheese, other studies have been carried out to investigate the genus *Pseudomonas* causing blue discoloration in dairy products (**Table 1**).

The issue of blue stains in fresh cheeses gained attention recently when Rodrigues et al. (2021) investigated isolates from Minas Frescal cheese that caused spoilage due to the production of blue pigment. The production of pigmentation was evaluated *in vitro* and, after 24 h of incubation, it was possible to observe the blue color in Potato Dextrose Agar (PDA) medium at storage temperatures of 5, 14 and 25 ºC. In this study, through complete genome sequencing, strain B157 was identified as *Pseudomonas carnis* and strain A006 as a potential new species, which was later identified as *Pseudomonas paracarnis* (Rodrigues et al., 2023). Furthermore, the genomic study demonstrated a common evolutionary development among isolates that are pigment producers. It is noteworthy that the species *P. carnis* sp. nov. and *P. paracarnis* sp. nov. were initially identified by genomic analysis of spoilage-associated meat microbiota by Lick et al. (2020, 2021).

The interest in acquiring new knowledge in relation to the blue pigment that causes deterioration led Andreani et al. (2015) carried out a study with genomic and transcriptomic data of pigmented and non-pigmented strains of *P. fluorescens* to identify the occurrence of gene expression associated with this phenotype. Through the analysis it was possible to identify that the pigment strains shared two copies of genes involved in the tryptophan biosynthesis pathway. Furthermore, the transcriptomic analysis revealed a high iron-depleted state in the blue pigment strains, which may indicate that iron is a cofactor for specific enzymes involved in pigment production. However, concrete data will be possible once the chemical structure of the pigment is determined.

Table 1. Reports of dairy products spoilage by blue discoloration produced by species belonging to the genus *Pseudomonas*.

Source	Locality	Species/strains	References
Latin-style fresh cheese (Queso Fresco)	USA	*P. fluorescens* biovar IV	Martin et al. (2011)
Mozzarella cheese	Italy	*P. fluorescens*	Nogarol et al. (2013)
Mozzarella cheese	Germany	*P. fluorescens* ps_2 22, ps_13 100	Andreani et al. (2014)
Mozzarella cheese	Italy	*P. fluorescens* ps_6 57, ps_22 MOZ3, ps_75 62	Andreani et al. (2014)
Mozzarella cheese	England	*P. fluorescens* ps_78 uk4	Andreani et al. (2014)
Raw milk	Spain	*P. fluorescens* biovar IV	Carrascosa et al. (2015)
Fresh cheese	Italy	*P. fluorescens*	Chierici et al. (2016)
Pasteurized milk and fresh cheese	USA	*P. fluorescens*	Reichler et al. (2019)
Pasteurized and raw milk and fresh cheese	Italy	*P. fluorescens* (No. 4693, 4710 and 4744)	Carminati et al. (2019)
Fresh cheese	Spain	*P. fluorescens*	Carrascosa et al. (2021)
Fresh soft cheese (Minas Frescal)	Brazil	*P. carnis* (B157) and *P. paracarnis* (A006)	Rodrigues et al. (2021)

In order to understand the biological function of the blue pigment, Andreani et al. (2019) used the transposon-induced random mutagenesis method in pigmented strains *P. fluorescens* Ps_77. When analyzing the phenotypic characteristics, it was possible to notice that the "white" (non-pigmented) mutants were completely inhibited in the presence of 5 mM hydrogen peroxide, while the wild Ps_77 strains maintained their growth. This feature demonstrated a strong relationship between the blue pigment and the resistance to oxidative stress. Thus, the hypothesis is raised that the pigmented strains of *Pseudomonas* are more tolerant to sanitizers used in industries than the non-pigmenting strains, which can be further investigated in future

studies. In addition, another factor that makes it difficult to eradicate this genus in dairy factories is the ability to form a biofilm.

5 Biofilm

In addition to their ability to grow under refrigeration conditions, the ability to form biofilm makes *Pseudomonas* spp. constitute up to 70 to 90% of the psychrotrophs in the raw milk microbiota (de Jonghe et al., 2011). Biofilm formation is a strategy for spoilage bacteria to survive in adverse environmental situations, and can grow and persist on abiotic surfaces even after sanitization. Cells aggregate to the surface and the production of an extracellular matrix of DNA, lipids, carbohydrates and proteins reinforces nutrient and cell trapping, representing a significant problem for food industries (Coughlan et al., 2016).

There are several studies that have focused on *Pseudomonas* spp. biofilm formation in the dairy industry (Kives et al., 2005; Aswathanarayan and Vittal, 2014; Rossi et al., 2016; Chiara Rossi et al., 2018; Santos Rosado Castro et al., 2021; Panebianco et al., 2022a; Deiab et al., 2023). The study by Rossi et al. (2016) demonstrated that the biofilm formation capacity of *P. fluorescens* strains depended on both temperature and strain. Low-temperature conditions favored biofilm formation by several isolates after 48 h. This result was associated with the adaptation of the strains to the low temperatures of the production environment and storage conditions. Additionally, Yuan et al. (2024) analyzed how calcium (Ca^{2+}), naturally present in raw milk, affects biofilm formation by *P. fluorescens* isolates. The supplementation with Ca^{2+} increased biofilm formation capacity and biomass. Moreover, RNA sequencing analysis showed significant regulation of 137 genes in *P. fluorescens* induced by the supplementation with 10 mM of Ca^{2+}.

To correlate the biofilm formation capacity of *Pseudomonas* spp. with the formation of blue pigment, Rossi et al. (2018) performed the crystal violet assay in 96-well polystyrene microplates and evaluated pigmentation *in vitro* (using *Pseudomonas* Isolation Agar, Potato Dextrose Agar and Mascarpone Agar culture media) and *in situ* (fresh mozzarella cheese discs) after incubation at 10 °C for 20 days and at 30 °C for 5 days. Among 16 blue pigment- producing strains of *P. fluorescens*, 11 also produced more biomass in the biofilm. The authors indicated that this correlation between pigmentation and *P. fluorescens* biofilm may be related to the "Blue Mozzarella event" (RASFF, 2010), although more studies are needed to better understand this phenomenon. Therefore, knowledge of *Pseudomonas* deterioration and resistance methods in the dairy industry shows the need to investigate control methods to avoid economic losses and waste.

6 Control methods

Aspects such as the production of thermostable hydrolytic enzymes, the blue discoloration, and the production of biofilms are characteristics of *Pseudomonas* spp. that can cause great losses in the food industry. The deterioration of food by microorganisms causes serious economic and social impacts due to the loss of billions of dollars worldwide, with more than 100 tons of dairy products being wasted every year because they are lost even before reaching commercialization (Quintieri et al., 2021). The socioeconomic impact and food insecurity caused by the COVID-19 pandemic have further increased the global demand for urgent solutions to reduce food loss and waste (Iranmanesh et al., 2022). Hence, methods of control and inhibition of microorganisms, such as members of the *Pseudomonas* genus, become even more necessary and urgent to be explored by researchers to find ways to reduce the negative impacts on the food industry.

The practice of thermal treatment in dairy products has been used for more than a century, with the pediatrician Henry Koplik being one of the pioneers in the thermal treatment of milk for babies in 1889 (Steele, 2000). Heat treatments, as well as the use of additives, are considered conventional methods currently used in the dairy industry. However, there is currently a need to seek alternative methods that are sustainable and efficient, the so-called "eco-friendly" methods.

6.1 Conventional methods

Heat treatment methods for raw milk are carried out to combat microbiological contamination, such as pasteurization and UHT treatment. However, even though these methods can inactivate microorganisms, some of them can produce enzymes capable of resisting these methods, which can cause reduced shelf life of dairy products and unpleasant taste (Stoeckel et al., 2016). In addition, studies report the development of blue pigment-producing *Pseudomonas* in foods that have already undergone heat treatment processes. This post-contamination can occur due to the presence of spoilage microorganisms on industrial processing equipment (del Olmo et al., 2018).

Pasteurization is a thermal treatment widely used in the food industry to eliminate heat-sensitive contaminating microorganisms. The name "pasteurization" was given in reference to the French scientist Louis Pasteur who, in the 19th century, became a pioneer in the investigation of the use of heat treatment as a preservation method (Bordenave, 2003). The best-known methods currently consist of varying the time and temperature used. The process known

as HTST (High Temperature, Short Time) consists of submitting food to a minimum temperature of 72 °C in a period of 15 s. The UHT method, on the other hand, consists of using higher temperatures, up to 150 °C, in a period with a duration of about 2 s (Qi et al., 2015). However, studies show the disadvantages of heat treatment of products such as milk, including damage to the protein quality of industrially treated milk (Cattaneo et al., 2008; Krishna et al., 2021; Morales et al., 2000).

Besides the use of heat treatments, the addition of synthetic preservatives to food products such as cheese is a strategy used to extend shelf life by preventing microbial deterioration. Additives may also be related to the intention of intensifying the flavor and improving the texture of the food, the most used being sodium acetate, sodium benzoate and potassium sorbate (Lima et al., 2021). The additives used must be permitted by law, since adulteration with prohibited products, such as hydrogen peroxide and salicylic and boric acids, can endanger the health of consumers (Hussain et al., 2020; Silva et al., 2012; Singh and Gandhi, 2015). Even in the face of risks and prohibitions, high demand and the need to generate profits increase fraudulent practices, making it necessary to establish different methods for detecting these substances (Nagraik et al., 2021). Furthermore, the appropriate levels must be respected by the industries, taking into account the cytotoxicity of the compounds, as tested by Mohammadzadeh-Aghdash et al. (2018). In this study, sodium acetate, sodium diacetate and potassium sorbate were considered safe at low concentrations regarding the risk of cytotoxicity and genotoxicity in human cells, demonstrating the importance of not exceeding the limit considered safe and the need to look for methods alternative and natural.

6.2 Alternative methods

The food industry seeks to follow market trends to attract a greater number of consumers. The interest in consuming foods that are closer to natural has increased the demand for clean label products (Asioli et al., 2017). Although there is no exact definition of this term, clean label products are those that do not contain synthetic preservatives that may be harmful to health (Aschemann-Witzel et al., 2019; Maruyama et al., 2021)**.** Thus, the application of natural additives and ecofriendly methods has become the target of several studies to evaluate their effectiveness against *Pseudomonas* (**Table 2**).

Table 2. Reports of alternative methods applied in dairy products against spoilage *Pseudomonas* species.

Strategies		Target microorganisms	Sources	Main results	References
Enzymes	Chitosan-lysozyme (active packaging)	*P. fluorescens*	Mozzarella cheese	0.40 to 1.40 log CFU/g reduction during 14-day storage	Duan et al. (2007)
	Pepsin-digested bovine lactoferrin	*P. fluorescens*	Mozzarella cheese	Neutralizing blue discoloration and delaying microbial growth	Caputo et al. (2015)
	Lactose oxidase	*P. fragi*	Pasteurized and raw milk	Bactericidal activity and reduced to undetectable concentrations within 24 h	Lara-Aguilar and Alcaine (2019)
	Lactose oxidase	*P. fluorescens*, *P. lundensis* and *P. fragi*	UHT milk	Gelation delay for 6 months	Rivera Flores et al. (2021)
Lactic acid bacteria and metabolites	*Lactobacillus acidophilus* producing hydrogen peroxide	*P. fragi*	Steam-pasteurized low-fat milk	Growth retardation under continuous agitation at 4 °C	Collins and Aramaki (1980)
	Nisin Z (*Lactococcus lactis* W8)	*P. fluorescens*	Pasteurized milk	Reduced to an undetectable level at 20 h	Mitra et al. (2011)
	Carnobacterium spp.	*P. fluorescens*	Ricotta *fresca* cheese	Reduction of 1.28 and 0.83 log CFU/g after 14 and 21 days, respectively	Spanu et al. (2018)
	Lacticaseibacillus rhamnosus	*P. fluorescens* and *P. putida*	Reconstituted powder milk	Growth retardation regardless of initial inoculum load (low, medium and high)	de Alcântara et al. (2019)
	Nisin-producing *Lactococcus lactis* (active packaging)	*Pseudomonas* spp.	Pastry cream (made with UHT-treated semi-skimmed milk)	6.36 log CFU/mL reduction after 20 days	Settier-Ramírez et al. (2021)
Bacteriophages	Cocktail with *Pedoviridae*, *Cystoviridae* e *Leviviridae*	*Pseudomonas* cocktail (*P. fragi*, *P. libanensis*, *P. veronii*, *P. psychrophila*, *P. rhodesiae*, *P. koreensis* and *P. gessardii*)	UHT milk	2 log CFU/mL reduction and delay of log phase growth at 4 °C and 25 °C	Hu et al. (2016)
	Podoviridae (HU1)	*P. lactis*	Raw milk	Viable cell numbers decreased by 1,000-fold	Tanaka et al. (2018)

Continuation of Table 2. Reports of alternative methods applied in dairy products against spoilage *Pseudomonas* species.

Strategies		Target microorganisms	Sources	Main results	References
Bacteriophages	*Podoviridae* (UFJF_PfDIW6 and UFJF_PfSW6)	*P. fluorescens*	Raw milk	Reduction in 3 log CFU/mL and inhibition in at least 2 days in the production of proteases	Nascimento et al. (2022)
Essential oils	*C. zeylanicum* (cinnamon bark) and *T. vulgaris* (thymus)	*P. fluorescens*	Mozzarella cheese	Inhibition with MIC values of 0.098%-0.195% and 0.195%-0.391% v/v	Pedonese et al. (2017)
	Origanum vulgare (Oregano)	*P. fluorescens*	Mozzarella cheese	Inhibition of biofilm formation and alteration of cell motility at 10 °C	C. Rossi et al. (2018)
High-pressure	150, 200 or 250 MPa	*P. fluorescens*	Raw milk	Undetectable levels at 200 MPa	Hayes et al. (2005)
	500 MPa	*Pseudomonas* spp.	Starter-free fresh cheese	Bactericidal activity and increased cheese shelf life from 7/8 days to up to 21	Evert-Arriagada et al. (2014)
	600 MPa	*Pseudomonas* spp.	Raw milk	Reduction below the detection limit and increased shelf life by 7 days compared to pasteurized milk	Stratakos et al. (2019)
Ozone	Gas and aqueous solution	*Pseudomonas* spp.	High moisture Mozzarella cheese	It was unable to reduce contamination and recover products with high microbial load. However, the reduction of 4 log CFU/mL in the cooling water demonstrates the usefulness of the method in the manufacturing steps.	Segat et al. (2014)
Ultraviolet radiation	200–280 nm, 6.0 kJ/m^2	*P. fluorescens* and *P. putida*	Fiordilatte cheese	Reduction of 1-2 log CFU/g and 80% shelf-life extension	Lacivita et al. (2016)
	254 nm, 6.54 J/cm^2	*P. fluorescens*	Ricotta cheese	Reduction of 1-3 log CFU/g and 50% shelf-life extension	Ricciardi et al. (2020)

MIC: Minimum Inhibitory Concentration

Continuation of Table 2. Reports of alternative methods applied in dairy products against spoilage *Pseudomonas* species.

Strategies		Target microorganisms	Sources	Main results	References
High-intensity light pulses (HILP)	200-1100 nm, frequency 3 Hz; pulse width of 360 µs; fluence 1.17 J/cm^2 /pulse	*P. fluorescens*	Mozzarella cheese	Reduction of 1 log CFU/g	Lacivita et al. (2018)
X-Ray irradiation	2 and 3 kGy	*Pseudomonas* spp.	Fiordilatte cheese	Complete inhibition. Increased shelf life by about 30 days	Lacivita et al. (2019)
	2 and 3 kGy	*Pseudomonas* spp.	Ricotta cheese	Complete inhibition. Increased shelf life by about 44 days	Ricciardi et al. (2019)
Light-emitting diode (LED)	460–470 nm (blue)	*P. fluorescens*	Packaged sliced cheese	Reduction of 3.60 log CFU/g after 7 days of treatment at 4 °C	Hyun and Lee (2020)
	λmax = 450 nm (blue) (LED + curcumin)	*P. fluorescens*	Minas Frescal cheese	Lower bacterial count and lower proteolysis in curcumin-treated samples	Saraiva et al. (2021)
Pulsed Electric Field	139,4 kJ/L, 2 µs pulse width, 200 pulses per second	*P. fluorescens*, *P. fragi* and *P. putida*	UHT milk	Reduction of 5 log CFU/mL at 55°C and shelf life extended by at least 8 days at 4°C	Craven et al. (2008)
	107 kJ/L, 3 µs pulse width, 5 to 100 pulses per second	*P. fluorescens*	Skim milk	Reduction of 3 log CFU/mL	Fernández-Molina et al. (2006)

6.2.1 Enzymes

The biocontrol is associated with the use of methods that allow consumers to safely consume fresh food. These methods may include microorganisms, plants and their metabolites, as well as enzymes and active packaging (**Table 2**). An example is bovine lactoferrin, which is a protein isolated from milk already known for its antibacterial ability against pathogenic microorganisms since the 1970s. The study by Law & Reiter (1977) demonstrated the bacteriostatic activity of active lactoferrin from bovine whey against *Escherichia coli*. Its effectiveness as an antimicrobial agent has also been investigated against food spoilage bacteria such as *Pseudomonas* genus, being able to inhibit microbial growth that deteriorates cheese, in addition to preventing the blue discoloration of mozzarella cheese caused by *P. fluorescens* (Caputo et al., 2015; Quintieri et al., 2012, 2013). The principle of action of lactoferrin is based on strong binding to free iron, which makes it unavailable for microorganisms that need iron to develop, such as *Pseudomonas* (Patil et al., 2022).

