ANDRESSA FUSIEGER

NEW INSIGHT INTO THE ANTIBACTERIAL ACTIVITY OF EMULSIFYING SALTS USED IN PROCESSED CHEESE: FROM *IN VITRO* TO *IN SITU* APPLICATION

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Food Science and Technology at the Department of Agriculture, Food and Environment of University of Catania, Italy, with co-tutelagem with the Post-Graduate Program of the Federal University of Viçosa, Brazil.

Adviser: Antonio Fernandes de Carvalho

Co-adviser: Luís Augusto Nero

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Adviser

To my parents, Nair Fank and Paulo Fusieger

To my grandmother, Maria Nelsi Führ Fank

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Frida.

“Tonight I'm gonna have myself a real good time
I feel alive
And the world I'll turn it inside out, yeah
I'm floating around in ecstasy
So, don't stop me now
Don't stop me
'Cause I'm having a good time, having a good time…”

Don't Stop Me Now, Queen

ABSTRACT

FUSIEGER, Andressa, PhD., Universidade Federal de Viçosa, December, 2022. **New insight into the antibacterial activity of emulsifying salts used in processed cheese: from *in vitro* to *in situ* application**. Adviser: Antonio Fernandes de Carvalho. Co-adviser: Luís Augusto Nero.

Processed cheese (PC) is a dairy product with multiple end-use applications, where emulsifying salts play a fundamental role in physicochemical changes during production. Moreover, some of these salts may be a strategy to control spoilage and pathogenic microorganisms, contributing to safety and shelf-life extension. In the construction of this thesis, four steps were employed to assess the antimicrobial activity of different emulsifying salts. (i) In the first step, the inhibitory activity of a commercial polyphosphate (JOHA® HBS, at different concentrations, from 0.2% to 3.0%) was tested *in vitro* against 21 bacterial strains by streak assay, agar-spot method, spot-on-the-lawn and well-diffusion method. Different culture medium (Brain Heart Infusion Broth, Nutrient, Plate Count, Trypticase Soy), either in liquid or solid form, were used. In all the protocols performed, the solid culture medium (Nutrient agar) was more effective for the inhibition of the target bacteria; results of the streak assay showed that 11 out the 21 tested strains were highly inhibited at 0.5% of JOHA® HBS. (ii) In this step, we selected *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124 to access the *in situ* inhibitory activity of two emulsifying salts (ESSP = short polyP and BSLP = long polyP). PC samples were produced through laboratory-scale and pilot-scale, inoculated and analyzed until 45 days of storage at 6 ± 2 ºC. *C. perfringens* ATCC 13124 growth was not affected ($p > 0.05$), but both of the treatments reduced *B. thuringiensis* CFBP 4376 counts in the tested conditions ($p < 0.05$); a higher and faster reduction in samples produced by the laboratory-scale method was obtained. (iii) In the third step, a total of 14 treatments (T1-T14) composed by mixtures of emulsifying salts (ESSP, BSLP and trisodium citrate) and antimicrobial additives (nisin and potassium sorbate) were evaluated against eight target strains. PC samples were produced through laboratory-scale, inoculated and analyzed until 90 days of storage at 6 ± 2 ºC. Most treatments resulted in some level of bactericidal or bacteriostatic effect against the target microorganisms. Bactericidal activity was evident against *Bacillus* spp., and bacteriostatic effect was clear against *C. perfringens* ATCC 13124, *Enterococcus faecalis*

FAIR-E 179, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* ATCC 6538 ($p < 0.05$) during the storage period. The treatments in which BSLP was applied alone showed greater inhibitory activity; that is, equal concentrations of BSLP result in less bacterial inhibition when in the presence of ESSP. (iv) In the last step, the bacteriostatic effect of six emulsifying salts, containing different concentrations of phosphorus pentoxide (P_2O_5), was investigated in PC samples deliberately contaminated with *B. cereus* spores. Also, the influence of PC processing methods (pasteurization and creaming), storage temperature (6 and 30 ºC) and storage time (until 120 days) were evaluated. Results showed that lower storage temperature and emulsifying salts significantly influenced bacterial growth. In addition, the creaming process and the P_2O_5 content enhanced the inhibitory effect of emulsifying salts; PC treated with the highest concentration of P_2O_5, the lowest average value of both vegetative cells and spore was detected. Finally, the four steps of this study reveals that: (i) Nutrient medium and agar-based tests are required to properly test the inhibitory activity of polyphosphates; (ii) the inhibitory effect of emulsifying salts against *B. thuringiensis* in PC obtained by two different methods was confirmed; (iii) the composition of emulsifying salts significantly influences bacterial inhibition and the phosphate interactions may infer such activity and; (iv) the creaming process and the P_2O_5 content can improve the inhibitory effect of emulsifying salts on *B. cereus* vegetative and spore growth in PC. Taken together, the results contribute to expanding the understanding of emulsifying salt application in PC, with a focus on microbiological safety and shelf-life. However, more detailed studies are needed to determine the specific emulsifying salt composition and the relation of P_2O_5 content responsible for bacterial inhibition.

Keywords: Dairy products. Emulsifiers. Microbiological stability. Pathogenic bacteria. Polyphosphates. Shelf-life.

RESUMO

FUSIEGER, Andressa, PhD., Universidade Federal de Viçosa, dezembro de 2022. **Nova percepção da atividade antibacteriana dos sais emulsificantes utilizados no queijo processado: da aplicação *in vitro* à aplicação *in situ*.** Orientador: Antonio Fernandes de Carvalho. Coorientador: Luís Augusto Nero.

O queijo processado (QP) é um produto lácteo com múltiplas aplicações finais, onde os sais emulsificantes desempenham um papel fundamental nas alterações físico-químicas durante a produção. Além disso, alguns desses sais podem ser uma estratégia para controlar a deterioração e microrganismos patogênicos, contribuindo para a segurança e extensão da vida de prateleira. Na construção desta tese, quatro etapas foram empregadas para avaliar a atividade antimicrobiana de diferentes sais emulsificantes. (i) Na primeira etapa, a atividade inibitória de um polifosfato comercial (JOHA® HBS, em diferentes concentrações, de 0,2% a 3,0%) foi testado *in vitro* contra 21 cepas bacterianas através dos métodos de estrias, *agar-spot*, *spot-on-the-law* e *well-diffusion*. Foram utilizados diferentes meios de cultura (Infusão de Cérebro e Coração, Nutriente, *Plate Count*, Tripticase de Soja), ambos na forma líquida e sólida. Em todos os protocolos realizados, o meio de cultura sólido (ágar Nutriente) foi mais eficaz para a inibição das bactérias alvo; os resultados do ensaio de estrias mostraram que 11 das 21 cepas testadas foram altamente inibidas a 0,5% de JOHA® HBS. (ii) Nesta etapa, selecionamos *Bacillus thuringiensis* CFBP 3476 e *Clostridium perfringens* ATCC 13124 para acessar a atividade inibitória *in situ* de dois sais emulsificantes (ESSP = polyP curto e BSLP = polyP longo). Amostras de QP foram produzidas em escala laboratorial e piloto, inoculadas e analisadas até 45 dias de armazenamento a 6 ± 2 ºC. O crescimento de *C. perfringens* ATCC 13124 não foi afetado ($p > 0,05$), mas ambos os tratamentos reduziram as contagens de *B. thuringiensis* CFBP 4376 nas condições testadas ($p < 0,05$); obteve-se uma redução maior e mais rápida nas amostras produzidas pelo método em escala laboratorial. (iii) Na terceira etapa, um total de 14 tratamentos (T1-T14) compostos por misturas de sais emulsificantes (ESSP, BSLP e citrato trissódico) e aditivos antimicrobianos (nisina e sorbato de potássio) foram avaliados contra oito cepas alvo. Amostras de QP foram produzidas em escala laboratorial, inoculadas e analisadas até 90 dias de armazenamento a 6 ± 2 ºC. A maioria dos tratamentos resultou em algum nível de efeito bactericida ou bacteriostático contra os microrganismos alvo. A atividade bactericida foi evidente contra *Bacillus* spp., e o efeito bacteriostático foi claro contra *C. perfringens* ATCC 13124, *Enterococcus faecalis* FAIR-E 179, *Listeria monocytogenes* Scott A e *Staphylococcus aureus* ATCC 6538 ($p < 0,05$) durante o período de estocagem. Os tratamentos em que o BSLP foi aplicado isoladamente apresentaram maior atividade inibitória; isto é, concentrações iguais de BSLP resultam em menos inibição bacteriana quando na presença de ESSP. (iv) Na última etapa, o efeito bacteriostático de seis sais emulsificantes, contendo diferentes concentrações de pentóxido de fósforo (P_2O_5), foi investigado em amostras de QP deliberadamente contaminadas com esporos de *B. cereus*. Também foi avaliada a influência dos métodos de processamento do QP (pasteurização e creme), temperatura de armazenamento (6 e 30 ºC) e tempo de armazenamento (até 120 dias). Os resultados mostraram que temperaturas de armazenamento mais baixas e os sais emulsificantes influenciaram significativamente o crescimento bacteriano. Além disso, o processo de cremificação e o teor de P_2O_5 potencializaram o efeito inibitório dos sais emulsificantes; QP tratado com a maior concentração de P_2O_5 apresentou o menor valor médio tanto de células vegetativas quanto de esporos. Por fim, as quatro etapas deste estudo revelam que: (i) o meio de cultura Nutriente e os testes baseados em ágar são necessários para avaliar adequadamente a atividade inibitória de polifosfatos; (ii) foi confirmado o efeito inibitório de sais emulsificantes contra *B. thuringiensis* em PC obtido por dois métodos diferentes; (iii) a composição dos sais emulsificantes influencia significativamente a inibição bacteriana e as interações do fosfato podem inferir tal atividade e; (iv) o processo de cremificação e o teor de P_2O_5 podem melhorar o efeito inibitório de sais emulsificantes sobre o crescimento vegetativo e de esporos de *B. cereus* em QP. Considerando tudo, os resultados contribuem para ampliar o entendimento da aplicação de sais emulsificantes em QP, com foco na segurança microbiológica e vida de prateleira. No entanto, estudos mais detalhados são necessários para determinar a composição específica do sal emulsificante e a relação do teor de P_2O_5 responsável pela inibição bacteriana.

Palavras-chave: Produtos lácteos. Emulsificantes. Estabilidade microbiológica. Bactérias patogênicas. Polifosfatos. Vida de prateleira.

RIASSUNTO

FUSIEGER, Andressa, PhD., Universidade Federal de Viçosa, Dicembre, 2022. **Nuove conoscenze sull'attività antibatterica dei sali emulsionanti utilizzati nei formaggi fusi: dall'applicazione *in vitro* a quella *in situ***. Advisor: Antonio Fernandes de Carvalho. Co-adviser: Luís Augusto Nero.