The enzymatic preservation method, therefore, has become one more alternative to prolong the shelf life of dairy products. When evaluating the activity of lactose oxidase in pasteurized milk and raw milk, Lara-Aguilar & Alcaine (2019) observed a reduction in the count of *P. fragi* and suggested that this enzyme is an alternative source of hydrogen peroxide that acts in the activation of the lactoperoxidase antimicrobial system. This enzyme was also effective in delaying the gelation of raw and sterile UHT milk caused by the activity of heat-resistant enzymes produced by different strains of *Pseudomonas* spp. isolated from facilities and dairy products (Rivera Flores et al., 2021).

The enzymes can be incorporated into food packaging through association with natural compounds, as was done with mozzarella cheese by Duan et al. (2007), who used chitosan-lysozyme as an active compound to inhibit *P. fluorescens*. Chitosan is a polymer derived from chitin that is part of the exoskeleton of crustaceans (Duan et al., 2008). Its principle of action has been associated with changing the permeability of the membrane, in addition to being able to form a polymeric film on the cell surface, causing the death of the microorganism (Macedo et al., 2022). Chitosan is even more effective in association with lysozyme, which is a naturally occurring enzyme derived from bacteria, plants, eggs or animal secretions (Lopes et al., 2019). Therefore, antimicrobial enzymes have proven efficacy, being a promising eco-friendly method. As with most proteins or enzymes, conformational changes can alter its activity, which makes it a challenge for researchers to seek strategies to circumvent its stability, such as using nanoencapsulation (Balcão et al., 2013).

6.2.2 Lactic acid bacteria and their metabolites

It has been shown that the active packaging method of preservation was also effective with the use of microorganisms and their metabolites, such as lactic acid bacteria (Settier-Ramírez et al., 2021). These microbial group had already been reported as effective bio-preservatives against *Pseudomonas* spp. in dairy products (**Table 2**). Nisin, for example, is a heat-stable antibacterial peptide produced by *Lactococcus lactis* approved for use in food preservation. Mitra et al. (2011) demonstrated the effectiveness of nisin Z from *L. lactis* W8 against *Pseudomonas*, which can replace the commercial nisin that is not effective against Gram-negative bacteria and in whole milk. However, the use of nisin is limited due to its instability at neutral pH, in addition to its effectiveness being influenced by the food matrix. Nisin can be adsorbed to fat globules, reducing their effectiveness. Furthermore, caseins can absorb nisin, decreasing its antimicrobial activity (Ibarra-Sánchez et al., 2020).

The ability of lactic acid bacteria to produce antimicrobial compounds enables their use as protective cultures in dairy products, as demonstrated by Spanu et al. (2018). In this study, *Carnobacterium* spp., known for producing bacteriocins, was artificially inoculated into ricotta cheese along with *P. fluorescens*. The results showed that *Carnobacterium* spp. significantly reduced the growth of *P. fluorescens* by 1.28 log after 14 days and by 0.83 log after 21 days of refrigerated storage. Similarly, de Alcântara et al. (2019) evaluated the inhibitory effect of *Lactobacillus rhamnosus* against *P. fluorescens* and *P. putida* in reconstituted powdered milk, observing that the production of organic acids was responsible for the delayed growth of these spoilage bacteria, regardless of the initial inoculum load.

It is important to emphasize that the efficacy of protective cultures depends on storage conditions, such as temperature, pH, moisture content, and the composition of the dairy product. Lactic acid bacteria may not grow or produce antimicrobial compounds under adverse or non-ideal conditions (Silva et al., 2018; Sionek et al., 2024). Additionally, protective cultures can affect the sensory characteristics of products through the production of organic acids and other metabolites, altering flavor, aroma, and texture (Carminati et al., 2015). These challenges highlight the need for continuous research and optimization to ensure the efficacy and economic viability of lactic cultures as protective cultures in dairy products.

6.2.3 Bacteriophages

The use of bacteriophages as preservatives in dairy products is also investigated. Bacteriophages are viruses that infect bacteria, presenting different modes of replication. Lytic bacteriophages, such as T4, insert their genetic material into the bacterial cell, using its

machinery to produce new viruses and eventually rupturing the cell to release the new viral particles. In contrast, lysogenic bacteriophages, such as T1, incorporate their DNA into the host bacteria's genome, replicating alongside it without causing its immediate destruction. Under certain conditions, this integrated viral DNA can activate, entering the lytic cycle and resulting in the production of new viruses and consequently causing cell lysis (Rogovski et al., 2021; Tanaka et al., 2018).

The use of bacteriophages has shown promise against *Pseudomonas* that contaminate milk, remaining active at neutral pH, similar to the pH of milk, and being subsequently inactivated during pasteurization (**Table 2**). However, studies highlight the disadvantages and problems that can arise with the constant use of phages in the industrial environment. First, lytic phages should be used, as the use of temperate phages can lead to an increase in the rate of bacteria transferring their virulence factors. Furthermore, the effectiveness of the bacteriophage may be reduced due to the possibility of selection of phage resistant mutants (Sommer et al., 2019; Sulakvelidze, 2013). The truth is that the biggest challenge for adding a virus to food is for the industry to face the consumer perception of it.

An alternative to the use of bacteriophages in food is the application of endolysins, which are enzymes encoded by bacteriophages that target bonds in the peptidoglycan structure of the bacterial cell wall. Although they are effective against Gram-positive bacteria, their action is limited against Gram-negative bacteria due to the outer membrane (Chang, 2020). However, Zhang et al. (2022) evaluated the use of endolysins against Gram-negative bacteria responsible for food spoilage. The study utilized an endolysin in which the *LysSTG2* gene from the lytic bacteriophage *STG2* of *Salmonella* was cloned into the *pET29b* vector and expressed in *E. coli*. The use of *E. coli* transformed with *pET29b-LysSTG2* resulted in the production of an endolysin that showed potential in controlling planktonic cells and biofilms of *Pseudomonas*.

6.2.4 Essential oils (EO)

With the tendency to use eco-friendly preservation methods, plants become attractive for use in dairy products, being carriers of nutraceuticals and with antimicrobial activities. The use of plants in food is traditional throughout human history for their therapeutic, medicinal and aromatic properties (El-Sayed and Youssef, 2019). One of the methods being explored by researchers is the use of essential oils, which can be incorporated into food products. Essential oils are secondary metabolites synthesized by almost all plant organs, mainly flowers, leaves, seeds, stems, and fruits, that play an important role in plant adaptation and protection. The International Organization for Standardization (ISO) defines essential oils as products obtained

from natural raw material of plant origin through vapor distillation, as well as products obtained by pressing citrus epicarps. There are several techniques available for extracting essential oils, including hydro-distillation, steam distillation, hydro-diffusion, and solvent extraction (Sharma et al., 2021).

For centuries, man has traditionally used EO for their pleasant odor, their taste or their antiseptic and/or preservative properties (Falleh et al., 2020; Tiwari and Rana, 2015). The antimicrobial properties due to the natural volatile compounds of EO have been tested on a wide range of microorganisms and reported in different studies (Chouhan et al., 2017; Murbach Teles Andrade et al., 2014; Smith-Palmer et al., 2001). Thus, it was possible to consider its use for the elimination of contaminating microorganisms in the food industry.

In addition to the need to avoid the use of synthetic preservatives in food, the increase in microbial resistance to traditional antimicrobial agents has aroused interest in better understanding the compounds and mechanisms of action of essential oils. The composition and functional groups of EO are factors that determine their activity, with terpenes and terpenoids as the main groups (Chouhan et al., 2017). Terpenes are hydrocarbons produced by combining various isoprene units. From acetyl-CoA, terpenes are synthesized through the mevalonic acid pathway, which occurs in the cytoplasm of plant cells. Terpenoids, on the other hand, occur by enzymatic biochemical modifications of terpenes, with modifications or removal of methyl groups and the addition of oxygen molecules. Examples of these compounds include menthol, limonene, eugenol, geraniol, cinnamyl alcohol, carvacrol, thymol, linalool, citronellal, and piperitone (Bakkali et al., 2008).

Studies report that EO act on the cell surface through their bioactive components that bind and penetrate the phospholipid bilayer of the cell membrane, which can impair its integrity (Bajpai et al., 2013). The consequent change in membrane permeability leads to the loss of vital intracellular contents such as proteins, reducing sugars, ATP and DNA, while inhibiting energy generation by the ability to inhibit ATP synthase activity, which leads to cell destruction and leakage of electrolytes (Issa et al., 2019; Lakehal et al., 2016). In addition, essential oils can act to induce bacterial oxidative stress with the production of reactive oxygen species (ROS) (Khan et al., 2017).

As presented in **Table 2**, EO were tested against bacteria belonging to *Pseudomonas* genus in the dairy matrix. The results showed the inhibitory potential of this plant metabolite. Rossi et al. (2018) evaluated the effects of *Origanum vulgare* L. EO (oregano) on biofilm formation and eradication, motility, and blue pigment production at 10 °C by *P. fluorescens* isolated from mozzarella cheese. The chemical composition evaluated by gas-chromatography-

mass spectrometry (GC/MS) showed carvacrol as the main component (69.1%). Minimum inhibitory and bactericidal concentrations ranged between 10 and 40 μL mL^{-1}. By confocal microscopy, it was possible to observe damage in cells stained with propidium iodide, in addition to a significant reduction in biofilm formation and thickness due to cell detachment. Bacterial motility was also affected by the presence of oil at a concentration of 5 μL mL^{-1}.

Bergamot, cinnamon, manuka and thyme EO were tested by Pedonese et al. (2017) to evaluate the antibacterial activity against strains of *P. fluorescens* isolated from mozzarella cheese with blue discoloration and strains of *Staphylococcus aureus* that produce biofilm isolated from milk. In *P. fluorescens*, the EO of thyme (carvacrol 30%, pcymene 20%, thymol 15%) and cinnamon (cinnamaldehyde 55%) exhibited the best activity with MIC (Minimum Inhibitory Concentration) values of 0.098%-0.195% and 0.195 %-0.391% v/v, respectively. The EO of manuka (leptospermone 23%) and thyme (carvacrol 30%, pcymene 20%, thymol 15%) showed the highest antibacterial activity against *S. aureus*. It is possible to identify in previous studies (Burt, 2004; Dorman and Deans, 2000; Shan et al., 2007) that Gram-positive bacteria may be more sensitive to essential oils and other antibacterial compounds than Gram-negative bacteria. The fact that Gram-negative bacteria have an outer membrane and the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict the entry of hydrophobic compounds into the cell may explain this lower sensitivity (Mith et al., 2014).

In addition to the challenge of Gram-negative bacteria being intrinsically more resistant to antibacterial compounds, when using essential oil as food components to fight spoilage microorganisms, food industries need to be aware of the fact that they have low water solubility, high volatility, low bioavailability and strong influences on the taste and aroma of food (Sharma et al., 2022). Therefore, EO can be preserved during their storage and processing in food through encapsulation in nanoemulsions. The nanometer size increases the effectiveness of the dosage to be used due to the increased surface area and stability. In addition, nanoemulsions slowly release EO components, make oils less volatile, and protect against environmental interactions (such as light, oxygen, humidity, pH), which ensures that the flavor of the food is preserved and the shelf life is extended. The better accessibility of EO to the microbial cell membrane makes it easier to compromise the integrity of the phospholipid bilayer or the active transport proteins incorporated into it (Barradas and de Holanda e Silva, 2021; Dima and Dima, 2015). Nanoemulsions are generally composed of three constituents, comprising an oil phase, an aqueous phase, and a surfactant (Pathania et al., 2018). Conventionally, the term "nanoemulsion" is used for dispersed droplets with a size between 100 and 200 nm (Amiri-Rigi et al., 2023).

Cumin essential oil nanoemulsion was tested by El-Sayed and El-Sayed (2021) as a natural antimicrobial agent in white cheese. In this study, its effectiveness against Gram-positive and Gram-negative pathogens was proven through the formation of an inhibition halo which varied from 3 to 26 mm. Thus, the nanoemulsion of different EO becomes a promising alternative to be tested against *Pseudomonas*, since there is still a lack of studies that evaluate EO against spoilage and in food matrices.

6.2.5 High pressure

Still looking for methods of inactivating microorganisms that do not use synthetic preservatives, physicochemical methods are applied to inactivate deteriorating bacteria such as *Pseudomonas* (**Table 2**). Oliveira et al. (2018) evaluated the use of high-pressure homogenization to reduce milk proteolysis caused by *P. fluorescens* protease. As a result, they observed reductions of 29% and 51% in proteolysis in standard milk with 3% fat in samples processed at 100 and 150 MPa, respectively. However, in skimmed milk, there was no protease inactivation in samples processed up to 150 MPa. Meanwhile, Stratakos et al. (2019) applied 600 MPa for 3 min to raw milk, observing significant reductions in *Pseudomonas* spp. counts, thus extending the microbiological shelf life of the milk by 1 week compared to pasteurized milk.

Some studies demonstrate the resistance of peptidases to high-pressure treatments, as observed by Pinto Júnior et al. (2017). The peptidases secreted in UHT milk artificially contaminated with *P. fragi* showed low susceptibility to the treatment, as a maximum inactivation of only 14% was achieved after treatment at 550 MPa for 15 min or 450 MPa for 20 min at 25°C. The authors attributed this result to the characteristics of the matrix, such as the presence of fat, and the conformational properties of the enzymes.

High-pressure processing is an example of a commercially used physical method. It has the advantage of not using high temperatures, allowing the preservation of the flavor of the food, in addition to minimizing the loss of nutrients (Stratakos et al., 2019). However, some disadvantages of applying high pressure to food limit its use. Physicochemical characteristics of milk, for example, can be compromised due to disruption of casein micelles. This is due to the effect of pressurization on protein conformation. Furthermore, as it requires water as a pressure transfer medium, this technology is not applicable to foods such as powdered milk and flour. The low efficacy against bacterial spores is also an important limitation to be considered (Huang et al., 2017).

6.2.6 Ozone

Another example of a physicochemical method of microbial inactivation used in the food industry is ozonation. Ozone is a highly effective oxidizing agent capable of diffusing through cell membranes. It decomposes into non-toxic products and thus does not leave residues in food and the environment (Guzel-Seydim et al., 2004). In the dairy industry, ozone has been effective in removing biofilm-forming bacteria and processing dairy products (Varga and Szigeti, 2016). Panebianco et al. (2022a) evaluated the effect of gaseous ozone at 50 ppm for 6 h on the inhibition and eradication of biofilms formed by 21 strains of *Pseudomonas* spp. isolated from dairy environments. Ozone demonstrated efficacy in inhibiting the biofilm in 13 of the tested strains, but no biofilms were eradicated. As an alternative to enhance the use of ozone, Tirpanci Sivri et al. (2023) evaluated the application of the Cleaning-in-place (CIP) process followed by an aqueous ozone stage (10 ppm for 10 minutes) on smooth and rough surfaces. This treatment proved effective, reducing the population of *P. fluorescens* biofilm cells to 0.9 CFU/100 cm² on smooth surfaces and 1.4 CFU/100 cm² on rough surfaces.

Some studies have sought to compare the antimicrobial activity of gaseous ozone and ozonated water. Segat et al. (2014) demonstrated that, although gaseous ozone did not show efficacy in the microbiological decontamination of cheese surfaces, the cooling water used in the mozzarella cheese production chain, when ozonated, resulted in a reduction of approximately 4 log CFU/mL of *Pseudomonas* spp. In turn, Okolo et al. (2023) evaluated the use of gaseous ozone and ozonated water through two different systems: the microbubble generator (MB) and the porous stone diffuser (PSD). The results indicated that ozonated water, both through the MB and PSD systems, was effective in removing *P. paracarnis* biofilm on stainless steel under static conditions.

However, ozone presents certain disadvantages. It can compromise the stability of high-fat foods due to its oxidative properties and is unstable under elevated temperature and pH conditions. Additionally, its antimicrobial effectiveness decreases in the presence of organic matter, which can act as a barrier. Moreover, when used in high concentrations, ozone can lead to equipment corrosion, which poses a challenge for long-term industrial use (Panebianco et al., 2022b).