Il formaggio fuso (FF) è un prodotto lattiero-caseario dalle molteplici applicazioni, in cui i sali emulsionanti aggiunti svolgono un ruolo fondamentale sui cambiamenti fisico-chimici che avvengono durante il processo produttivo. Inoltre, alcuni di questi sali possono rappresentare una promettente strategia di controllo dei microrganismi patogeni e deterioranti, contribuendo alla sicurezza e all'estensione della shelf-life del prodotto finale. Il disegno sperimentale della presente tesi ha previsto l'impiego di quattro fasi per valutare l'attività antimicrobica di diversi sali emulsionanti. (i) Nella prima fase, l'attività inibitoria di un polifosfato commerciale (JOHA® HBS, a diverse concentrazioni, dallo 0.2% al 3.0%) è stata testata *in vitro* contro 21 ceppi batterici mediante differenti metodi, quali striscio su piastra, *agar-spot*, *spot-on-the-lawn* e metodo *well-diffusion*. Sono stati utilizzati diversi terreni di coltura (Brain Heart Infusion Broth, Nutrient, Plate Count, Trypticase Soy), sia in forma liquida sia in forma solida. In tutti i protocolli eseguiti, il terreno di coltura solido (Nutrient agar) è risultato il più efficace nell'inibizione dei batteri bersaglio; I risultati del test di striscio hanno mostrato che 11 dei 21 ceppi testati sono stati altamente inibiti dallo 0.5% di JOHA® HBS. (ii) In questa fase, per valutare l'attività inibitoria *in situ* di due sali emulsionanti (ESSP = short polyP e BSLP = long polyP), sono stati selezionati due ceppi target quali, *Bacillus thuringiensis* CFBP 3476 e *Clostridium perfringens* ATCC 13124, che sono stati inoculati nei campioni sperimentali. In dettaglio, campioni di FF, prodotti su scala di laboratorio e su scala pilota, sono stati inoculati e analizzati fino a 45 giorni di conservazione a 6 ± 2 ºC. I risultati hanno mostrato che la crescita di *C. perfringens* ATCC 13124 non è stata influenzata ($p > 0.05$) da nessuna delle variabili considerate, ma entrambi i trattamenti hanno ridotto le conte di *B. thuringiensis* CFBP 4376 nelle condizioni testate ($p < 0.05$) ed è stata ottenuta una riduzione maggiore e più rapida nei campioni prodotti con il metodo su scala di laboratorio. (iii) Nella terza fase, un totale di 14 trattamenti (T1-T14), costituiti da miscele di sali emulsionanti (ESSP, BSLP e citrato trisodico) e additivi antimicrobici (nisina e sorbato di potassio) differenti, sono stati valutati contro otto ceppi target. I campioni di FF sono stati prodotti su scala di laboratorio, inoculati e analizzati fino a 90 giorni di conservazione a 6 ± 2 ºC. I risultati ottenuti hanno mostrato che la maggior parte dei trattamenti ha prodotto un effetto battericida e/o batteriostatico contro i microrganismi bersaglio. L'attività battericida è stata più evidente contro *Bacillus* spp., mentre l'effetto batteriostatico nei confronti di *C. perfringens* ATCC 13124, *Enterococcus faecalis* FAIR-E 179, *Listeria monocytogenes* Scott A e *Staphylococcus aureus* ATCC 6538 ($p < 0,05$) durante il periodo di conservazione analizzato. I trattamenti in cui il BSLP è stato applicato da solo hanno mostrato una maggiore attività inibitoria, mettendo in evidenza che la presenza di ESSP, influenza l’attività inibitoria di BSLP, riducendola. (iv) Nell'ultima fase, è stato studiato l'effetto batteriostatico di sei sali emulsionanti, contenenti diverse concentrazioni di pentossido di fosforo (P_2O_5), in campioni di FF deliberatamente contaminati da spore di *B. cereus*. È stata, inoltre, valutata l'influenza dei metodi di lavorazione del FF (pastorizzazione e scrematura), della temperatura di conservazione (6 e 30 ºC) e del tempo di conservazione (fino a 120 giorni). I risultati hanno mostrato che la temperatura di conservazione più bassa e i sali emulsionanti hanno influenzato significativamente la crescita batterica. Inoltre, il processo di creaming e il contenuto di P_2O_5 hanno potenziato l'effetto inibitorio dei sali emulsionanti. Infatti, il FF trattato con la concentrazione più alta di P_2O_5 ha fatto registrare il valore medio più basso sia di cellule vegetative, sia di spore. In definitiva, le quattro fasi di questo studio hanno rilevato: (i) l’efficacia maggiore del terreno Nutrient, preferibilmente agarizzato, per testare correttamente l'attività inibitoria dei polifosfati; (ii) l'effetto inibitorio dei sali emulsionanti contro *B. thuringiensis* in FF ottenuti con entrambi i metodi utilizzati; (iii) l’influenza della composizione dei sali emulsionanti sull'inibizione batterica e sulle interazioni con i fosfati; (iv) l’aumento dell'effetto inibitorio sulla crescita vegetativa e delle spore di *B. cereus* dei sali emulsionanti nel FF sottoposti a processo di scrematura e sulla base del contenuto di P_2O_5. Nel complesso, i risultati ottenuti contribuiscono ad ampliare la comprensione dell'applicazione dei sali emulsionanti nei FF, con particolare attenzione alla sicurezza microbiologica e alla shelf-life. Tuttavia, sono necessari studi più approfonditi per determinare la composizione specifica del sale emulsionante e il suo rapporto con il contenuto di P_2O_5, responsabile dell'inibizione batterica.

Parole chiave: Prodotti lattiero-caseari. Emulsionanti. Stabilità microbiologica. Batteri patogeni. Polifosfati. Shelf-life.

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SUMMARY

CHAPTER I.

GENERAL BACKGROUND

General introduction

Dairy industry has been investing in new trends to meet consumers' demand for convenient, easy-to-prepare or ready-to-eat products, but still able to deliver high nutritional value, healthiness, meeting desirable sensory standards. Stability is also an important attribute, especially in adverse food supply contexts, as experienced in the Covid-19 pandemic. Processed cheese fits this product profile, as it is easily prepared food products that can be stored at room temperature, availability of various kinds of brands in supermarkets, being the leading cheese varieties in the world that are used as ingredient in various food preparations. Processed cheese can be characterized as a viscoelastic dairy matrix obtained by mixing and heating natural cheeses and emulsifying salts (usually sodium phosphate, polyphosphates, citrates and/or their combinations). It is smooth, uniform in color, melts uniformly, slices uniformly, has a "compact body" and in relation to cream cheese, has a milder, savory taste with less acidity.

Compared to natural cheeses, processed cheese has a relatively short history. The first patent was granted to a German cheese company in 1899 and at the beginning of the twentieth century, the first processed cheese with emulsifying salts was developed by Walter Gerber and Fritz Stettler in Switzerland. The aim in processing cheese was to produce a finished product that had sufficient fluidity for convenient packaging, long keeping qualities, improving shelf-life of cheese shipped to warmer climates. Currently, processed cheese is widely consumed around the world and has a market of around 2.2 billion kilos annually, with a compound annual growth rate (CAGR) between 2017 and 2021 of 3.4%. For the future projections, the CAGR from 2022 to 2032 is estimated to be 3.8% and the market value to increase is from USD 15.9 Bn in 2022 to USD 23.2 Bn by 2032.

The application of emulsifying salts in processed cheese is one of the most studied among cheese scientists; however, most of the published papers are related to the mechanical properties, physicochemical properties, microstructure, rheology, matrix stability, formation of casein fibrils and sensory quality. The basic function of emulsifying salts is the ability to cleave the calcium ions bounded into the natural cheese protein matrix and its exchange for sodium ions and, as result, less insoluble calcium para-caseinate is converted to the more soluble sodium para-caseinate. The added emulsifying salts influence the pH, structure and stability of the product. In fact, the main purpose of this ingredient is not to perform a bioconservative role.

Although emulsifying salts are not considered antimicrobial agents around the word, e.g., the U.S. Food and Drug Administration (FDA), the Brazilian Department of Agriculture, Livestock and Food Supply (MAPA), Gram-positive bacteria have been reported to be susceptible to inhibition by polyphosphates. These papers are mainly related to the *in vitro* inhibitory activity, demonstrating emulsifying salts effects on the growth and in the increase of the length of the lag phase of several bacterial groups and species. On the other hand, the microbiological application of emulsifying salts in processed cheese was only explored in a few *in situ* studies against spore-forming bacteria. None of these studies investigated the survival and growth of vegetative bacteria that have already been isolated from this dairy matrix, or related to secondary contamination and worse hygienic conditions during the production. There is a lack of information on the inhibitory effect of emulsifying salts in processed cheese, especially when different emulsifying salts are applied together. Therefore, studying the inhibitory effect of emulsifying salts was of interest, as it may be an important key to controlling bacterial growth in processed cheese.

Importance of the study

Processed cheeses are made around the world and their variety is increasing. Phosphates and polyphosphates are used as emulsifier additives in order to improve the technological and functional qualities of processed cheese. Their antimicrobial effect has been reported in some *in vitro* and *in situ* studies. However, emulsifying salts have different solubilities and *in vitro* protocols can influence the results of the inhibitory effect. The *in situ* application to evaluate a possible interaction between emulsifying salts and the influence of different processing methods of processed cheese has not been studied against vegetative cells. The feasibility of emulsifying salts with technological and microbiological characteristics has potential financial benefits for companies, as well for consumers who are looking for more stable processed products. This research has commercial and scientific importance, as while ingredient and dairy companies are interested in applying emulsifying salts in processed cheese to inhibit bacterial growth and increase shelf-life, few scientific studies on the evaluation of the inhibitory activity through the application of emulsifying salts in the dairy matrix are available.

Research problem

Previously, InovaLeite team performed a project where the main objective was to investigate the effect of an emulsifying salt against *Bacillus* spp. in processed cheese. Two emulsifying salts were used: i. long-chain polyphosphate with 69 ± 1% P_2O_5 and ii. poly- and sodium phosphates with 59.7 ± 1% P_2O_5. To evaluate the *in vitro* inhibitory effect, the long-chain polyphosphate with 69 ± 1% P_2O_5 was added in liquid culture medium and the minimum inhibitory concentration was obtained by the drop-plate method and 96-well microtiter plates; concentrations between 2.86% (w/v) and 5.71% (w/v) were required to inhibit the targets. To evaluate the *in situ* inhibitory effect, the poly- and sodium phosphates with 59.7 ± 1% P_2O_5 (used as emulsifies, 1.5% w/w) plus the long-chain polyphosphate with 69 ± 1% P_2O_5 (used as antimicrobial agent, 0.5% w/w) were applied processed cheese; this concentration was not enough to inhibit the growth of the bacterial targets. Furthermore, the minimum inhibitory concentration obtained in the *in vitro* test was higher than that allowed by Brazilian legislation and, in general, the results were not consistent and compatible with the scientific data available in the literature. In this way, many questions were created and new strategies were employed to find answers and solutions. Critical thinking in the search for the "how" and "why" enabled the construction of this thesis.

Research hypotheses and aims

The hypothesis for this research was that the emulsifying salts polyphosphates applied in processed cheese can provide inhibitory effects against Gram-positive bacteria. In order to verify this hypothesis, the following aims were defined:

i. To assess the *in vitro* inhibitory activity of an emulsifying salts polyphosphate against a panel of twenty-one target bacteria using different culture media, polyphosphate concentration and protocols.
ii. To evaluate the inhibitory activity of two emulsifying salts against two selected target bacteria and to compare the effects on processed cheeses obtained through laboratory-scale and pilot-scale production.

iii. To determine the effectiveness of emulsifying salts and compare with selected preservatives applied in processed cheese and to report how mixtures of emulsifying salts can influence different bacterial populations recognized for compromising the quality of processed cheese.

iv. To characterize the effect of six emulsifying salts based on the P_2O_5 content and the influence of creaming process, storage temperature and storage time using *B. cereus* as a target model.

Thesis outline

Chapter I gives an overview of processed cheese and emulsifying salts, the importance of this work, the research problem, as well as the hypotheses and objectives of the thesis.

Chapter II describes an *in vitro* study to access the inhibitory effect of emulsifying salt. Different target strains, protocols, culture medium and emulsifying salt concentration were applied, and the results compared to treatments containing nisin. The pH effect was also evaluated. The results were important to determine the possible inhibitory concentrations needed in the dairy matrix.

Chapter III investigates the inhibitory activity of two emulsifying salts in processed cheese obtained through different processes methods: laboratory-scale and pilot-scale. *B. thuringiensis* CFBP 3476 and *C. perfringens* ATCC 13124 were used as target strains and processed cheese samples analyzed until 45 days of storage at 6 ± 2 ºC. The data were useful in outlining the following chapters.

Chapter IV reports the *in situ* performance of emulsifying salts against eight target strains inoculated in processed cheese produced by laboratory-scale. Different combinations of emulsifying salts were explored and the interaction between them was investigated until 90 days of storage at 6 ± 2 ºC. To fulfill the objective, 2,520 samples were produced. The findings were important to open our minds and explore other types of emulsifying salts.

Chapter V focusses on *B. cereus* spores inoculation in processed cheese. Six combinations of emulsifying salts based on the P_2O_5 content were evaluated and the samples were analyzed until 120 days of storage at 6 ± 2 ºC and 30 ± 2 ºC. In addition, the creaming process was applied to verify if it influences the inhibitory effect. Approximately 770 samples were produced. The results were important to answer questions that we had raised in previous studies and to generate new perspectives for the future.

Chapter VI provides the overall experiments, summaries the main findings of this research and provides recommendations for future study.

Chapter VII brings a survey of the scientific production (papers and conference abstracts) carried out during the PhD (March 2019 to December 2022), either through this research or through parallel projects.

CHAPTER II.

RESEARCH ARTICLE ONE

Inhibitory activity of an emulsifying salt polyphosphate (JOHA HBS®) used in processed cheese: an *in vitro* analysis of its antibacterial potential

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Chapter presented according to the final format of the journal.

All analyzes described in this chapter were conducted at InovaLeite, Department of Food Technology, Federal University of Viçosa, Viçosa, MG, Brazil.

Title page

Inhibitory activity of an emulsifying salt polyphosphate (JOHA HBS®) used in processed cheese: an *in vitro* analysis of its antibacterial potential

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Abstract

In the present study, the inhibitory activity of a commercial polyphosphate (JOHA® HBS) was tested in vitro against 21 bacterial strains. Firstly, the antimicrobial activity of JOHA® HBS, at different concentrations (from 0.5 to 2.0%) was evaluated in different agar and broth media (namely, Brain Heart Infusion: BHI; Nutrient: NT; Plate Count: PCA; Trypticase Soy: TSA/B), using streak assay (agar) and culture enumeration (broth). Furthermore, the bacterial inhibition of JOHA® HBS (at different concentrations, from 0.2% to 3.0%) was evaluated both on NT agar and broth medium, by streak assay, agar-spot method, and by spot-on-the-lawn and well-diffusion method. Finally, the JOHA® HBS antimicrobial activity was tested on NT agar at pH 6.3. Results of the streak assay on NT agar showed that 11 out the 21 tested strains were highly inhibited at 0.5% of JOHA® HBS. Interestingly, at adjusted pH levels, JOHA® HBS concentrations of 1.0% (w/v) were able to inhibit the same targets, confirming the antimicrobial effect of JOHA® HBS. This study reveals that NT medium and agar-based tests are required to properly test the inhibitory activity of polyphosphates. Furthermore, results confirmed the antimicrobial activity of JOHA® HBS, at low concentrations, against target bacteria of interest to the dairy industry.