6.2.7 Electromagnetic waves

Electromagnetic waves are oscillations of electric and magnetic fields that propagate through space, carrying energy. They are generated by moving charged particles and can travel through a vacuum, air, and other media. These waves have been studied for their promising

antimicrobial activity, which vary according to frequency and intensity (Yadollahpour et al., 2014). Examples of electromagnetic waves include ultraviolet (UV) radiation, visible light, and X-rays. All of them are part of the electromagnetic spectrum, classified based on their wavelengths and frequencies (Aydogan, 2014).

The photodynamic inactivation phenomenon was first observed to be effective over a century ago (Downing, 1878), leading to the use of technologies such as UV light as a decontamination method (Kim and Kang, 2018). UV radiation is part of the electromagnetic spectrum and has a shorter wavelength than visible light, ranging from 100 to 400 nm (Dai et al., 2012). The inactivation of microorganisms by UV irradiation occurs mainly through the formation of pyrimidine dimers in the strands of nucleic acids, which causes DNA deformations and interferes with replication and transcription, resulting in cell death (Ou and Petersen, 2021).

To control surface contamination and extend the shelf life of Fiordilatte cheese, Lacivita et al. (2016) tested UV light with an irradiance of 20 W/m² on the cheese surface, treating the samples for progressively longer times, up to 750 s. As a result, they observed a reduction of approximately 1–2 log cycles in *Pseudomonas* spp., along with an 80% extension of the cheese's shelf life. Additionally, no changes in the product’s appearance, texture, or color were detected. The effectiveness of this method was also confirmed by Ricciardi et al. (2020), who reported a reduction of 1–3 log cycles in *P. fluorescens* in ricotta cheese, as well as an 80% increase in shelf life.

Another method for using photodynamic inactivation is through High-Intensity Light Pulses (HILP). This method can employ a wide range of wavelengths, covering various parts of the electromagnetic spectrum, as demonstrated by Lacivita et al. (2018). In this study, a HILP system was used with a xenon lamp that emits light with wavelengths between 200 and 1100 nm, including UV to infrared. The method was applied to mozzarella cheese with a frequency of 3 Hz, pulse width of 360 µs, and fluence of 1.17 J/cm²/pulse. As a result, a reduction of 1 log CFU/g of *P. fluorescens* was observed in the inoculated mozzarella after 4 s of exposure.

X-rays are also a type of electromagnetic wave, classified as ionizing radiation, with wavelengths ranging from 0.01 to 10 nm (Ou et al., 2021). Due to their high penetration capability, provided by their shorter wavelength and higher energy compared to UV radiation, Lacivita et al. (2019) evaluated the antimicrobial activity of X-rays with doses of 2 and 3 kGy on packaged Fiordilatte cheese. As a result, complete inhibition of *Pseudomonas* spp. was observed, along with an extension of the cheese's shelf life by approximately 30 days. Similar results were obtained by Ricciardi et al. (2019), who applied the same X-ray dose to ricotta

cheese, resulting in complete inhibition of *Pseudomonas* spp. and an extension of approximately 44 days in shelf life.

Although UV light, HILP, and X-rays have proven efficacy in decontamination processes, these technologies face certain limitations. In the case of X-rays, while irradiation does not induce radioactivity in food nor leave harmful or toxic radioactive residues, consumer acceptance can still be a challenge due to the negative perception associated with irradiated food, which may impact its widespread use (Mshelia et al., 2023). Additionally, the use of UV light presents potential health risks to industry workers, such as skin and eye damage from prolonged exposure, leading to the search for safer alternatives (Csapó et al., 2019).

This concern for operator safety, combined with economic and environmental advantages, has driven the adoption of visible wavelength light-emitting diodes (LEDs) as a promising decontamination method. The benefits of LEDs include lower costs, higher energy efficiency, and greater durability, without posing the same radiation risks to workers. These characteristics make LEDs an increasingly attractive solution for the food industry, addressing both safety requirements and demands for sustainable and cost-effective methods (Lu et al., 2021).

Aiming to investigate the antibacterial activity of 460-470 nm light-emitting diode (blue) illumination, Hyun et al. (2020) tested this method against pathogens and spoilage in culture media and on packaged cheese slices. Inhibition of bacterial growth by LED $_{460\text{-}470nm}$ was more effective at 4 °C than at 25 °C, and the populations of *Listeria monocytogenes* and *P. fluorescens* were significantly reduced by 2.72 and 2.01 log CFU/mL respectively, compared to controls (total dose 286.8 J/cm^2 at 4 °C). It was observed that there were no significant differences in color between unlit and lit sliced cheese after storage for 7 days at 4 °C. It was possible to observe that the use of LED $_{460\text{-}470nm}$ associated with refrigeration caused injury to bacterial cells due to rupture of the cell membrane with the leakage of cytoplasmic content observed in transmission electron microscopy.

To better understand the effects caused by LED $_{460\text{-}470nm}$ on pathogenic and spoilage bacteria, Hyun et al. (2021) tested this method on *E. coli* O157:H7, *P. fluorescens* and *L. monocytogenes* in buffered saline solution with phosphate at 4 and 25 ° C. Again, a greater effectiveness of the inhibitory potential of LED $_{460\text{-}470nm}$ at refrigeration temperature was observed. This may be due to the increase in the proportion of unsaturated fatty acids in the bacterial membrane in response to changes in ambient temperature to maintain adequate membrane fluidity and thus becoming more sensitive to damage caused by LED (Ghate et al., 2013). The consequences of the incidence of light observed in bacterial cells were higher levels

of intracellular ROS, lipid peroxidation, membrane depolarization induced by oxidative stress, and DNA damage (Jung and Yoon, 2023).

To expand the study of the use of visible light emitting diodes against spoilage, violet (400 nm), blue (420 nm), green (570 nm), yellow (584 nm) and red (698 nm) LEDs were used against *P. fluorescens* by Angarano et al. (2020). The tests showed that only violet light could inactivate planktonic cells of *P. fluorescens*. Both violet and blue light were able to achieve high inactivation of individual cells on a surface when there was a salt stress condition in the environment. This study indicates the effectiveness of adding a violet or blue light treatment as a decontamination step in industrial settings.

Despite having advantages and proven effectiveness, the use of LED may present limitations in the industrial environment due to the characteristics of food, which may hinder the depth of light penetration due to thickness and opacity. Furthermore, large-scale use can cause the "shadow effect", where treatment can be uneven due to parts of the food that may not receive direct exposure to light and are therefore treated unevenly (Poonia et al., 2022).

6.2.8 Pulsed Electric Field (PEF)

Pulsed Electric Fields (PEF) are a non-thermal food preservation technology that utilizes short bursts of electricity to inactivate microorganisms. One of the main advantages of this technology is its ability to maintain the physical, sensory, and nutritional attributes of food, which are often altered in conventional thermal processes (Abbas Syed, 2017). The antimicrobial mechanism of PEF is based on the phenomenon of electroporation, in which cells are subjected to a critical voltage that creates pores in the cell membranes, potentially leading to cell death. Irreversible electroporation makes PEF an effective method for the decontamination of both liquid and solid foods (Arshad et al., 2021; Zhang et al., 2023).

The application of PEF was effective in the inactivation of *P. fluorescens* in skim milk, as demonstrated by Fernández-Molina et al. (2006). The average energy used to achieve a 3 log CFU/mL reduction was 107 kJ/L. The authors considered this amount of energy advantageous, arguing that it is lower than that required for heating in the HTST (high-temperature short-time) pasteurization process of milk. Craven et al. (2008) investigated the use of PEF in combination with mild heat to inactivate and control the growth of spoilage-causing *Pseudomonas* spp. in milk, aiming to extend its shelf life. With an energy input of 139.4 kJ/L, combined with heating at 55ºC, an inactivation greater than 5 log CFU/g was achieved.

Despite the proven efficacy of PEF in the inactivation of *Pseudomonas* spp. in dairy products, there are still some limitations that need to be addressed for the full utilization of this

technology in the food industry. One of the main challenges involves electrochemical reactions that occur at the interface between the electrodes and the food within the PEF treatment chamber. These reactions can result in electrode corrosion and fouling, the formation of residues, and even chemical alterations in the food (Pataro and Ferrari, 2020). Therefore, for the PEF method to become more effective, it is necessary to control these reactions to avoid negative impacts on product quality.

7 Challenges and future remarks

Despite the success of various alternative methods against *Pseudomonas* spp., such as enzymes, lactic acid bacteria and their metabolites, EO, and electromagnetic waves, each of these approaches faces specific challenges that limit their large-scale application in the food industry. For instance, although PEF preserves the sensory and nutritional qualities of products, electrochemical reactions inside the treatment chamber can cause electrode corrosion and chemical alterations in the food. Similarly, ozone, while effective and safe, has the potential to corrode equipment, despite its advantage of decomposing into non-toxic products, leaving no residues in food or the environment. Another important obstacle is consumer perception, particularly regarding the use of bacteriophages and X-rays. The addition of viruses to food and food irradiation still generates distrust, which may limit their market acceptance.

Technologies such as high pressure and LED use may have limited effectiveness depending on the type of food. High-pressure technology, for example, requires water as a transfer medium, making it impractical for products like powdered milk. The use of visible light LEDs, although a more economical and safer alternative compared to UV light, faces challenges in opaque and thick foods that hinder light penetration. In the case of EOs, the main challenges are their low water solubility and high volatility, which reduce their efficacy. Techniques such as nanoencapsulation can help overcome these limitations by increasing the stability and bioactivity of EO.

For these technologies to advance, technical improvements are essential to identify the ideal food matrices for each method and to ensure the preservation of industrial equipment. Additionally, clear regulations must be established to ensure operator safety. Greater dissemination of alternative methods could help improve consumer perception, reducing skepticism and promoting the acceptance of these technologies. Further studies that combine different technologies could enhance their effectiveness without compromising the texture or flavor of the food. This will result in safer dairy products and increase the use of eco-friendly methods as alternatives to thermal processes and the addition of synthetic additives.

8 CONCLUSIONS

Knowledge of the forms of deterioration caused by *Pseudomonas* in food products leads to the search for methods that guarantee food safety to extend the shelf life of products and reduce losses and waste. However, consumers are awakening interest in more natural products, free of synthetic additives, due to concerns about health, and sustainability, in addition to greater access to nutrients offered by food. In this way, the use of eco-friendly methods may become more and more of a trend in the food industry. This review demonstrates the proven effectiveness of several alternative methods against *Pseudomonas* spp. in dairy products, highlighting their promising potential for the food industry. However, it is essential to pay attention to the disadvantages and limitations to optimize its use. Future studies are needed to expand knowledge of the action of these methods *in vitro* and directly in the food matrix. Furthermore, it is necessary to better understand their influence on the production of blue pigment by different strains of *Pseudomonas* spp. and the mechanisms that lead to the inhibition of its cells and biofilms.

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Chapter 2: Application of cinnamon essential oil and nanoemulsions for controlling *Pseudomonas paracarnis* A006 and pigment inhibition in fresh cheese model matrix

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Application of cinnamon essential oil and nanoemulsions for controlling *Pseudomonas paracarnis* A006 and pigment inhibition in fresh cheese model matrix

Letícia Elisa Rossi[1] et al.

[1]Department of Microbiology, Federal University of Viçosa, Av. P.H. Rolfs, 365700-900, Viçosa- Brazil

ABSTRACT

The control of spoilage bacteria in dairy products, especially in fresh cheeses, is crucial for ensuring food safety and quality, stimulating the exploration of alternative methods such as the use of natural preservatives. This study evaluated the use of essential oils (EO) and nanoemulsion in inhibiting *Pseudomonas paracarnis* A006, both *in vitro* and *in situ*, using fresh cheese-mimicking matrix. Cinnamon essential oil (CEO) stood out as the most effective among the 20 oils tested, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.625 µL/mL. The main component identified by gas-chromatography-mass spectrometry (GC-MS) was cinnamaldehyde, which was also tested separately. Response surface methodology, using central composite design, optimized the ultrasound time at 4.26 min and the soy lecithin percentage at 2.79%, resulting in a nanoemulsion with an average size of 164.33 nm. In the *in situ* trials, the highest concentrations (4xMIC) of cinnamaldehyde, free cinnamon essential oil, and nanoemulsion treatments reduced bacterial growth by 2.24, 1.55, and 1.46 log CFU/g, respectively, after 7 days of storage comparing to the control model cheeses. However, the nanoemulsion showed lower antibacterial efficacy, possibly due to interaction with the protein and lipid matrix of the cheese, which contributed to the early appearance of blue spots in the model cheeses. Headspace GC-MS analysis revealed lower cinnamaldehyde release in the nanoemulsion treatment. While cinnamon essential oil proved to be an effective "clean label" preservative, its application in the form of a nanoemulsion may be limited in protein- and lipid-rich foods, such as cheese, requiring optimizations to ensure its efficacy.

Keywords: cinnamaldehyde; soy lecithin; Response Surface Methodology; antimicrobial activity; pigment inhibition; food preservation.

1 Introduction

The genus *Pseudomonas* is widely studied for its importance in the food industry and healthcare. These bacteria are aerobic, exhibit rapid growth, and have simple nutritional requirements, allowing them to survive in various environmental conditions. Some species are psychrotrophic, enabling them to grow at temperatures below 7°C, making them common in refrigerated foods (Radovanovic et al., 2020). The pigments production and hydrolytic enzymes by these bacteria is associated with food spoilage. The spoilage caused by their thermotolerant proteolytic and lipolytic enzymes reduces the shelf life of food, altering its texture, appearance, and producing unpleasant odors (Martin et al., 2011).

The spoilage of raw milk and dairy products by *Pseudomonas* is frequently favored by their high moisture content, neutral pH, abundant nutrients, and refrigerated storage conditions. Additionally, the enzymes produced by these bacteria withstand the heat treatment, which promotes the dairy spoilage (Tanaka et al., 2018). The regulation of fresh raw milk refrigeration practices immediately after milking is essential to reduce spoilage caused by the acidifying activity of mesophilic bacteria. These standards ensure the maintenance of milk quality by preventing microbial growth at an early stage. However, they can also present technological challenges, particularly related to spoilage caused by psychrotrophic bacteria, which thrive at low temperatures (Reguillo et al., 2018).

In recent years, various *Pseudomonas* species have been implicated in cheese spoilage, particularly due to causing blue discoloration. The species involved include *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas lactis*, *Pseudomonas carnis*, and *Pseudomonas paracarnis* (Carrascosa et al., 2021; Reichler et al., 2019; Rodrigues et al., 2024; Rodrigues, Machado, de Carvalho, et al., 2023). *P. paracarnis* was initially isolated and identified as a meat-spoiling bacterium in beef (Lick et al., 2021). However, phylogenetic analyses have identified isolates of this species, including *P. paracarnis* A006, in Minas Frescal cheese in Brazil, where they produce blue pigment at low temperatures (Rodrigues, Machado, Carvalho, et al., 2023).

Thermal treatment methods for raw milk, such as pasteurization and UHT (Ultra High Temperature) treatment, are carried out to combat microbiological contamination. However, heat sensitivity can trigger protein denaturation, loss of amino acids and vitamins, calcium phosphate precipitation, Maillard reaction-induced browning, and flavor changes (van den Oever & Mayer, 2021). Furthermore, studies report the growth of blue pigment-producing *Pseudomonas* after exposure to air, highlighting its potential as a contaminant in raw foods like

fresh cheeses and meats, as well as in thermally processed foods due to post-processing contamination (del Olmo et al., 2018).

New investigations into methods for controlling food spoilage microorganisms are, therefore, necessary to improve the quality of dairy products and extend their shelf life. With the growing demand for “clean label” foods, consumers are increasingly prioritizing minimally processed products with a minimal number of ingredients and free from artificial additives (Roobab et al., 2021). This shift highlights the need for alternative preservation methods that maintain product quality while avoiding the use of synthetic preservatives.

With the aim of reducing the use of synthetic preservatives and antimicrobials that may be harmful to health, the use of essential oils (EO) is promising and desirable. EO are secondary metabolites produced by plants and can be extracted from leaves, bark, seeds, flowers, and roots. Their natural origins and the fact that some possess Generally Recognized As Safe (GRAS) status make them an alternative of great interest for the food industry. (Pandey et al., 2017).

Studies report the antimicrobial activity of EO on the cell surface through their bioactive components, which bind to and penetrate the phospholipid bilayer of the cell membrane, causing its disintegration, increased fluidity, leakage of potassium ions and protons, increased levels of reactive oxygen species (ROS), as well as inhibition of enzymatic activity and cell lysis (Angane et al., 2022; Bajpai et al., 2013; Luo et al., 2022). An alternative that can further enhance the effectiveness of this method is the formulation of EO nanoemulsions, which reduces their volatility, improves access to the bacterial cell membrane, and minimizes the impact on flavor and aroma for food applications (Prakash et al., 2018).

Given the relevance of the topic and the need to find effective clean label methods to inhibit the development of blue-pigment producing *Pseudomonas* in fresh cheeses, the objective of this study was to evaluate EO and its nanoemulsion to inhibit the growth and blue pigmentation of *Pseudomonas paracarnis* A006 both *in vitro* and *in situ* conditions.