Keywords: standardization, dairy industry, pathogenic bacteria.

Highlights

- Influence of solubility and different culture media on the inhibitory potential of JOHA® HBS.
- *In vitro* characterization by different approaches against 21 target strains.
- Description of effective *in vitro* agar protocol for screening inhibitory activity of JOHA® HBS.
- Indication of application of JOHA® HBS in processed cheese to increase shelf-life.

1. Introduction

Emulsifying salts are of major importance to processed cheese production where they promote physico-chemical changes, such as the dispersion of proteins and lipids. Serious technological issues during homogenization of processed cheese mass would occur without the addition of emulsifying salts during processing (Buňka et al., 2014; Fox, Guinee, Cogan, & McSweeney, 2016; Vollmer, Kieferle, Youssef, & Kulozik 2021). From a technological point of view, processed cheese is a dairy product produced by heating a mixture of natural cheeses, under partial vacuum conditions and constant stirring, in the presence of an emulsifying salt capable of chelating calcium; in order to disrupt the structural network and solubilize casein (Kapoor & Metzger, 2008; Buňka et al., 2014). The ability of emulsifying salts to sequester calcium phosphate is relevant to the characteristics of processed cheese, resulting in the conversion of insoluble calcium paracaseinate into soluble sodium paracaseinate (Kapoor & Metzger, 2008; Salek et al., 2019; Hammam, Beckman, Sunkesula, & Metzger 2022).

In processed cheese production, monophosphates, polyphosphates and citrates are the emulsifying salts commonly used (Buňka, Černíková, & Salek, 2022). Sodium salts of polyphosphate, a long-chain phosphate, are a class of compounds derived from the condensation of phosphate ions, polyanions forming complexes with metal ions and with positively charged macromolecules, such as proteins (Iammarino et al., 2020; Buňka et al., 2022). These salts can inhibit microbial growth and, consequently, are relevant for assuring the safety of processed cheeses (Buňková & Buňka, 2017; Martinez-Rios, Jørgensen, Koukou, Gkogka, & Dalgaard, 2019). The antimicrobial activity of phosphates is mainly described against Gram-positive bacteria, and the inhibitory effect is positively related to chain length and level of condensation (Lee, Hartman, Stahr, Olson, & Williams, 1994; Buňková, Pleva, Buňka, Valášek, & Kráčmar, 2008; Ramos, Alves, Spadoti, Zacarchenco, & da Cruz, 2022).

Management strategies that carry out the microbiological control of processed cheeses are based on conservative agents, biopreservatives, control of extrinsic factors and predictive microbiology (Ramos et al., 2022). Therefore, *in vitro* and *in situ* studies have been carried out to assess the antimicrobial effects of commercial emulsifying salts; in order to determine their application as food additives in processed cheeses (Zaika, Scullen, & Fanelli, 1997; Maier, Scherer, & Loessner, 1999; Buňková et al., 2008; Lorencová, Vltavská, Budinský, & Koutný, 2012; Martinez-Rios et al., 2019). However, no standard method has been properly described for the antimicrobial evaluation of long-chain sodium phosphates, in which the polyphosphate condensation ratio can highly affect solubility (Buňka et al., 2022).

In this regard, the present study aims to evaluate the inhibitory activity of a commercial sodium polyphosphate, JOHA® HBS (ICL Food Specialties, Ladenburg, Germany), against a panel of 21 target bacteria using different culture media, polyphosphate concentration and *in vitro* tests, in order to select the best protocol.

2. Materials and methods

2.1. Bacterial strains and food additives

Twenty-one bacterial strains (n = 21) were included in the present study as targets: *Bacillus cereus* INV 10(3); *Bacillus subtilis* ATCC 19659; *Bacillus thuringiensis* CFBP 3476; *Enterococcus faecalis* FAIR-E 77, FAIR-E 179; *Escherichia coli* ATCC 25922; *Lactiplantibacillus plantarum* ATCC 8014; *Latilactobacillus sakei* ATCC 15521; *Listeria innocua* ATCC 33090; *Lactococcus lactis* subsp. *lactis* ATCC 13675, ATCC

19435; *Listeria monocytogenes* ATCC 7644, ATCC 19112, Scott A; *Pseudomonas fluorescens* ATCC 13525, 07A; *Salmonella enterica* subsp. *enterica* ATCC 13076; *Staphylococcus aureus* ATCC 6538, ATCC 14458, ATCC 43300; *Streptococcus salivarium* 20P3. The strains were stored at -80 ºC in Brain Heart Infusion (BHI; Kasvi, São José dos Pinhais, PR, Brazil) broth or in Trypticase Soy broth (TSB; Kasvi), both supplemented with glycerol at 20% (v/v; Exôdo Cientifica, Sumaré, SP, Brazil). For microbiological assays, strains were inoculated in BHI, Nutrient (NT; Kasvi) broth or TSB, incubated overnight at 25, 30 or 37 °C and diluted in NaCl 0.85% (w/v) until a turbidity equivalent to 1 MacFarland Equivalence Turbidity Standard (ref. R20411; Remel™, Lenexa, KS, USA) was achieved; corresponding to approximately 3×10^8 colony forming units per mL (cfu/mL). The cultures were diluted in decimal scale to 3×10^6 cfu/mL, to obtain concentrations previously used by Buňková et al. (2008).

A commercially available blend, food grade, long-chain sodium polyphosphate (JOHA® HBS, poly- and sodium phosphates, E 452 and E 339; 69 ± 1% P2O5, white powder, ICL Food Specialties, Ladenburg, Germany) was used for microbiological assays. Nisin (Nisaplin®, 2.5% w/w nisin A, Danisco, Copenhagen, Denmark), a commercial antimicrobial agent commonly used in the production of processed cheese, was used as a control for the inhibitory assays.