2 Materials and methods

2.1 Bacterial strain and growth conditions

P. paracarnis A006 was isolated from Minas fresh cheese that showed blue discoloration and identified by PCR and whole genome sequencing by Rodrigues, Machado, Carvalho, et al. (2023). The pigment-producing strain was maintained at −80 °C in Brain Heart Infusion (BHI) broth added with glycerol 20% (v/v).

To carry out the experiments, the bacterial suspension was prepared and standardized. Briefly, 100 µL of stock cultures were inoculated into Mueller-Hinton (MH) broth and incubated at 25 ºC for 24 h. Refreshed culture was placed in sterilized Falcon tubes and subjected to centrifugation at 4500 rpm for 10 min at 25 °C. The pellet was washed twice and resuspended in 0.85 % (w/v) saline solution and thus the bacterial suspension concentrations were monitored by measuring the optical density (OD) on a spectrophotometer (Biospectro, SP-22, Brazil) at 600 nm. The cell suspension was diluted until reaching an OD of approximately 0.200, which is equivalent to a population of 10^8 CFU/mL. The cell population of the standardized suspension was analyzed by microdroplet plating (Machado et al., 2024) on Muller-Hinton agar, followed by incubating the plates at 25 °C for 24 h.

2.2 Essencial oils (EO)

Twenty commercial EO were purchased from Laszlo (Belo Horizonte, Brazil) and Ferquima (São Paulo, Brazil). The EO from Ferquima include Cinnamon (*Cinnamomum cassia*), Clove (*Syzygium aromaticum*), Copaiba (*Copaifera officinalis*), Oregano (*Origanum vulgare*), Rosemary (*Rosmarinus officinalis*), Melaleuca (*Melaleuca alternifolia*), White thyme (*Thymus vulgaris*), Thyme bela-luz (*Thymus mastichina*), Ho Wood (*Cinnamomum camphora*), Patchouli (*Pogostemon cablin*) and two species of mint, Spearmint and Wild Mint (*Mentha spicata* and *Mentha arvensis*, respectively). The EO from Laszlo include Green coffee (*Coffea arabica*), Marjoram (*Origanum majorana*), Dalmatian sage (*Salvia officinalis*), Java citronella (*Cymbopogon winterianus*), Sweet orange (*Citrus sinensis*), Nutmeg (*Myristica fragrans*), Bay laurel (*Laurus nobilis*) and Sicilian lemon (*Citrus limon*). To carry out the tests, the EO were diluted in Tween 80 solution 1% (v/v).

2.3 Screening of EO antibacterial activity

The antibacterial activity of the 20 EO was initially tested using the agar well diffusion method, as described by Balouiri et al. (2016). The previously standardized bacterial suspensions were spread on the surface of the MH agar using a sterile swab. Then, the 6 mm wells on the agar were filled with 20 µL of each EO. For this test, all EO were diluted in Tween 80 1% (v/v) to a final concentration of 160 µL/mL. The plates were incubated at 25 °C for 24 h, and then the zones of inhibition were measured using a caliper. The tests were performed in duplicate and repeated three times. The positive control was performed with tetracycline (50 µg/mL) and the negative control with Tween 80 1% (v/v). Only the EO that presented an

inhibition halo were selected for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) analysis.

2.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The evaluation of EO was carried out using resazurin, based on the method described by Nima et al. (2021), with adaptations. Briefly, in a 96-well microtiter polystyrene plate, 80 µL of MH broth (2.5 times concentrated), 100 µL of the different EO concentration and 20 µL of the cell suspension adjusted to 5.0 x 10^8 CFU/mL were added to each well. The double dilution method was used to prepare the EO in solutions of 40, 20, 10, 5, 2.5, 1.25 and 0.625 µL/mL. Tween 80 1% (v/v) and tetracycline (50 µg/mL) was used as the negative and the positive control, respectively. The plates were incubated at 25 °C for 24 h. To confirm the result, 50 µL of resazurin solution 0.1% (w/v) was added to the wells. The microtiter plates were further incubated under the dark conditions (Elshikh et al., 2016) at 25 ºC for 4 h to allow resazurin reduction to take place. The wells with no resazurin color change confirmed the MIC values. The MIC was recorded as the value of the minimum concentration of EO to inhibit the *P. paracarnis* A006. The tests were performed in triplicate and repeated three times.

Subsequently, 10 µL of the well contents without significant turbidity (before adding resazurin) and 10 µL of the wells that had EO concentrations 2 times higher than the MIC value were plated in triplicate on MH agar microdroplet method. After incubation at 25 °C for 24 h, the EO concentration of the group exhibiting no bacterial growth was recorded as the MBC value.

2.5 Characterization of the selected EO

The identification of the components of the EO with the lowest MIC and MBC was carried out using gas chromatography. The sample of EO was analyzed by gas chromatography with a flame ionization detector (GC-FID) (GC-2010 Plus, Shimadzu, Japan) and by gas chromatography coupled with mass spectrometry (GC-MS) (GC-MS-2010, Shimadzu, Japan). The following chromatographic conditions were used for both analyses: fused silica capillary column (30 m x 0.25 mm) with SH-Rxi®-5HT stationary phase (0.25 µm film thickness); nitrogen (N_2) for GC-FID analysis or helium (He) for GC-MS analysis as the carrier gas with a flow rate of 3.0 mL/min; the oven temperature program started at 40 °C for 1 min and then increased by 5°C/min until reaching 220 °C, holding at this temperature for 10 min; injector

temperature at 240 °C; detector temperature at 240 °C; split ratio of 1:30. A volume of 1.0 µL of a 2% (v/v) solution of EO dissolved in ethanol PA was injected for both GC-FID and GC-MS analyses. The GC-MS analyses were performed on a device operating with electron impact at 70 eV; scan speed of 1,000; scan range of 0.50 fragments/second and detected fragments from 29 to 400 (m/z).

The identification of EO components was carried out by comparing the mass spectra available in the spectral library database (Wiley Registry of Mass Spectral Data and NIST Mass Spectral Data) and by the Adjusted Retention Indices calculated (RIcalc) using Equation 1 with a chromatogram obtained from the injection of a C7-C30 n-alkane mixture (Sigma-USA). The calculated retention index was compared with the retention index (Eq. 1) described in the literature (R. P. Adams, 2007). Compounds with a relative area percentage ≥ 1% were considered for the EO composition study.

$$\textbf{Eq.1:} \quad \mathrm{RIcal} = 100\mathrm{n} + 100\left\{\left[\frac{(t\prime_{\mathrm{Ri}})-(t\prime_{\mathrm{Rn}})}{(t\prime_{\mathrm{Rn}+1})-(t\prime_{\mathrm{Rn}})}\right]\right\}$$

Where: **i** is the compound of interest; **n** is the number of carbon atoms in the hydrocarbon with a retention time immediately before the retention time of **i**; $\mathbf{t'_{Ri}}$ is the adjusted retention time for the compound of interest; $\mathbf{t'_{Rn}}$ is the adjusted retention time for the hydrocarbon with a retention time immediately before the retention time of **i**; $\mathbf{t'_{Rn+1}}$ is the adjusted retention time for the hydrocarbon with a retention time immediately after the retention time of **i**.

After identifying the main compound of the EO, the MIC and MBC of the compound against *P. paracarnis* A006 were also determined, as described in item 2.4.

2.6 Optimization of nanoemulsion preparation by response surface methodology (RSM)

EO nanoemulsions were prepared using soy lecithin as a surfactant, which has naturally occurring amphiphilic phospholipids (Klang & Valenta, 2011). The emulsion was made with 1% (v/v) EO, soy lecithin ranging from 0.5 to 3% (w/v) and sterile distilled water. The coarse emulsion was vortexed for 1 min and subsequently subjected to probe ultrasound (VCX 400, Sonics & Materials Inc., Danbury, CT, USA, power 400 W and frequency 20 kHz) to reduce droplet size using an ice bath to control temperature, as described by Torres Neto et al. (2023). The sonication time varied from 0.1 to 5 min. The formed EO nanoemulsion was kept at room temperature at 25 °C till use.

The parameters used for the production of the EO nanoemulsion were defined using response surface methodology (RSM) (Bezerra et al., 2008). RSM was employed using R

software (v. 4.3.1) through central composite design (CCD). The independent variables used were sonication time (X1) and lecithin percentage (X2), with 13 runs. The units and coded levels of the independent variables are shown in **Table 1**.

Table 1. Coded variables with central composite design for optimization of essential oil (EO) nanoemulsions.

Run order	X1: Sonication time	X2: Lecithin percentage
1	3 min (0)	3.41% (1.414)
2	5.83 min (1.414)	2% (0)
3	5 min (1)	3% (1)
4	3 min (0)	2% (0)
5	0.17 min (-1.414)	2% (0)
6	1 min (-1)	3% (1)
7	5 min (1)	1% (-1)
8	3 min (0)	2% (0)
9	3 min (0)	0.59% (-1.414)
10	1 min (-1)	1% (-1)
11	3 min (0)	2% (0)
12	3 min (0)	2% (0)
13	3 min (0)	2% (0)

Numbers in parentheses indicate the coded level of the factors in the experimental design.

2.6.1 Droplet size, polydispersity index (PDI) and MIC

As carried out by Hou et al. (2019), droplet size and polydispersity index (PDI) were determined by dynamic light scattering and electrophoretic mobility scattering in a Zetasizer meter (Nano ZS90, Malvern, UK). According to Gurpreet et al. (2018), to measure the zeta potential, the nanoemulsion was diluted at a 1:100 ratio by adding 10 µL to 1 mL of distilled water and its value is estimated from the electrophoretic mobility of the oil droplets.

To identify antibacterial activity, the MIC of each nanoemulsion formulation against *P. paracarnis* A006 was performed, as described in section 2.4.

2.6.2 Validation of the central composite design (CCD) model

After testing the different combinations generated by the model and obtaining the response variables, it was possible to obtain an estimate of the coefficients in a mathematical model and thus, by predicting the response, evaluate the effectiveness of the model. For two independent variables, X_1 and X_2, the quadratic regression model can be written as:

Eq. 2: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon,$

where Y is the response variable (in this case, “droplet size prediction”), β_0, β_1, β_2, β_{12}, β_{11}, β_{22} the coefficients estimated by the model, X_1, X_2 the independent variables (time and lecithin, respectively) and ε is the residual error (Bhattacharya, 2021).

2.7 Time kill assay

The bactericidal activity time of EO against *P. paracarnis* A006 was evaluated as described by Li et al. (2022), with some modifications. Briefly, 100 µL of the cell suspension with 10^8 CFU/mL was added to a 1.5 mL microtube containing 400 µL of MH broth and 500 µL of the treatments with cinnamaldehyde (C), free EO (F), and with EO in nanoemulsion (N). The tested concentrations for each treatment were 4 x MIC, 2 x MIC, MIC, and ½ MIC. The mixture was incubated at 25 °C for 30 min (T30), 90 min (T90), and 150 min (T150). Then, 20 µL of each treatment was collected and plated in microdroplets on plates containing MH agar to identify bacterial colony growth or lack thereof after 24 hours of incubation at 25 ºC.

2.8 Reactive oxygen species (ROS) assay

The intracellular ROS level was investigated by fluorescent probe as described by DU et al. (2020). The treatments chosen for this test were C 2xMIC, C MIC, F 2xMIC, F MIC, N 2xMIC, and N MIC. Cells, standardized to a concentration of 10^8 CFU/mL, were added to 1.5 mL microtubes containing MH medium along with the respective treatments. The tubes were vortexed for 1 min and kept at room temperature at 25 ºC. The tested exposure times were 30 and 150 min. As a negative control, tubes with inocula that were not exposed to the C, F, and N were prepared. After exposure, *P. paracarnis* A006 cells were washed twice by centrifugation at 8,000 rpm for 5 min and resuspended in phosphate-buffered saline (PBS) solution. After addition of 100 µL of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 10 µM, the cell suspension was incubated for an additional 1 h in the dark at 37 °C, followed by two washes with PBS (centrifugation at 12,000 × g for 5 min). The pellet was resuspended in PBS and 200 µL of each sample was transferred to 96-well microplates. Relative fluorescence intensity was detected using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, USA), at 525 nm wavelength with 488 nm excitation (Hyun et al., 2021; Mao et al., 2019). The tests were performed in duplicate and repeated three times.

2.9 Application of essential oil (EO) in cheese

2.9.1 Model cheese production

To test the antibacterial activity of EO *in situ*, the high-throughput screening method suggested by Garnier et al. (2018), using a cheese-mimicking matrix distributed in 6-well plates, was used. This matrix forms a model cheese that was inoculated with *P. paracarnis* A006. Briefly, pasteurized skimmed milk was subjected to ultrafiltration using a membrane system (HFM 180, Koch Membrane Systems Inc., USA) with a molecular weight cut-off of 80 kDa and a membrane porosity of 0.22 µm, under a temperature of 45 °C. Fresh cream containing 54% fat was added to the retentate to adjust the fat content to 20 g/kg. Additionally, sodium chloride was incorporated into the mixture at a concentration of 0.7% (w/v). The mixture was then subjected to heat treatment at 95 °C for 2 min, with constant stirring. After heating, the mixture was cooled to 45 °C for the addition of rennet (Maxiren XDS, France), previously diluted at a ratio of 1 part rennet to 5 parts sterilized distilled water.

The model cheeses were made with three different treatments: C, F and N. Three different concentrations of each treatment were tested, based on the MIC of C, F and N determined *in vitro*. Thus, the MIC (0.625 µL/mL), 2xMIC (1.25 µL/mL), and 4xMIC (2.5 µL/mL) were added to the cheese. The main compound of the EO will be added to the cheese according to the percentage it represents in the EO. As the nanoemulsion was formulated with 1% EO, the nanoemulsion amount added to the cheese was also equivalent to the EO MIC. As controls, cheeses were made with the treatments (C, F, N) without the inoculum (NIn) and cheeses inoculated without any treatment (WEO). This way, the mixture was ready to be transferred to 6-well plates, adding the different treatments and 200 µL of the *P. paracarnis* A006 cell suspension at 10^8 CFU/mL. The 6-well plates were incubated for 1 h at 30 °C and then stored at 4 °C.

2.9.2 Evaluation of cheese shelf life

2.9.2.1 Microbiological analysis

Microbiological analyses were performed at T0 and T7. A 10 g portion of cheese was aseptically removed and homogenized in 90 mL of 1% sodium citrate buffer using a Stomacher (BagMixer 400, Interscience, France) for 2 min. Serial dilutions were performed for plating on selective medium for *Pseudomonas*, the *Pseudomonas* Agar Base supplemented with cefalotin, fucidin, and cetrimide (PAB + CFC), using the microdroplet method (Machado et al., 2024) in

duplicate. PAB is composed of peptone gelatin (16 g/L), tryptone (10 g/L), potassium sulfate (10 g/L), magnesium chloride (14 g/L), lactose (10 g/L), bromothymol blue (0.02 g/L), and agar (11 g/L). The plates were incubated at 25 °C for 24 h for subsequent colony enumeration. For aerobic mesophilic counting, 1 mL of the serial dilutions was plated by pour plate method on Plate Count Agar (PCA), and incubated at 37 °C for 48 h for colony counting.

2.9.2.2 Colorimetry

The model cheeses of the different formulations (C 4xMIC, C 2xMIC, C MIC, F 4xMIC, F 2xMIC, F MIC, N 4xMIC, N 2xMIC, N MIC, In WEO, and NIn) were monitored in duplicate for pigmentation caused by *P. paracarnis* A006 at immediately after cheese production (T0) and after 7 days of storage at 4 ºC (T7). The blue pigment production was evaluated using a colorimeter (Minolta CR 400, Minolta Corporation, Osaka, Japan) to measure two random points on the surface of the cheeses. This equipment measures the CIELab color coordinates (*Commission Internationale de l'Eclairage*). The colors identified were expressed by the coordinates *L** (lightness, black to white), *a** (green to red), and *b** (blue to yellow).

The color intensity or saturation, represented by Chroma (*C**), the color hue represented by the Hue angle (*h**), and the total visible color difference (*ΔE*) were calculated according to **Eq. 3**, **4**, and **5**, respectively (Hill et al., 1997). For the *ΔE* calculations, the coordinates of the inoculated cheeses were compared with the coordinates of the non-inoculated cheeses. The *L**, *a**, and *b** coordinates for the *C** and *h** calculations refer to the inoculated cheeses.

$$\textbf{Eq. 3: } C^* = \sqrt{((a^*)^2 + (b^*)^2)}$$

$$\textbf{Eq. 4: } h^* = arctg\ (\frac{b^*}{a^*})$$

$$\textbf{Eq. 5: } \Delta E^* = \sqrt{((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)}$$

Additionally, after determining the MIC for each *in situ* treatment, the cheeses containing these concentrations were monitored through photographs every 24 h up to the 7th day of storage at 4 °C, to assess how much the shelf life was extended compared to the untreated cheese.

2.9.3 Volatile composition

The treatments with the best performance in the shelf-life evaluation, as well as the control WEO were selected for Headspace solid-phase micro extraction coupled to GC-MS. Samples of 8 g from each uninoculated cheese were added to 20 mL sealed septum glass crimp vial, and inert nitrogen gas was added to preserve the sample until analysis. The samples were

analyzed by GC-MS, according to Moro et al. (2015) with some modifications, using the GC-2010 gas chromatograph and the QP2020 mass spectrometer (Shimadzu) available at the Central Analítica – Instituto de Química - USP (IQUSP).

The samples were heated to 80 °C for 60 min to allow the volatile compounds to equilibrate in the vial's headspace with agitation. An appropriate capillary column ZB 5msHT, measuring 30 meters by 0.25 mm by 0.25 µm thickness, was used, and the injection mode was split, with the injector temperature maintained at 250 °C. The heating ramp was 2 °C/min, starting at 40 °C for 1 min up to 100 °C, and then with a ramp of 20 °C/min up to 250 °C. In the mass spectrometer, electron ionization was performed with the ion source temperature set at 250 °C at 70 eV. The identification of volatile compounds was carried out by comparing the obtained mass spectra with the reference spectra from the NIST14 library.

2.10 Statistical analyses

Statistical analyses were performed using R Studio version 1.2.2. Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was employed. When the data did not meet the assumption of normality, the non-parametric Wilcoxon test was applied. The level of significance was set at $p < 0.05$.

3 Results

3.1 Screening of essential oil (EO) antibacterial activity

From the screening using the agar well diffusion method, only 10 out of the 20 EO tested against *P. paracarnis* A006 showed antibacterial activity (**Figure 1**). The cinnamon EO (CEO) exhibited the best activity, showing an inhibition zone with a mean diameter of 18.5 ± 0.71 mm. Furthermore, using Tukey's multiple comparison test, it was the only EO that showed a significant difference ($p < 0.05$) when compared to all other tested EO (**Figure 1**). Clove EO had the second largest inhibition zone, with a mean diameter of 14.0 ± 1.41 mm, but it did not show a significant difference compared to the mean diameter of the wild marjoram EO, which was 11.5 ± 0.71 mm.

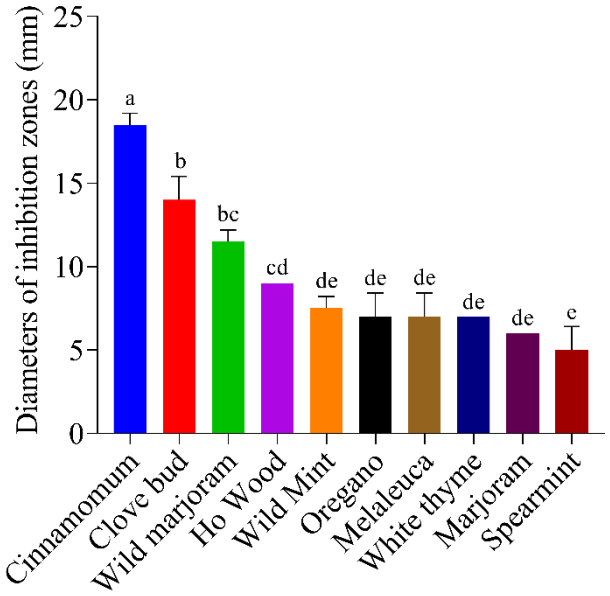


Figure 1. Diameters of inhibition zones formed by essential oils (EO) against *P. paracarnis* A006. Each bar represents the mean diameter (mm) and the standard deviations. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

The MIC and MBC data confirmed that cinnamon EO exhibited the best antibacterial activity, with the lowest MIC and MBC values of 0.625 µL/mL (**Table 2**). The second best activity was observed for clove bud EO, with a MIC of 2.5 µL/mL and an MBC of 3.333 µL/mL corroborating the results of agar well diffusion assay (**Figure 1**).

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each tested essential oil (EO).

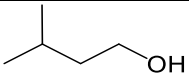
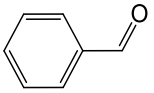
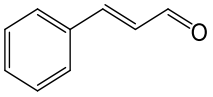
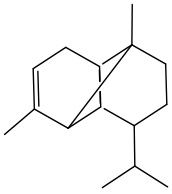
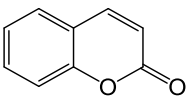
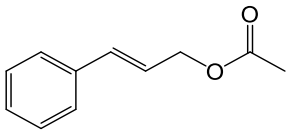
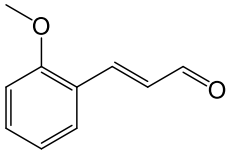
Essential oil	**MIC** (µL/mL)	**MBC** (µL/mL)
Cinnamomum	0.625 ± 0	0.625 ± 0
Clove bud	2.5 ± 0	3.333 ± 1.443
Melaleuca	5 ± 0	6.667 ± 2.887
Wild marjoram	5 ± 0	5 ± 0
Ho Wood	5 ± 0	10 ± 0
Oregano	8.333 ± 2.887	8.333 ± 2.887
Marjoram	20 ± 0	20 ± 0
White thyme	20 ± 0	40 ± 0
Wild Mint	40 ± 0	80 ± 0
Spearmint	40 ± 0	80 ± 0

Values represent mean ± standard deviation.

3.2 Characterization of the cinnamon essential oil (CEO)

The CEO, having demonstrated the best antibacterial activity against *P. paracarnis* A006, was selected to proceed with the experiment. Using GC-FID and GC-MS, seven components of the CEO were identified (**Table 3**), with cinnamaldehyde being the major component, accounting for 82.55% of the relative area (**Figure 2**). The MIC and MBC of cinnamaldehyde were tested in vitro, obtaining a value of 0.625 µL/mL.

Table 3. Identification of the chemical composition of cinnamon essential oil (CEO) by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS).

Peak*	RI-Cal	RI-Tab.**	Substance name	Structure	Relative area (%) GC-FID	Similarity (%) GC-MS
1	732	731	isopentyl alcohol		2.05	96
2	957	952	benzaldehyde		1.31	96
3	**1271**	**1267**	**cinnamaldehyde**		**82.55**	**97**
4	1380	1374	α-copaene		1.18	91
5	1424	1432	coumarin		2.05	-
6	1441	1443	cinnamil (E)-acetate		2.57	96
7	1524	1527	o-methoxy-cinnamaldehyde		8.29	96

*The peaks correspond to compounds with a relative area ≥ 1% and are listed in the order of elution using the SH-Rxi®-5HT column. RT; RICal: Retention Index calculated through data obtained from a sample of saturated n-alkanes (C7-C30); RITab: Tabulated Retention Index; Data used from the Wiley 7, NIST 05, and NIST 05s libraries.

** The tabulated indices (RITab) are from R. P. Adams (2007).

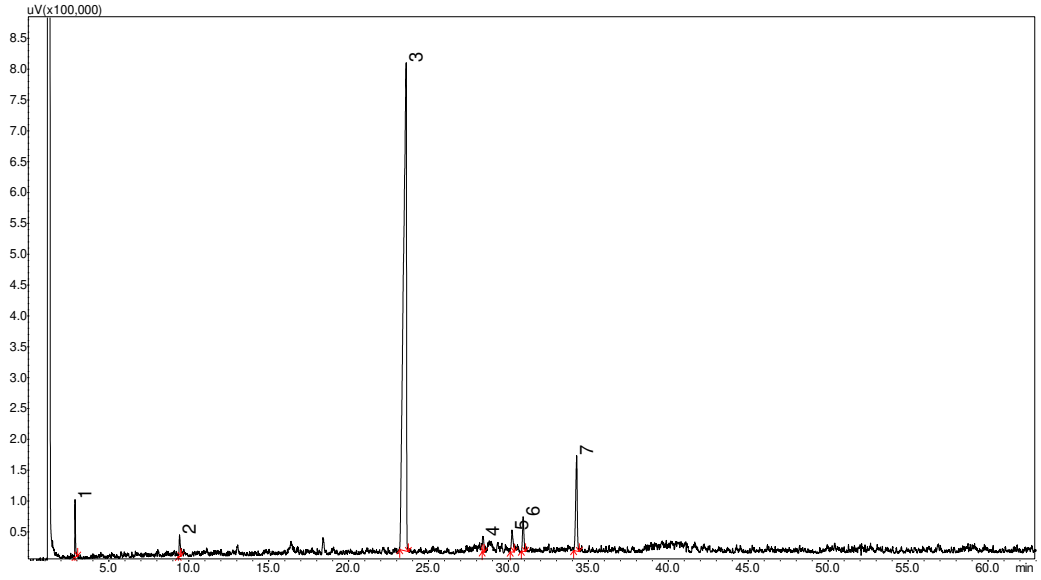


Figure 2. Chromatographic profile of cinnamon essential oil (CEO) obtained by gas chromatography with flame ionization detection (GC-FID) analysis. The numbers of each peak correspond to the following compounds: 1- isopentyl alcohol; 2- benzaldehyde; 3- cinnamaldehyde; 4- α-copaene; 5- coumarin; 6- Cinnamil (E)-acetate; 7- o-methoxy-cinnamaldehyde.

3.3 Optimization of cinnamon essential oil (CEO) nanoemulsion using response surface methodology (RSM)

The experimental design used to optimize the CEO nanoemulsion production, employing RSM through CCD, included two independent variables, sonication time (X1) and lecithin percentage (X2), and 13 runs. The MIC of each combination, the droplet size, and the PDI of the nanoemulsions measured by the Zetasizer are listed in **Table 4**. The MIC values remained constant for all formulations, while the PDI values showed no significant variations, ranging from 0.2 to 0.577. In contrast, the droplet size varied from 132.5 to 319.4 nm. Based on these results, droplet size was used as a parameter to fit a response surface model to the data.

The model coefficients revealed that the X1 had a significant negative effect on droplet size ($p < 0.05$), while the quadratic term of X1 ($X1^2$) was also significant, indicating a nonlinear relationship with droplet size. This suggests the existence of an optimal sonication time, after which droplet size begins to increase again. In contrast, the X2 did not show a significant effect ($p > 0.05$) on droplet size. Additionally, the interaction effect between X1:X2 did not have a relevant impact on the droplet size of the CEO nanoemulsions (**Table 5**).

Table 4. Experimental combinations from response surface methodology (RSM) with central composite design (CCD) for cinnamon essential oil (CEO) nanoemulsions.

Run order	X1: Sonication time	X2: Lecithin percentage	MIC (µL/mL)	Droplet size (nm)	PDI
1	3 min (0)	3.41% (1.41)	0.625	185.7	0.230
2	5.83 min (1.414)	2% (0)	0.625	164.5	0.260
3	5 min (1)	3% (1)	0.625	167.0	0.211
4	3 min (0)	2% (0)	0.625	178.3	0.214
5	0.17 min (-1.414)	2% (0)	0.625	319.4	0.577
6	1 min (-1)	3% (1)	0.625	204.8	0.316
7	5 min (1)	1% (-1)	0.625	132.5	0.224
8	3 min (0)	2% (0)	0.625	155.4	0.240
9	3 min (0)	0.59% (-1.41)	0.625	141.8	0.228
10	1 min (-1)	1% (-1)	0.625	178.2	0.322
11	3 min (0)	2% (0)	0.625	169.2	0.200
12	3 min (0)	2% (0)	0.625	171.7	0.213
13	3 min (0)	2% (0)	0.625	168.5	0.208

Numbers in parentheses indicate the coded level of the factors in the experimental design. MIC: minimum inhibitory concentration; PDI: polydispersity index.

Table 5. Regression analysis of the coefficients from the quadratic equations fitted for the droplet size of cinnamon essential oil (CEO) nanoemulsions.

	Estimate	Std. Error	p-value
Intercept	168.620	11.529	0.001 *
X1	-37.820	9.114	0.004 *
X2	15.398	9.114	0.135
X1:X2	1.975	12.890	0.882
$X1^2$	28.608	9.774	0.022 *
$X2^2$	-10.491	9.774	0.318

X1: ultrasound time; X2: lecithin percentage; *Indicates values that were significant ($p < 0.05$);

The second-order polynomial model fitted to the data presented a coefficient of determination (R^2) of 0.8149, indicating that 81.49% of the variation in droplet size was explained by the independent variables. The ANOVA indicated that the lack of fit test was significant ($p = 0.006$), suggesting that the model did not adequately capture all the variation in the data, which may indicate the need for additional terms in the model.

The second-order polynomial equation fitted for droplet size (Y) is expressed as:

Eq. 6: $Y = 168.620 - 37.820\mathbf{X1} + 15.398\mathbf{X2} + 1.975\mathbf{X1X2} + 28.608\mathbf{X1^2} - 10.491\mathbf{X2^2}$

Based on the coefficients and the ANOVA, contour plots (**Figure 3-a**) and three-dimensional (3D) surface plots (**Figure 3-b**) were generated to visualize the relationship between X1, X2, and droplet size. The contour plot identifies the regions of smaller droplet sizes, with darker colors representing the lowest values. The 3D surface plot shows the interaction between X1 and X2, revealing a minimum point for droplet size, confirming the nonlinear relationship suggested by the significant quadratic coefficient of X1 ($X1^2$).

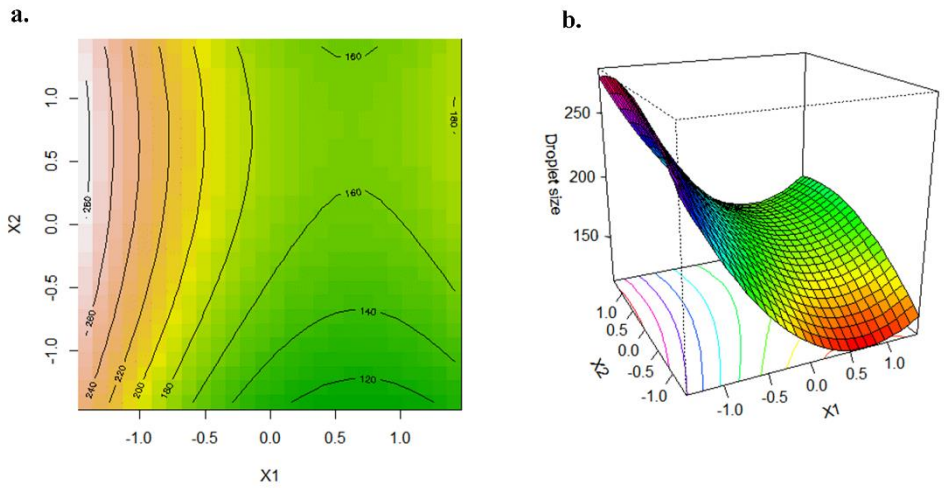


Figure 3. Two-dimensional (2D) contour plot (A) and three-dimensional (3D) response surface plot (B), showing interactions between the independent variables, sonication time (X1) and lecithin percentage (X2), in relation to droplet size (nm).

Based on the response surface analysis, the stationary point was identified at X1 = 0.633 and X2 = 0.793, corresponding to 4.267 min of ultrasound time and 2.793% lecithin, respectively.

3.3.1 Validation of the central composite design (CCD) model

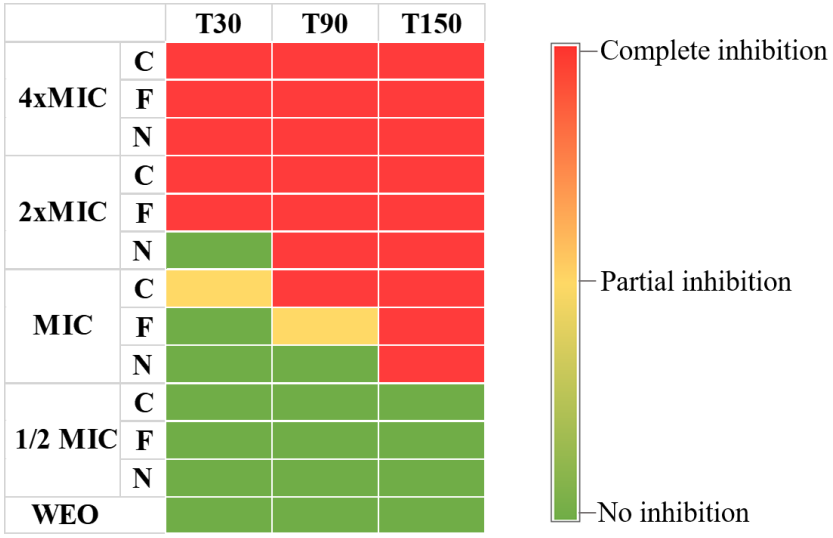
To validate the developed response surface model, we used the **Eq. 6** to predict the droplet size. The predicted size was 162.74 nm. To verify the accuracy of the model, a CEO nanoemulsion was prepared using the optimal values of lecithin percentage (2.79%) and ultrasound time (4.26 min) provided by the model. The experimentally observed average droplet size, measured with the zetasizer, was 164.33 ± 11.91 nm.