2.2. Screening of culture media and plating protocols

Culture media and plating methods were assessed to detect the inhibitory activity of polyphosphate, and to select the best combination for further antimicrobial assays. A protocol based on culture streaking (streak assay) was performed on BHI agar, NT agar, Plate Count agar (PCA; Kasvi) and Trypticase Soy agar (TSA; Kasvi), supplemented with JOHA® HBS (at 0.5, 1.0 and 2.0%, w/v). Each agar medium (supplemented with agar at

1.5%, w/v) was prepared, according to the manufacturer's instructions, autoclaved at 121 ºC for 15 min, cooled to 45 ºC and then added with JOHA® HBS, at the indicated concentrations. This resulted in the following treatments (for each culture media): A1 (0.5% of HBS); A2 (1.0% of HBS); A3 (2.0% of HBS); and AC (no HBS: control). To simulate processed cheese manufacture (Kapoor & Metzger, 2008), the agar suspensions were homogenized then heated at 85 ºC for 15 min in a water bath (MA156/6, Marconi, Piracicaba, SP, Brazil), and then poured into Petri dishes. Seven selected target strains (*E. faecalis* FAIR-E 179, *E. coli* ATCC 25922, *L. plantarum* ATCC 8014, *L. innocua* ATCC 33090, *L. monocytogenes* Scott A, *S. aureus* ATCC 6538, and *S. salivarium* 20P), at a final concentration of 3 × 10^6 cfu/mL, were then streaked and plates incubated at 37 ºC for 48 h; sterile water was used as negative control. Absence of bacterial growth indicated the inhibitory activity of the combined culture medium and JOHA® HBS. The assay was conducted in duplicate and in three independent repetitions.

An additional inhibitory assay, based on broth culturing and then plating (broth assay) was also assessed. Three of the target strains (*E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *S. aureus* ATCC 6538) were inoculated into BHI, NT, TSB and a formulated PCA (no agar added) supplemented with JOHA® HBS (at 0.5, 1.0 and 2.0%, w/v), resulting in the following treatments for each culture medium: A1 (0.5% of HBS); A2 (1.0% of HBS); A3 (2.0% of HBS); and AC (no HBS: control). Inoculated tubes were incubated at 37 ºC for 24 h, ten-fold diluted in NaCl 0.85% (w/v; Exôdo Cientifica) and drop plated in PCA, and incubated at 37 °C for 48 h. Growth colonies were counted and results expressed as log cfu/mL. This assay was conducted in duplicate and in three independent repetitions.

2.3. Determination of JOHA® HBS inhibitory activity

Based on the previous results, NT was selected as the culture medium for the evaluation of JOHA® HBS inhibitory activity. For this, all 21 selected strains were considered.

The streak assay, as described above, was designed considering NT as the culture medium, supplemented with different concentrations of JOHA® HBS; from 0.2 to 3.0% (w/v), resulting in 8 treatments: T1 (0.2%); T2 (0.4%); T3 (0.6%); T4 (0.8%); T5 (1.0%); T6 (1.5%); T7 (2.0%); T8 (3.0%). In addition, NT supplemented with commercial nisin (Nisaplin®) at 0.0012% (w/v) (Brasil, 1996) and NT (without HBS or nisin) were included as positive (TNis) and negative controls (target growth control, TG), respectively. The same procedures described above were carried out for the 21 selected target strains. This assay was conducted in duplicate and in three independent repetitions.

JOHA® HBS inhibitory activity was also assessed utilizing three diffusion assay-based protocols: spot-on-the-lawn (adapted from Lewus & Montville, 1991), agar-spot (adapted from Moon, Park, & Lee, 2011) and well-diffusion (adapted from Lewus & Montville, 1991). For these assays, JOHA® HBS and nisin were added to sterile distilled water, heated at 85 ºC for 15 min, cooled to room temperature, and diluted up to the previously described concentrations (JOHA® HBS from 0.2 to 3.0%, w/v, and nisin at 0.0012%, w/v); sterile distilled water was used as negative control.

For the spot-on-the-lawn assay, 20 µL aliquots of JOHA® HBS prepared solutions and controls were spotted onto plates containing 15 mL of semi-solid NT (0.8 g/100 mL of bacteriologic agar). After absorption (4 ºC for 2 h), plates were covered with an overlay of semi-solid NT (8 mL, 0.8 g/100 mL of bacteriologic agar) inoculated with 10^6 cfu/mL of each target strain (n = 21) and incubated at 25, 30 or 37 °C for 48 h. Inhibition zones with diameters higher than 1 mm were considered as indicative of inhibitory activity (Lewus & Montville, 1991).

For the agar-spot assay, JOHA® HBS and controls were added to semi-solid NT (0.8 g/100 mL of bacteriologic agar) at the above reported concentrations, treated at 85 ºC for 15 min, and poured into Petri dishes. The plates were spotted with 10 µL of each target bacterial suspension (n = 21) at 3 × 10^6 cfu/mL and incubated at 25, 30 or 37 °C for 48 h. The absence of microbial growth was considered as indicative of inhibitory activity (Moon et al., 2011).

For the well-diffusion assay, plates containing 20 mL of semi-solid NT (0.8 g/100 mL of bacteriologic agar) previously inoculated with 3 × 10^6 cfu/mL of each target strain (n = 21) were prepared. Wells with a diameter of 3 mm were cut on the plates, and 50 µL aliquots of JOHA® HBS and controls solutions were used at the described concentrations. After absorption (4 ºC for 2 h), the plates were incubated at 25, 30 or 37 °C for 48 h. Inhibition zones with diameters higher than 1 mm were considered as indicative of inhibitory activity (Lewus & Montville, 1991).

Finally, the selected strains (n = 21) were subjected to a broth assay to measure the effects of JOHA® HBS on them at specific conditions. Bacterial cultures were diluted up to approximately 3 × 10^6 cfu/mL, ten-fold diluted in NaCl 0.85% (w/v) and drop-plated into plates containing NT supplemented with agar (1.5%, w/v) and JOHA® HBS at different concentrations, as indicated above (from 0.2 to 3.0%, w/v); nisin (0.0012%, w/v) and sterile distilled water as positive and negative control, respectively. Agar NT supplemented with JOHA® HBS and controls were prepared and pH was corrected to 6.3 with 1M NaOH (Exôdo Cientifica). Plates were incubated at 25, 30 or 37 °C for 24 h (based on selected strain), and growth colonies enumerated. Results were expressed as log cfu/mL.

2.4. Statistical analysis

Counts obtained in broth assay for the screening of culture media were compared based on treatments through ANOVA and Tukey's honest significant difference (HSD) test ($p < 0.05$). Counts obtained in broth assay for the determination of JOHA® HBS inhibitory activity were subjected to Normality tests (Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera) and then to a nonparametric comparison based on Kruskal-Wallis and Dunn, after Bonferroni correction ($p < 0.005$). All statistics were conducted using the software XLSTAT software (Addinsoft, New York, NY, USA).

3. Results and Discussion

Although heat treatment is commonly applied (at temperature between 75 ºC and 95 ºC for standard processes, and from 110 ºC to 140 ºC for UHT processes) in processed cheese manufacture (Kapoor & Metzger, 2008; Fox, et al., 2016), microbial contamination can still occur, resulting in faster product deterioration, health risks for consumers and shortened shelf life (Ramos et al., 2022). Therefore, the use of emulsifying salts, such as polyphosphates, with antimicrobial activity represents a convenient strategy in processed cheese manufacturing. Thus, the first aim of the present study was to assess the inhibitory effect of the commercial polyphosphate salt, JOHA® HBS, in different culture media and through different assays against a panel of selected target strains.

Overall, when JOHA® HBS was added to BHI and TSA medium, lower bacterial inhibition was recorded, compared to results obtained from NT or PCA through the streak assay protocol (Table 1 and Figure 1). Although sensitive strains, such as *L. plantarum* ATCC 8014 and *S. aureus* ATCC 6538, were inhibited under all evaluated conditions; *E. faecalis* FAIR-E 179, *E. coli* ATCC 25922, and *S. salivarium* 20P3 were inhibited exclusively by NT and PCA medium at the highest tested salt concentration (as 2.0% w/v), as shown in Table 1. Focusing on medium composition for all tested conditions and for all tested strains, it is interesting to note that results were comparable between BHI and TSA, and between NT and PCA; except for *L. innocua* ATCC 33090 and *L. monocytogenes* Scott A strains, for which a lower minimum salt concentration was determined in NT (1.0% and 0.5% w/v, respectively) when compared to the 2.0% and 1.0% w/v in PCA, respectively (Table 1 and Figure 1).

Results of the enumeration assay, performed for *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A and *S. aureus* ATCC 6538, were in accordance with those obtained by streak assay, highlighting high levels of inhibition in PCA (formulated) and in NT broth, as reported in Table 2. For *E. faecalis* FAIR-E 179, the different concentrations of JOHA® HBS significantly affected the growth counts in both PCA and NT media, whereas no significant differences were observed among all tested concentrations in BHI and TSB media ($p > 0.05$). This highlights that the polyphosphate did not affect the growth of this strain. It is also interesting to note that no significant differences between the control (0%) and JOHA® HBS at 0.5% (w/v) were observed for all tested culture media and that the lowest *E. faecalis* FAIR-E 179 cell density was detected in PCA with the highest salt concentration (2% w/v); as shown in Table 2. Focusing on *L. monocytogenes* Scott A, the data indicates that the salt inhibited bacterial growth, with the lowest viable counts detected for all tested media, at a concentration of 2% (w/v). Furthermore, the data highlighted that in BHI medium, a significant difference was observed only at the 2% (w/v) salt concentration. Among the tested culture media, a significant difference was detected for NT medium, where the lowest cell densities were revealed for all tested salt concentrations (Table 2). These results support that *L. monocytogenes* Scott A growth is influenced by both culture medium and JOHA® HBS addition. Results from *S. aureus* ATCC 6538 confirmed that the presence of JOHA® HBS influenced growth, with the highest inhibitory activity detected at the highest salt concentration (2.0% w/v); whereas at 0.5% and 1.0% (w/v) concentrations, no significant differences were revealed for any tested media ($p > 0.05$). In addition, no differences in any media, between the control and the lowest salt concentration (0.5% w/v), was observed; however, at 1.0% (w/v) salt concentrations, differences among media were revealed, with the highest cell densities detected in PCA (Table 2). At 2% (w/v) salt concentration, viable counts were significantly different only in BHI, where the highest count of *S. aureus* ATCC 6538 was enumerated (Table 2). Based on the enumeration assay (in broth), it was confirmed that *E. faecalis* FAIR-E 179 and *S. aureus* ATCC 6538 were not inhibited by the highest concentration of JOHA® HBS (2% w/v); whereas *L. monocytogenes* Scott A was completely inhibited in NT broth, starting from 1.0 % (w/v) of JOHA® HBS. Finally, comparing the two protocols, the agar streak assay revealed the highest inhibitory activity.

According to López-Malo et al. (2020), several factors can influence antimicrobial tests, such as interaction between the antimicrobial compounds, pH and components present in medium substrate. Furthermore, combinations of different effects can cause an apparent increase or decrease of susceptibility, and the inhibitory activity of polyphosphates can be reduced by the presence of multivalent metal ions in culture medium (Zaika et al., 1997; Lee, Hartman, Stahr, Olson, & Williams, 1994; Maier et al., 1999; Buňková et al., 2008). Another important point concerns the heat treatment to which the polyphosphates underwent with the possibility of hydrolysis and loss of antimicrobial potential. JOHA® HBS is a long-chain phosphate and susceptible to hydrolysis. However, hydrolysis of polyphosphates is negligible in water at neutral or slightly acidic pH (5.6) at temperatures below 100 ºC and only in the presence of calcium and temperatures above 120 ºC its composition is significantly affected (Rulliere et al., 2012).

Results of the qualitative evaluation of inhibitory effects of JOHA® HBS at the ten different concentrations (from 0.2 to 3.0% w/v) against the 21 selected target bacteria, through streak assay, spot-on-the-lawn, agar spot and well-diffusion assays, are shown in Table 3. In detail, the inhibitory effect of spot-on-the lawn assay was detected only for the two strains of *L. lactis* subsp. *lactis*, only when 3% (w/v) of JOHA® HBS was considered. The well-diffusion assay inhibitory effects were detected for the two *S. aureus* strains, starting from 0.8% (w/v) of salt concentration (Table 3). However, for *L. plantarum* ATCC 8014, *L. monocytogenes* Scott A, and *S. aureus* ATCC 43300 the inhibitory effect was revealed starting from 1.0% (w/v); and for *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *L. sakei* ATCC 15521, *L. lactis* subsp. *lactis* ATCC 13675, *L. monocytogenes* ATCC 7644 and ATCC 19112 starting from 1.5% (w/v) salt concentration (Table 3). It should be noted that in the well-diffusion assay, a higher volume of treatment solution was used (50 µL), which can have an effect, increasing the sensitivity of the assay. Overall, the inhibition activity was higher in assays performed in agar culture medium, confirming the results obtained in the first step. In spot-on-the-lawn and well-diffusion assays, performed in aqueous solutions, the lower inhibitory rate observed can be related to the low solubility of polyphosphates. These protocols, based on the diffusion of antimicrobial substances in culture media (Moraes et al., 2010), are commonly used for the preliminary screening of bacteriogenic strains for assessing the inhibition spectrum of bacteriocins, medicinal herbal extracts, and plant extracts (Moraes et al., 2010; Balouiri, Sadiki, & Ibnsouda, 2016; Ullah et al., 2016). Although the two assays failed in revealing the inhibitory effect of JOHA® HBS, the well-diffusion assay proved to be more effective than the spot-on-the-lawn assay.