Residuals were calculated as the difference between the observed values and the predicted droplet size, resulting in a value of 1.582381. The measured droplet size diameter was close to the predicted value, indicating that the proposed model was reliable with accurate prediction results.

3.4 Time kill assay

A time-kill assay was conducted to investigate the antibacterial activity of cinnamaldehyde, free CEO, and CEO in nanoemulsion against *P. paracarnis* A006. As shown in **Table 6**, treatments C, F, and N exhibited bactericidal activity in a time- and concentration-dependent manner. While all 4xMIC treatments achieved complete inhibition from T30 onward, the ½ MIC treatments showed no inhibition at any time point. It is noteworthy that at the 2xMIC concentration, only treatment N required more time (90 min) to achieve complete inhibition.

Table 6. Times of complete, partial or no inhibition of *P. paracarnis* A006 exposed to different treatments and exposure times.



C: cinnamaldehyde; F: free cinnamon essential oil; N: cinnamon essential oil in nanoemulsion; WEO: control without treatment. MIC: minimum inhibitory concentration.

3.5 Reactive oxygen species (ROS) assay

Based on the results observed in the time-kill assay, the level of intracellular ROS was investigated using a fluorescent probe to understand the influence of time and treatments on ROS production. The 4xMIC concentration used in the time kill assay was excluded from this test because, as it showed complete inhibition from T30 (**Table 6**), it may represent a concentration so high that it could compromise the assay by causing extrusion of cellular contents. Similarly, the ½ MIC concentration was also excluded for showing no antibacterial efficacy. Regarding the time points, T30, was chosen for already demonstrating inhibition in some treatments, and T150, which was effective for all chosen treatments.

All treatments showed significantly lower levels of ROS ($p<0.05$) compared to the WEO control (**Figure 4** and **Supplementary Table S1**). Although all treatments showed a reduction from T30 to T150, the reduction was only significant for C MIC ($p = 0.016$), indicating a time-dependent effect in this specific treatment.

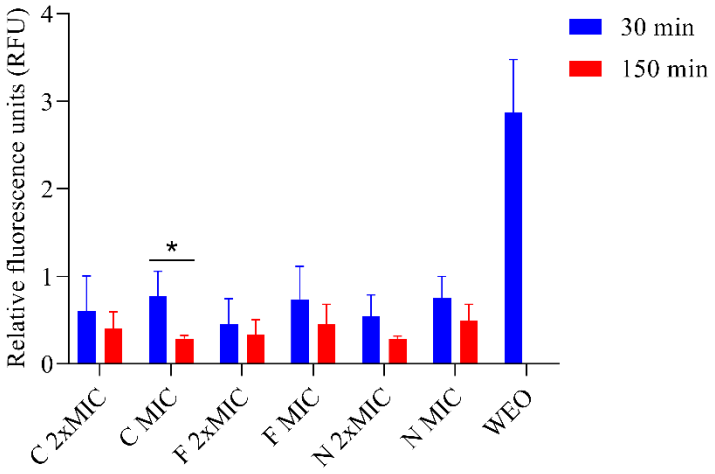


Figure 4. Impact of different treatments and times on intracellular reactive oxygen species (ROS) accumulation in *P. paracarnis* A006. Relative fluorescence units (RFU) detected as an indicator of ROS formation by the fluorescence of the DCFH-DA dye. RFU was measured with a plate reader (λex = 488 nm; λem = 525 nm). The values represent means ± standard deviation for the different treatments after 30 min (blue bars) and 150 min (red bars) of incubation. WEO represents the untreated control. C: cinnamaldehyde; F: free cinnamon essential oil; N: cinnamon essential oil in nanoemulsion; MIC: minimum inhibitory concentration. * Indicates significant difference ($p<0.05$) between times.

3.6 Application of cinnamon essential oil (CEO) in fresh cheese

3.6.1 Microbiological analysis

By using the selective culture medium PAB + CFC, it was possible to quantify, through colony counting, how much each treatment was able to inhibit the growth of *P. paracarnis* A006 inoculated in each model cheese. The ANOVA statistical analysis indicated that both treatment and time had significant effects on the logarithmic reduction of microbial counts, with $p < 0.001$ for both factors. However, the interaction between treatment and time was not significant ($p = 0.319$), suggesting that differences between times do not depend on the type of treatment applied.

Direct comparison between T0 and T7 for each treatment individually, through paired t-tests, showed that all treatments, except the WEO control, presented significant differences between times ($p < 0.05$). **Figure 5** (and **Supplementary Table S2**) presents the logarithmic

reduction (log CFU/g) of the bacteria in the different treatments analyzed at 0 (T0) and 7 days (T7). Observing the treatments with a 4xMIC concentration, cinnamaldehyde, free CEO, and nanoemulsion, all showed a significant reduction in the initial bacterial load, maintaining a considerable reduction after 7 days. The highest efficacy was observed with the C 4xMIC treatment (5.58 ± 0.92 log CFU/g at T0 to 2.24 ± 0.54 log CFU/g at T7), followed by F 4xMIC (5.21 ± 1.63 log CFU/g at T0 to 1.55 ± 0.68 log CFU/g at T7), and then N 4xMIC (4.81 ± 0.66 log CFU/g at T0 to 1.46 ± 0.55 log CFU/g at T7).

For all treatments and concentrations, there was a reduction in bacterial growth at T0. However, after 7 days, the treatments F 2xMIC, F MIC, N 2xMIC, N MIC, and the WEO control showed negative values for log reduction, suggesting possible increase in bacterial populations after an initial reduction.

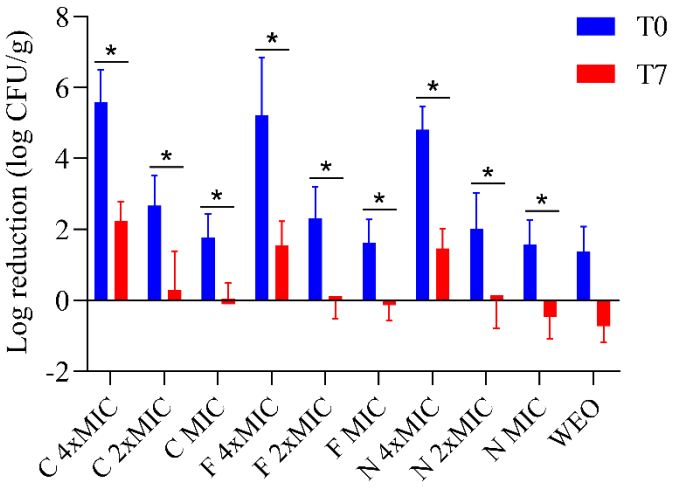


Figure 5. Logarithmic reduction (log CFU/g) in the count of *P. paracarnis* A006 inoculated in the model cheeses in the different treatments analyzed at 0 (T0) and 7 days (T7). C: cinnamaldehyde; F: free cinnamon essential oil; N: cinnamon essential oil in nanoemulsion; WEO: control without treatment. MIC: minimum inhibitory concentration. *Indicates significant difference ($p<0.05$) between times.

To evaluate the impact of treatments and time on the CFU/g count of mesophilic bacteria, we used the colony counting method on PCA culture medium. The ANOVA statistical analysis indicated that both factors, treatment and time, have significant effects on bacterial growth ($p < 0.05$). However, the interaction between these factors was not significant ($p=0.1347$), suggesting that the effect of time was consistent across the different treatments.

The treatments with a concentration of 4xMIC showed the lowest counts at both T0 and T7 (**Figure 6** and **Supplementary Table S3**). Similar to the count of *P. paracarnis* A006, the highest efficacy for inhibiting mesophilic bacteria was observed with treatment C 4xMIC (2.17

± 0.30 log CFU/g at T0 to 5.66 ± 0.11 log CFU/g at T7), followed by F 4xMIC (2.76 ± 0.30 log CFU/g at T0 to 6.08 ± 0.01 log CFU/g at T7) and then N 4xMIC (4.40 ± 1.05 log CFU/g at T0 to 7.42 ± 0.12 log CFU/g at T7). Treatments with a MIC concentration showed no efficacy, as demonstrated by Tukey's multiple comparisons test, which indicated no significant differences ($p < 0.05$) in the WEO control count.

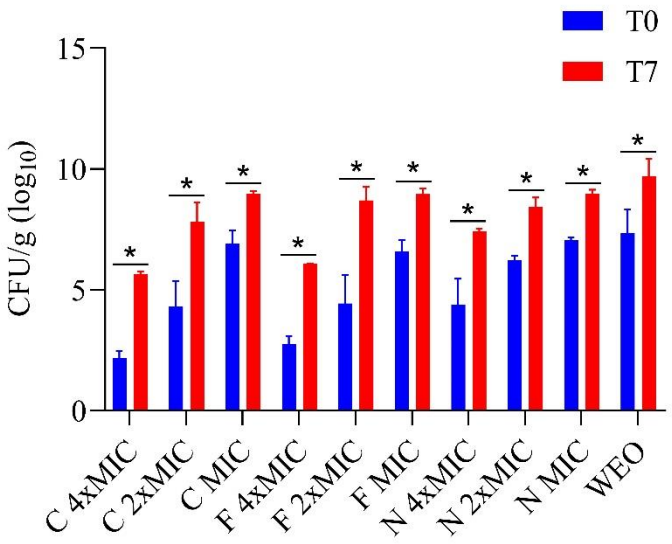


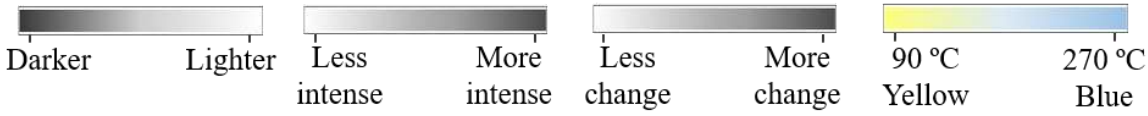
Figure 6. Mesophilic bacteria count (log CFU/g) of model cheeses in the different treatments analyzed at 0 (T0) and 7 days (T7). C: cinnamaldehyde; F: free cinnamon essential oil; N: nanoemulsified cinnamon essential oil; WEO: control without treatment. MIC: minimum inhibitory concentration. * Indicates significant difference ($p < 0.05$) between times.

3.6.2 Colorimetry

When comparing storage times for treatments C, F, and N 4xMIC, no significant differences ($p > 0.05$) were observed for all coordinates and calculated values (**Table 7** and **Supplementary Table S4**). Since positive b^* values indicate a tendency towards yellow and negative values indicate a tendency towards blue, the persistence of positive values for these treatments after 7 days indicated effectiveness of tested treatments. In contrast, C, F, and N MIC, as well as WEO, showed lower b^* values after 7 days, indicating that they are less effective treatments. The reduction in C^* demonstrated that the color lost its vibrancy and became closer to gray, while the increase in ΔE indicated significant color changes, mainly influenced by changes in b^* values.

Table 7. Colorimetry using CIElab coordinates of cheeses inoculated with *P. paracarnis* A006 and treated with different concentrations of cinnamaldehyde, free CEO, and CEO in nanoemulsion.

		T0	T7	T0	T7	T0	T7	T0	T7
		L*		C*		ΔE		h*	
4xMIC	C	70.18	67.05	9.12	9.81	2.02	0.64	108.80	108.79
	F	70.32	67.40	10.21	11.06	2.68	1.80	108.91	108.37
	N	69.53	66.63	10.44	10.69	2.27	0.74	107.58	107.59
2xMIC	C	69.64	60.88	8.60	5.62	2.18	9.08	110.14	154.33
	F	70.26	56.95	9.41	5.51	2.65	12.77	110.39	164.16
	N	69.12	55.85	9.48	5.93	0.51	13.52	109.77	172.83
MIC	C	70.03	53.18	8.47	3.70	1.06	16.53	110.20	184.73
	F	70.47	53.85	9.18	3.72	0.61	15.75	109.80	179.67
	N	70.16	54.19	8.46	3.78	1.20	15.55	111.15	191.22
WEO		70.79	53.81	7.89	1.84	0.98	14.25	110.45	160.35



*L**: CIELab coordinate, representing lightness; *C**: Chroma; *ΔE*: total difference in visible colors; *h**: Hue angle. C: cinnamaldehyde; F: free cinnamon essential oil; N: cinnamon essential oil in nanoemulsion; WEO: control without treatment. MIC: minimum inhibitory concentration. Values indicate mean ± standard deviation.

The cheeses containing the 4xMIC concentrations of the three treatments showed the least color variation during the period analyzed by the colorimeter. Therefore, photographs of these cheeses were taken every 24 h (**Figure 7**). From T0 to T3 there was no change in any of the treatments. Starting on the 4th day (T4), the control WEO began to show a grayish color, while the treated cheeses remained uncolored. On T5, the N 4xMIC treatment started to develop a small dark spot in the corner, which gradually spread over the following days. Only on T7 did a slight blue spot appear on the edge of the C 4xMIC cheese. For the F 4xMIC treatment, no color formation was observed during the analyzed period.

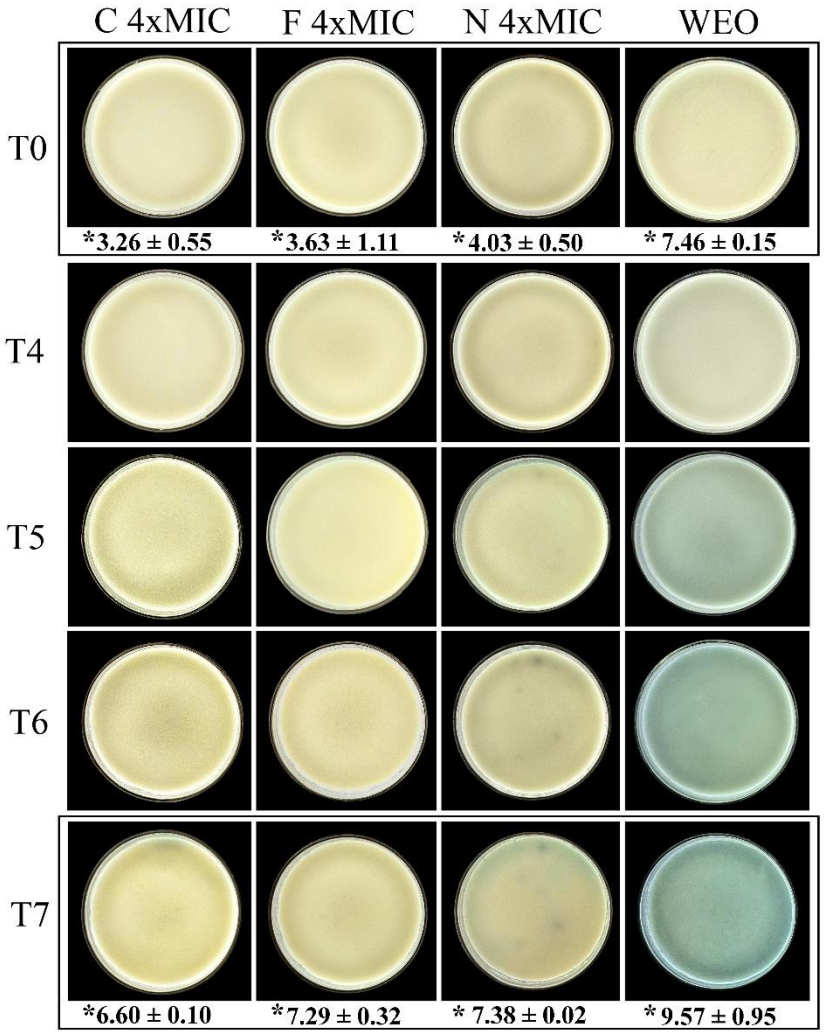


Figure 7. Cheeses treated with cinnamaldehyde (C 4xMIC), free cinnamon essential oil (F 4xMIC), and in nanoemulsion (N 4xMIC). WEO represents the control cheese inoculated without treatments. The photos were taken on the day of cheese production (T0) and from the 4th to the 7th day (T4 to T7). *Indicates the means ± standard deviation of *Pseudomonas* counts in log CFU/g.

3.6.3 Headspace - gas chromatography-mass spectrometry (GC–MS)

The treatments with the best performance from the evaluation of shelf life, C 4xMIC, F 4xMIC, and N 4xMIC, as well as the control WEO, were selected for volatile compound analysis by Headspace GC-MS. Cinnamaldehyde, identified as “2-Propenal, 3-phenyl-“ or “cinnamaldehyde, (E)-“ (**Supplementary Figure S1**), was the predominant compound in all treated cheese samples. In the C 4xMIC cheeses, the observed area for cinnamaldehyde was 14,355,649 units, corresponding to 63.38% of the total volatile compound area. In the F 4xMIC treated cheeses, the area was 13,265,648 units, representing 50.83% of the total area. Finally, in the cheeses treated with N 4xMIC, the cinnamaldehyde area was 9,628,768 units,

corresponding to 45.28% of the total area. The WEO control did not present cinnamaldehyde as a volatile compound.

4 Discussion

Alternative antimicrobial methods, such as using EO to reduce contamination by *Pseudomonas* spp. in dairy products, offer several advantages. They can help industries reduce food waste caused by contamination and may also decrease the reliance on chemical additives. These additives often raise concerns in the food industry, particularly regarding product quality and consumer satisfaction. (Burt, 2004). Milk, one of the most important dairy products since ancient times, has significant nutritional value, being an excellent source of vitamins, minerals, and proteins. Additionally, milk is an ideal medium for the cultivation of probiotics, which play a crucial role in promoting gut health (Parvez et al., 2006).

Previous studies have indicated the use of EO in foods to combat pathogenic bacteria, such as *L. monocytogenes*, *Salmonella* spp., and *E. coli*, in foods like meats, fresh fruits and vegetables, and dairy products (Coimbra et al., 2022; Osaili et al., 2021; Santos et al., 2022; Saraiva et al., 2021). Specifically, bacteria of the genus *Pseudomonas*, known for their ability to spoil foods, have been the focus of various studies involving EO to preserve dairy products (Maggio et al., 2023; Pedonese et al., 2017; Rossi et al., 2018). These studies have shown that EO, such as those of thyme, clove, Ceylon cinnamon, and oregano, exhibited significant antibacterial efficacy against *P. fluorescens* originating from dairy products like milk, ricotta, and mozzarella cheese.

This study focused on identifying EO capable of inhibiting *P. paracarnis* A006, which is associated with blue discoloration in Brazilian soft fresh cheese (Minas Frescal cheese) (Rodrigues et al., 2021; Rodrigues, Machado, Carvalho, et al., 2023). To date, there are no studies in the literature that have used this antimicrobial method against spoilage bacteria that produce blue pigment.

In this study, we began screening with 20 EO, and only 10 exhibited antibacterial activity against *P. paracarnis* A006, the blue-pigment producer. The tolerance of this strain to 50% of the tested oils may be related to the fact that it is a Gram-negative bacterium. The presence of an additional outer membrane of lipopolysaccharides, which acts as a barrier to restrict the entry of hydrophobic compounds into the cell, can make these bacteria less susceptible to EO (Mith et al., 2014). However, it was possible to identify oils capable of combating this strain, with CEO being the most effective, as it showed the largest inhibition zone and the lowest MIC and MBC values (**Figure 1**, **Table 2**). These results are consistent

with previous studies that have demonstrated the potent antibacterial activity of CEO against Gram-negative bacteria, such as *E. coli*, *Klebsiella* sp., and *Pseudomonas aeruginosa* (Gupta et al., 2008; Oulkheir et al., 2017; Zhang et al., 2016).

The CEO used in this study was extracted by the manufacturer (Ferquima) using the steam distillation method. The choice of extraction method directly influences the yield and chemical composition, impacting its bioactivity and safety (Khan et al., 2023). Steam distillation prevents direct contact between liquid water and plant material, avoiding unwanted reactions like hydrolysis or degradation of volatile compounds, preserving the EO chemical integrity (Souiy, 2024). This quality preservation is a key factor in the EO obtaining GRAS status, ensuring its safety in food applications (Falleh et al., 2020).

The characterization of the CEO demonstrated that cinnamaldehyde is the most abundant component (**Table 3**, **Figure 2**). When evaluating the efficacy of this compound *in vitro* in the same proportion as it is found in the CEO (82.55%), it was observed that it exhibited the same MIC as the free CEO. This finding reinforces the attribution of this compound to the antimicrobial activity of the oil. Li et al. (2018) demonstrated that cinnamaldehyde has a significant inhibitory capacity on several critical functions of *P. fluorescens* associated with quorum sensing. Specifically, cinnamaldehyde can interact with the LuxR-type protein and thus inhibit the production of extracellular proteases, swimming and swarming motility, as well as biofilm formation. Additionally, other studies have reported CEO antimicrobial and antibiofilm properties against pathogenic bacteria such as *P. aeruginosa* and *E. coli* (Firmino et al., 2018; Utchariyakiat et al., 2016).

After confirming the efficacy of CEO against *P. paracarnis* A006, efforts focused on enhancing its activity and overcoming challenges like low water solubility, high volatility, low bioavailability, and strong sensory impact (Sharma et al., 2022). Nanoemulsions were chosen as a promising approach. Research highlights the importance of formulation parameters, including surfactant type and concentration, and ultrasound probe time, for effective antimicrobial activity (Donsì et al., 2012; Salvia-Trujillo et al., 2014). Therefore, RSM was utilized for optimization.

The evaluation of different percentages of soy lecithin surfactant and ultrasound times revealed that the last significantly influenced droplet size reduction, whereas lecithin concentration did not show a significant effect (**Table 5**). This finding aligns with studies by Pongsumpun et al. (2020) and Torres Neto et al. (2023) who also used RSM to optimize EO nanoemulsion production and observed that longer sonication times typically reduce droplet size. In contrast, Kaur et al. (2021) demonstrated an inverse relationship between surfactant

concentration and both average droplet size and PDI of nanoemulsions. It is crucial to determine the optimal surfactant concentration, as excessive amounts can negatively impact emulsion stability and appearance (Mahmud et al., 2024).

Using 2.79% soy lecithin and 4.26 min of ultrasound time, as optimized by RSM, resulted in an average droplet size of 164.33 nm. This size is within the ideal range for nanoemulsions, typically defined as having droplet sizes between 100 nm and 200 nm (Amiri-Rigi et al., 2023). Additionally, the low residual value found (1.58) obtained during model validation confirms the accuracy and robustness of the response surface model, demonstrating its reliability in optimizing the nanoemulsion formulation. Although the second-order model showed a good fit to the data ($R^2 = 0.8149$), the Lack of fit test was significant ($p < 0.05$), indicating that the model did not fully capture the variability in the data. This suggests that other factors or terms not considered in the model may be influencing the response, and future studies could explore more complex fitting models.

Soy lecithin was selected as surfactant for this study do to its status as a natural, GRAS-approved emulsifier widely used in the food industry, known for enhancing texture, stability, and shelf life in food products (Klang & Valenta, 2011). Despite the common use of synthetic surfactants like Tween 80, which can achieve smaller droplet sizes in nanoemulsions (Singh et al., 2023) lecithin offers stability, with no phase separation observed even after three months (Kaur et al., 2021). Therefore, lecithin presents significant advantages in applications where stability is crucial, even if it may limit the extent of droplet size reduction.

To understand how the treatments used in this study, C, F, and N, can inhibit *P. paracarnis* A006, a ROS detection assay was performed to identify whether cell death is induced by oxidative stress. The detection of RFU demonstrated that treated cells exhibited lower levels of intracellular ROS compared to the WEO control (**Figure 4** and **Supplementary Table S1**). These results contradict several studies that have shown higher levels of ROS in cells treated with CEO (Niu et al., 2022; Z. Zhang et al., 2022; Zhao et al., 2023).

However, several studies have shown that EO can have antioxidant activity, as demonstrated by Piechowiak et al. (2022). In their study, it was shown that the addition of CEO to an edible fruit coating resulted in the inhibition of ROS production, which was associated with the neutralization of free radicals. Additionally, Mathew & Abraham (2006) had previously identified antioxidant activities in the methanolic extract of *Cinnamomum verum* barks. In that study, the antioxidant capacity was confirmed through hydrogen donation, evaluated by both the reducing power and the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity.

Thus, it is necessary to associate other mechanisms of action CEO with cell death. Zhang et al. (2016) demonstrated that CEO was effective against Gram-negative pathogenic bacteria by inducing changes in membrane structure, cellular swelling, and increasing membrane permeability. These alterations, in turn, may lead to the loss of essential intracellular components, compromising cell viability and leading to bacterial death.

In situ analysis using a model cheese and colorimetric measurements confirmed treatments C, F, and N at 4xMIC effectively inhibited the blue pigment production by *P. paracarnis* A006, as evidenced by the stable color coordinates between T0 and T7 (**Table 7**, **Supplementary Table S4**). This inhibition aligns with the observed antibacterial efficacy, further supported by microbiological analysis, which showed significant log reductions in *Pseudomonas* counts for these treatments (**Figure 5**, **Supplementary Table S2**). While no prior studies have reported similar pigment inhibition in cheese, Kalia et al. (2015) demonstrated that increasing concentrations of CEO reduced pyocyanin production in *P. aeruginosa*. Although the exact mechanism behind the blue pigment production in *P. paracarnis* remains unclear, it may be linked to oxidative stress tolerance and metabolic regulation under stress conditions (Andreani et al., 2015, 2019). These findings suggest that the inhibition observed in this study could be due to both direct antimicrobial action and suppression of specific metabolic pathways involved in pigment production.

The absence of similar studies on CEO nanoemulsions in cheese matrices highlights the novelty of this research. However, comparable antimicrobial activity have been observed when CEO was combined with TiO_2 in active films, indicating potential for further application in cheese preservation (Sharma et al., 2023).

Notably, treatment N exhibited the lowest *Pseudomonas* log reductions across all concentrations, as evidenced by the progressive appearance of blue spots on the model cheeses from T5 to T7 (**Figure 7**). This observation suggests that the interaction between soy lecithin, used as a surfactant in the nanoemulsion, and the cheese matrix may hinder antimicrobial efficacy. The amphiphilic nature of lecithin, with its phospholipid structure (Klang & Valenta, 2011), likely interacts with casein micelles, potentially obstructing the direct contact of the nanoemulsion with bacterial cells. Electrostatic forces and hydrogen bonds may facilitate these phospholipid-protein interactions (Gallier et al., 2012). Additionally, the presence of fat in the cheese matrix further impairs the effectiveness of natural antimicrobials, as seen in other studies where higher fat content reduced the efficacy of EO (Cava et al., 2007; Gouvea et al., 2017). These factors necessitated higher concentrations in the cheese model (2.5 µL/mL) compared to *in vitro* tests (0.625 µL/mL) to achieve comparable microbiological inhibition.

Building on the microbiological results discussed earlier, an additional analysis of aerobic mesophilic bacteria counts in the model cheeses was conducted. The findings aligned with those observed on the *Pseudomonas*-selective medium, where the most effective treatments were C, F, and N at 4xMIC, with counts ranging from 2.17 $\log_{10}$ CFU/g (C 4xMIC at T0) to 7.06 $\log_{10}$ CFU/g (N MIC) (**Figure 6**, **Supplementary Table S3**). The non-sterilized milk used in cheese preparation likely contributed to the significant presence of mesophilic bacteria, as these microorganisms are commonly introduced during milking through contamination from the udder, the milker's hands, and the equipment (Mikulec et al., 2024).

The final analysis, conducted via Headspace GC-MS, revealed that the cheese treated with N 4xMIC had the lowest cinnamaldehyde area (Supplementary Figure S1). This result is consistent with the controlled release properties of essential oils in nanoemulsions, which can prolong antimicrobial activity and improve bioavailability (Bilia et al., 2014). However, this controlled release may also explain the lower immediate antimicrobial efficacy observed in the nanoemulsions compared to other formulations, as the gradual release might delay the full antimicrobial impact. Similar findings were reported by Da Silva Abreu et al. (2020), where *Eucalyptus citriodora* EO in chitosan-based nanoemulsions exhibited a sustained release profile, following a zero-order kinetic model, potentially reducing the initial burst of activity necessary for maximum antimicrobial effect.

5 Conclusion

The findings of this study confirm that CEO and its major compound, cinnamaldehyde, are promising clean label food preservatives, demonstrating significant antibacterial activity against *P. paracarnis* A006 both *in vitro* and *in situ*. However, the CEO nanoemulsion showed lower antibacterial activity in the model cheese. Although the advantage of CEO nanoemulsion in less interference with food aroma has been described in the literature, their controlled release may limit immediate antibacterial efficacy in protein- and lipid-rich foods, such as cheese. Future research should explore optimized nanoemulsion formulations and conduct sensory analyses to ensure that antimicrobial effectiveness does not compromise food flavor and aroma.

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SUPPLEMENTARY MATERIAL

Table S1. RFU detected as an indicator of ROS formation by *P. paracarnis* under different treatment conditions and time. Lowercase letters indicate statistically significant differences between treatments ($p < 0.05$); treatments with the same letter do not differ significantly. Values indicate mean ± standard deviation.

	T30	T150
C 2xMIC [a]	0.601 ± 0.403	0.403 ± 0.193
C MIC [a]	0.770 ± 0.290	0. 289 ± 0.036
F 2xMIC [a]	0.455 ± 0.289	0.337 ± 0.166
F MIC [a]	0.739 ± 0.377	0.454 ± 0.226
N 2xMIC [a]	0.543 ± 0.245	0.283 ± 0.036
N MIC [a]	0.750 ± 0.250	0.499 ± 0.181
WEO [b]	2.870 ± 0.608	

Table S2. *P. paracarnis* A006 count in model cheeses under different treatments and times (T0 and T7). Lowercase letters indicate statistically significant differences between treatments ($p < 0.05$); treatments with the same letter do not differ significantly. Values indicate mean ± standard deviation.

	Log reduction (log CFU/g)	
	T0	T7
C 4xMIC [a]	5.58 ± 0.92	2.24 ± 0.54
C 2xMIC [abc]	2.67 ± 0.84	0.29 ± 1.10
C MIC [abc]	1.78 ± 0.65	0.04 ± 0.45
F 4xMIC [ab]	5.21 ± 1.63	1.55 ± 0.68
F 2xMIC [abc]	2.32 ± 0.88	-0.03 ± 0.50
F MIC [bc]	1.63 ± 0.65	-0.14 ± 0.43
N 4xMIC [abc]	4.81 ± 0.66	1.46 ± 0.55
N 2xMIC [abc]	2.02 ± 1.01	-0.01 ± 0.79
N MIC [bc]	1.58 ± 0.69	-0.47 ± 0.62
WEO [c]	1.38 ± 0.70	-0.73 ± 0.46

Table S3. Mesophilic bacteria count in model cheeses under different treatments and times (T0 and T7). Values indicate mean ± standard deviation.

	CFU/g (log10)	
	T0	**T7**
C 4xMIC	2.17 ± 0.30	5.66 ± 0.11
C 2xMIC	4.32 ± 1.03	7.82 ± 0.80
C MIC	6.91 ± 0.56	8.98 ± 0.12
F 4xMIC	2.76 ± 0.30	6.08 ± 0.01
F 2xMIC	4.44 ± 1.17	8.71 ± 0.55
F MIC	6.59 ± 0.47	8.96 ± 0.23
N 4xMIC	4.40 ± 1.05	7.42 ± 0.12
N 2xMIC	6.22 ± 0.20	8.44 ± 0.39
N MIC	7.06 ± 0.12	8.99 ± 0.15
WEO	7.35 ± 0.97	9.70 ± 0.72

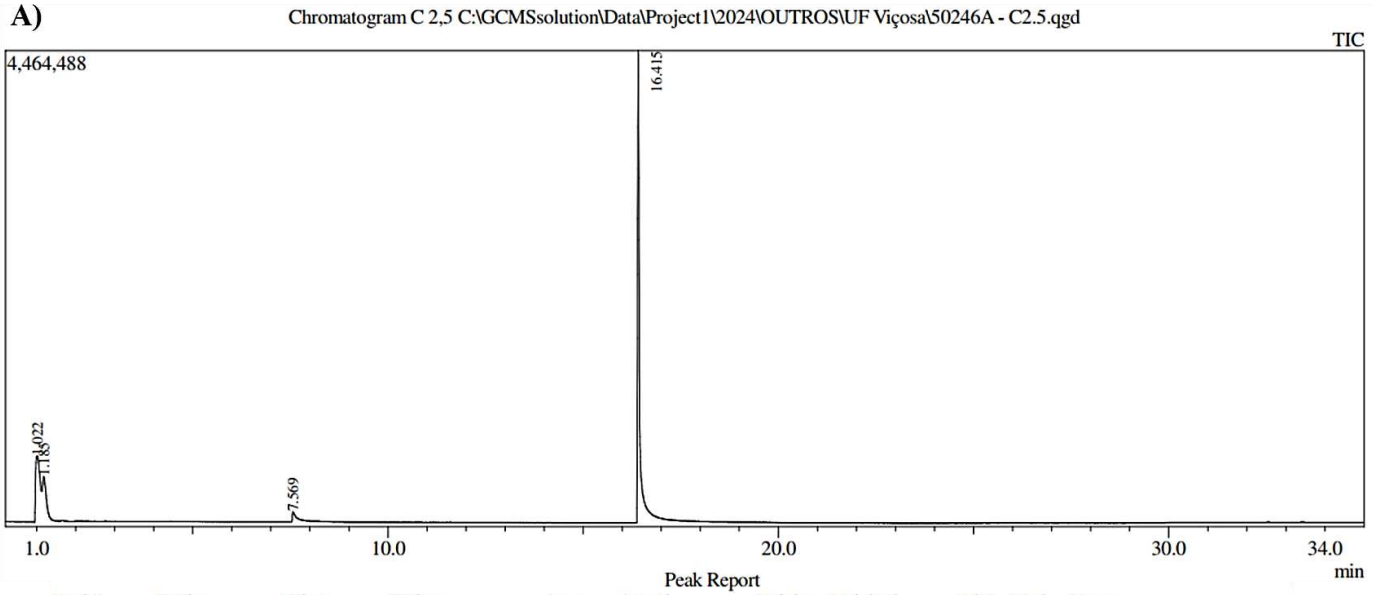
Table S4. CIElab coordinates of cheeses inoculated with *P. paracarnis* A006 and treated with different concentrations of cinnamaldehyde, free CEO, and CEO in nanoemulsion.

		L*	***a****	***b****	***C****	***h****	***ΔE***
C 4xMIC	**T0**	70.18 ± 0.22	-2.94 ± 0.29	9.63 ± 0.24	9.12 ± 0.32	108.80 ± 1.24	2.02 ± 0.49
	T7	67.05 ± 2.89	-3.16 ± 0.06	9.29 ± 0.09	9.81 ± 0.07	108.79 ± 0.49	0.64 ± 0.03
C 2xMIC	**T0**	69.64 ± 0.34	-2.96 ± 0.09	8.07 ± 0.02	8.60 ± 0.05	110.14 ± 0.48	2.18 ± 0.19
	T7	60.88 ± 6.77	-3.67 ± 0.11	3.14 ± 4.89	5.62 ± 3.40	154.33 ± 35.89	9.08 ± 7.10
C MIC	**T0**	70.03 ± 0.10	-2.92 ± 0.16	7.95 ± 0.09	8.47 ± 0.14	110.20 ± 0.82	1.06 ± 0.10
	T7	53.18 ± 7.05	-3.56 ± 0.43	-0.23 ± 1.15	3.70 ± 0.23	184.73 ± 19.38	16.53 ± 4.03
F 4xMIC	**T0**	70.32 ± 0.41	-3.31 ± 0.12	9.66 ± 0.20	10.21 ± 0.15	108.91 ± 1.00	2.68 ± 0.23
	T7	67.39 ± 4.39	-3.48 ± 0.07	10.50 ± 0.59	11.06 ± 0.59	108.37 ± 0.59	1.80 ± 0.09
F 2xMIC	**T0**	70.26 ± 0.37	-3.28 ± 0.12	8.83 ± 0.36	9.41 ± 0.38	110.39 ± 0.06	2.65 ± 0.75
	T7	56.95 ± 7.93	-3.96 ± 0.32	2.04 ± 4.56	5.51 ± 2.28	164.16 ± 41.56	12.77 ± 7.27
F MIC	**T0**	70.47 ± 0.02	-3.11 ± 0.24	8.64 ± 0.19	9.18 ± 0.26	109.80 ± 1.00	0.61 ± 0.10
	T7	53.85 ± 8.17	-3.57 ± 0.84	0.22 ± 1.20	3.72 ± 0.76	179.67 ± 21.19	15.75 ± 4.95
N 4xMIC	**T0**	69.53 ± 0.11	-3.15 ± 0.12	9.95 ± 0.06	10.44 ± 0.09	107.58 ± 0.52	2.27 ± 0.47
	T7	66.63 ± 3.81	-3.22 ± 0.07	10.19 ± 0.91	10.69 ± 0.88	107.59 ± 1.18	0.74 ± 0.31
N 2xMIC	**T0**	69.12 ± 0.10	-3.21 ± 0.08	8.93 ± 0.15	9.48 ± 0.17	109.77 ± 0.16	0.51 ± 0.13
	T7	55.84 ± 10.81	-4.10 ± 0.40	1.57 ± 5.46	5.93 ± 2.45	172.83 ± 49.71	13.52 ± 8.70

N MIC	**T0**	70.16 ± 0.11	-3.05 ± 0.01	7.89 ± 0.16	8.46 ± 0.16	111.15 ± 0.32	1.20 ± 0.08
	T7	54.18 ± 4.79	-3.55 ± 0.87	-0.56 ± 1.27	3.78 ± 0.47	191.22 ± 23.65	15.55 ± 0.82
WEO	**T0**	70.79 ± 0.18	-2.76 ± 0.19	7.39 ± 0.08	7.89 ± 0.01	110.45 ± 1.50	0.98 ± 0.09
	T7	53.81 ± 5.03	-1.38 ± 0.04	0.62 ± 1.30	1.84 ± 0.15	160.35 ± 44.50	14.25 ± 1.92

*L**, *a** and *b**: CIELab coordinates; *C**: Chroma; *ΔE*: total difference in visible colors; *h**: Hue angle. C: cinnamaldehyde; F: free cinnamon essential oil; N: cinnamon essential oil in nanoemulsion; WEO: control without treatment. MIC: minimum inhibitory concentration. Values indicate mean ± standard deviation.

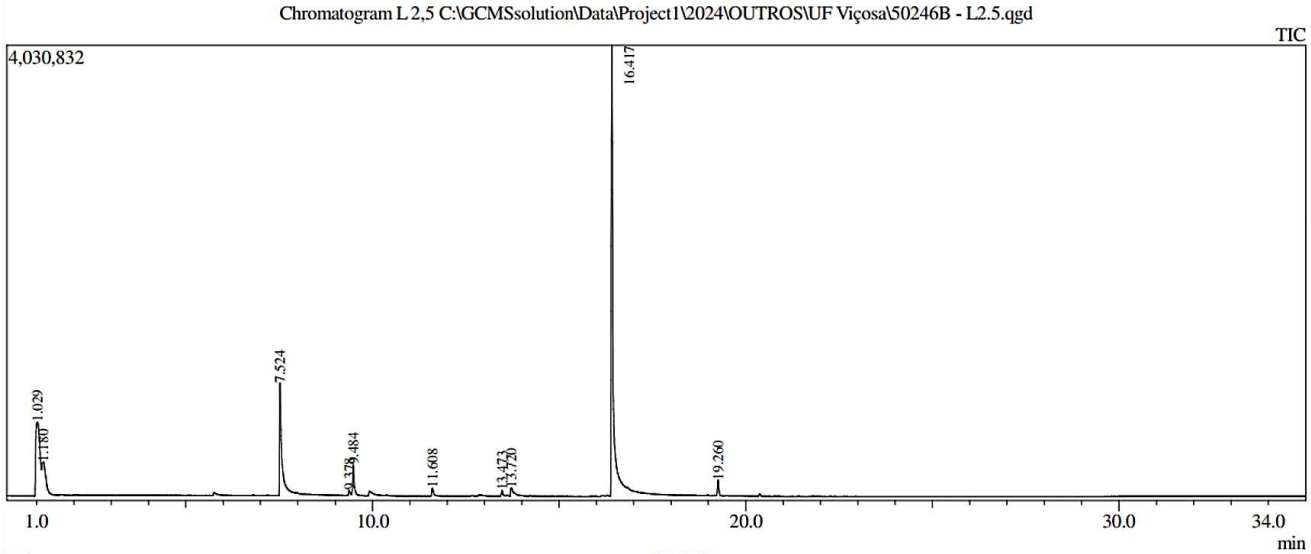
A)



Peak Report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.022	0.950	1.137	4948380	21.85	616593	11.16	8.03		(R)-(-)-2-Amino-1-propanol
2	1.185	1.137	1.423	2891715	12.77	417418	7.55	6.93	V	o-Allylhydroxylamine
3	7.569	7.537	7.737	454315	2.01	90428	1.64	5.02		Benzaldehyde
4	16.415	16.370	16.810	14355649	63.38	4401832	79.65	3.26		2-Propenal, 3-phenyl-
				22650059	100.00	5526271	100.00			

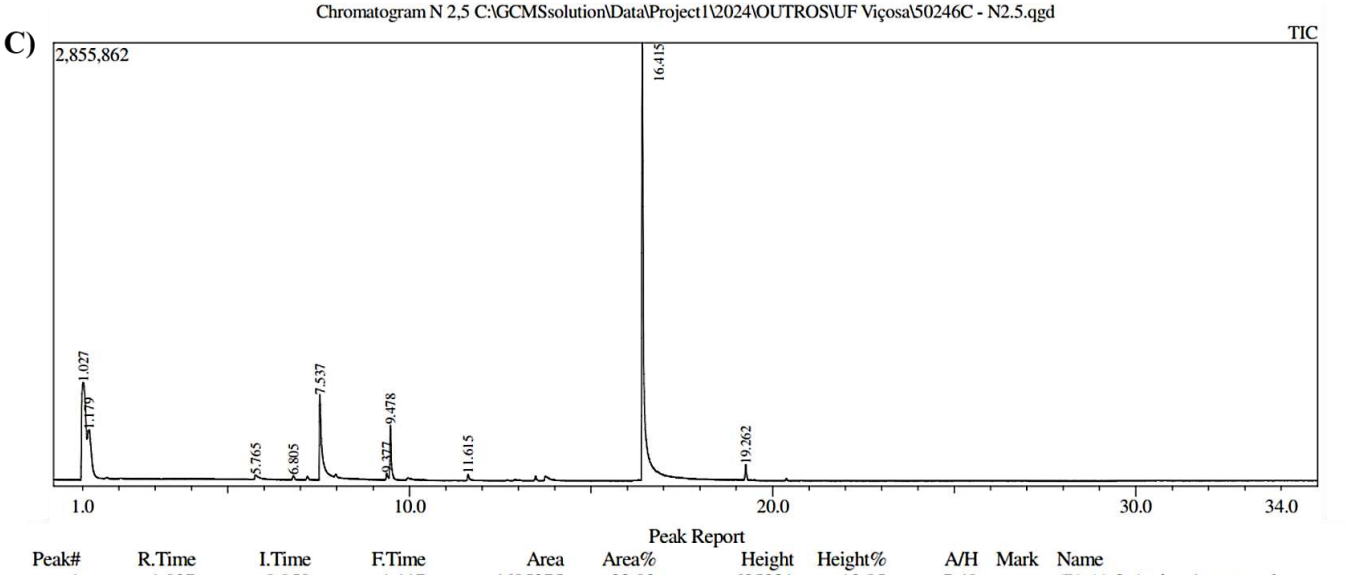
B)



Peak Report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.029	0.950	1.130	5041769	19.32	652631	9.96	7.73		(R)-(-)-2-Amino-1-propanol
2	1.180	1.130	1.410	2159277	8.27	295172	4.51	7.32	V	Ethanol
3	7.524	7.497	7.790	3790579	14.52	993949	15.17	3.81		Benzaldehyde
4	9.378	9.323	9.443	128470	0.49	42988	0.66	2.99		o-Cymene
5	9.484	9.443	9.617	782163	3.00	267498	4.08	2.92	V	D-Limonene
6	11.608	11.557	11.703	214949	0.82	71525	1.09	3.01		Linalool
7	13.473	13.417	13.543	133989	0.51	47156	0.72	2.84		endo-Borneol
8	13.720	13.683	13.797	213098	0.82	65614	1.00	3.25		Benzofuran, 2-methyl-
9	16.417	16.377	16.803	13265648	50.83	3978604	60.73	3.33		2-Propenal, 3-phenyl-
10	19.260	19.190	19.343	366995	1.41	136616	2.09	2.69		Copaene
				26096937	100.00	6551753	100.00			

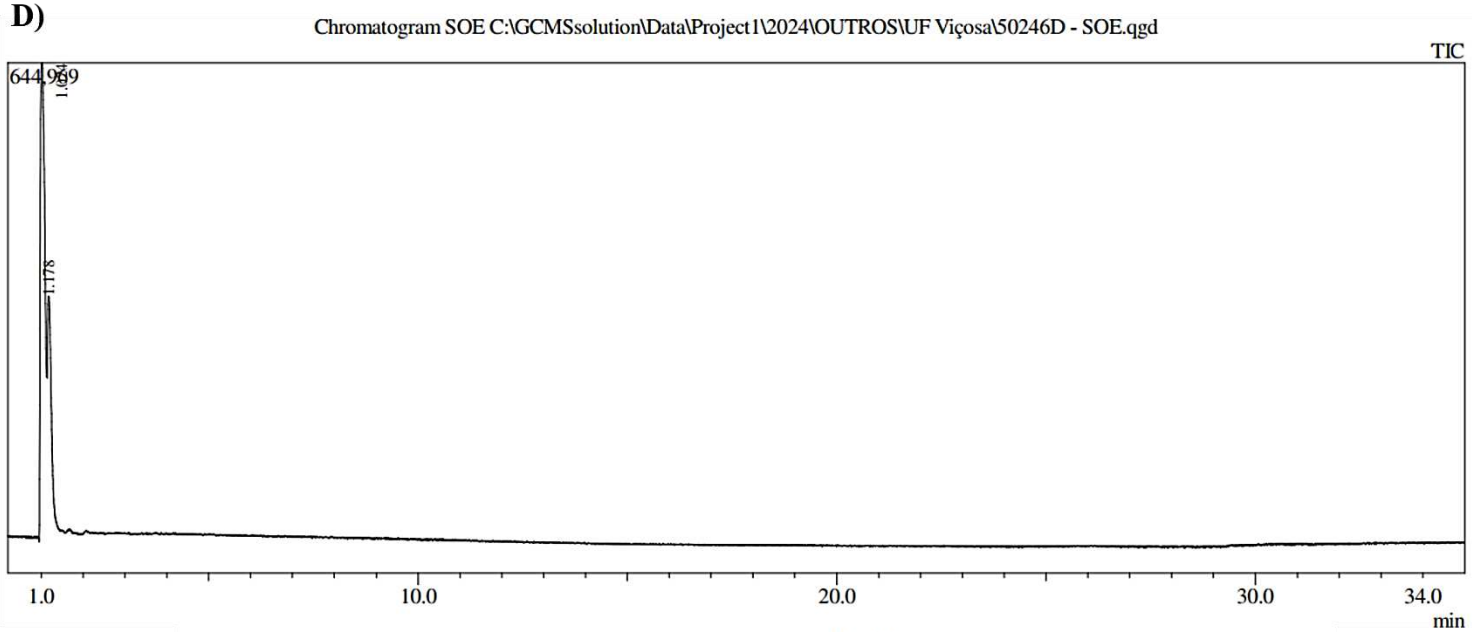
C)



Peak Report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.027	0.950	1.117	4685275	22.03	625231	12.85	7.49		(R)-(-)-2-Amino-1-propanol
2	1.179	1.117	1.430	2453517	11.54	317801	6.53	7.72	V	Ethanol
3	5.765	5.723	5.917	136762	0.64	26569	0.55	5.15		1,3,5,7-Cyclooctatetraene
4	6.805	6.757	6.903	95546	0.45	26223	0.54	3.64		Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-
5	7.537	7.510	8.083	2753061	12.95	547229	11.25	5.03	S	Benzaldehyde
6	9.377	9.330	9.437	128932	0.61	42191	0.87	3.06		Benzene, 1-methyl-3-(1-methylethyl)
7	9.478	9.437	9.643	1008180	4.74	348224	7.16	2.90	V	D-Limonene
8	11.615	11.563	11.730	120511	0.57	37869	0.78	3.18		Linalool
9	16.415	16.377	16.830	9628768	45.28	2794507	57.44	3.45		Cinnamaldehyde, (E)-
10	19.262	19.210	19.350	256186	1.20	99184	2.04	2.58		Copaene
				21266738	100.00	4865028	100.00			

D)



Peak Report

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.024	0.950	1.143	4684856	72.07	599046	66.81	7.82		Alanine
2	1.178	1.143	1.377	1815248	27.93	297547	33.19	6.10	V	Ethanol
				6500104	100.00	896593	100.00			

Figure S1. Representative chromatogram of volatile compounds found in model cheese samples, analyzed by Headspace GC-MS. The peaks correspond to different volatile compounds, and their relative abundance is represented by the area (%). A) C 4xMIC; B) F 4xMIC; C) N 4xMIC; D) WEO.

GENERAL CONCLUSION

This work highlights the importance of developing and optimizing alternative and eco-friendly methods for controlling *Pseudomonas* spp. in food products, in response to the growing demand for natural products free from synthetic additives. The review presented in the first chapter demonstrates the proven effectiveness of several alternative methods for the food industry, while also emphasizing the need for further studies to better understand their limitations and optimize their use, especially in complex food matrices. It is also necessary to deepen the investigation into the influence of these methods on blue pigment production by different *Pseudomonas* strains.

In the second chapter, the antimicrobial efficacy of cinnamon essential oil (CEO) was confirmed, both *in vitro* and *in situ*, demonstrating its potential as a natural preservative in dairy products. However, the application of its nanoemulsion showed limitations in protein- and lipid-rich foods, suggesting the need for future optimizations. Thus, further investigations are needed to adjust formulations and ensure that antimicrobial effectiveness is maintained without compromising the sensory properties of food.

Therefore, this work contributes to advancing knowledge about the use of natural preservation methods and highlights the challenges that need to be addressed for their efficient application in the food industry.