In the present work, streak assay and agar spot assays showed better efficacy in revealing inhibitory effects and the two methods gave similar results (Table 3). By these assays, inhibition was confirmed in at least two treatments (at 2.0 and 3.0% w/v of salt) against all 21 bacterial targets (Table 3). Low concentrations of JOHA® HBS, up to 0.4% (w/v), were able to inhibit 11 out of the 21 targets, namely *B. cereus* INV 10(3), *B. thuringiensis* CFBP 3476, *L. plantarum* ATCC 8014, *L. sakei* ATCC 15521, *L. lactis* subsp. lactis ATCC 13675 and ATCC 19435, *L. monocytogenes* ATCC 19112 and Scott A, *S. aureus* ATCC 6538, ATCC 14458 and ATCC 43300 (Table 3). These results corroborate with those reported by Buňková et al. (2008), who performed an *in vitro* study on the antimicrobial effects of three commercial phosphates (JOHA® HBS, JOHA® S9 and CFB 690) against reference microorganisms and bacteria isolated from long-stored processed cheeses. In this study, Buňková et al. (2008) reported the positive inhibitory activity of JOHA® HBS at 0.3% (w/v) against *B. cereus* CCM 3953, *B. subtilis* CCM 2216 and *S. aureus* CCM 3953; at 0.5% of JOHA® HBS (maximum tested concentration); no inhibitory activity was verified against *E. coli* CCM 180.

Although the inhibitory effects of phosphates on Gram-negative bacteria are rarely reported (Buňková et al., 2008; Buňková & Buňka, 2017); in the present work, JOHA® HBS was found to inhibit, even if at higher tested concentrations, *P. fluorescens* ATCC 13525 and 07A, *E. coli* ATCC 25922, and *S. enterica* subsp. *enterica* ATCC 13076 (at 1.0, 1.5 and 2.0% w/v, respectively; Table 3). Compared to Gram-negative, Gram-positive bacteria highlighted higher sensitivity to polyphosphates and these results could be related to the cell-wall structure and, in particular, to the presence of teichoic acids (Buňková et al., 2008; Wang et al., 2021). Among the Gram-positive target strains, *S. salivarium* 20P3, *E. faecalis* FAIR-E 77, and *E. faecalis* FAIR-E 179 were inhibited at higher concentrations (at 1.5, 1.5 and 2.0% w/v, respectively; Table 3). Lorencová et al.

(2012) carried out a study on the antibacterial effect of seven phosphates at different chain lengths (different phosphates compared to those used in this study) and less effective at the suppression of bacterial growth than salts obtained from JOHA® HBS. Against *E. faecalis* CCM 4224, a positive inhibition at 2% (w/v) of HEXA68 polyphosphate and > 2% (w/v) of HEXA70 polyphosphate was reported. However, a minimum concentration of 2% (w/v) of HEXA68 and HEXA70 polyphosphate was required to inhibit *B. subtilis* subsp. *subtilis* CCM 2216 and *E. coli* CCM 3954 (Lorencová et al.; 2012); while a lower concentration of JOHA® HBS inhibited *B. subtilis* ATCC 19659 (0.6%; Table 3) and *E. coli* ATCC 25922 (1.5% w/v; Table 3).

These differences might be related to the divergence in the structure of polyphosphates, as the inhibitory effect of phosphates is dependent on the length of their chains. Long-chain phosphates, as in the case of JOHA® HBS, revealed a higher inhibitory effect compared to short-chain phosphates (Lee, Hartman, Olson, & Williams, 1994; Lee, Hartman, Stahr, Olson, & Williams, 1994; Buňková & Buňka, 2017). In the study performed by Lorencová et al. (2012) with phosphates at different chain lengths, the authors pointed out that the inhibitory effect can be affected by intrinsic factors, such as number of phosphorus atoms and acid basic properties of phosphates in aqueous solutions. In addition, the inhibitory mechanisms of long-chain phosphates are mainly based on the chelation of divalent metal ions (Ca^{2+} and Mg^{2+}), which are involved in the transverse bridges among teichoic acids in the Gram-positive cell wall (Lee, Hartman, Olson, & Williams, 1994; Lee, Hartman, Stahr, Olson, & Williams, 1994). Chelation of divalent ions can also affect some essential physiological growth processes (Maier et al., 1999).

Comparing the effect of JOHA® HBS with those of commercial nisin (Table 3), JOHA® HBS was more effective against Gram-positive bacteria. As expected, nisin did not inhibit Gram-negative bacteria, where their outer membrane acts as a protective barrier against nisin (Małaczewska, & Kaczorek-Łukowska, 2021). Nisin is a natural antimicrobial peptide produced by *L. lactis* subs. *lactis*, has been approved for use in over 50 countries and was granted generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA) in 1988 (Ibarra-Sánchez et al., 2020). In our tests, we used the maximum allowable concentration of nisin in processed cheese. According to the Codex Committee on milk and milk products from Food and Agriculture Organization and World Health Organization (FAO/WHO), nisin can be used as a food additive for processed cheese at a concentration of 12.5 mg/kg product (Ibarra-Sánchez et al., 2020). In Brazil, nisin is used as a preservative in the manufacture of pasteurized cheese, processed cheese and "requeijão", with a maximum limit of 12.5 mg/kg (Brasil, 1996).

Results obtained in broth assay on NT agar with different concentrations of JOHA® HBS (from 0.2 to 3.0% w/v), and with adjusted pH against the bacterial panel (n = 21) are shown in Figure 2. In detail, prior to adjustment, the pH values of the substrates were: T1: 6.05 (0.2% of HBS); T2: 5.51 (0.4% of HBS); T3: 5.49 (0.6% of HBS); T4: 5.26 (0.8% of HBS); T5: 5.18 (1.0% of HBS); T6: 4.87 (1.5% of HBS); T7: 4.53 (2.0% of HBS); T8: 4.04 (3.0% of HBS); TNis: 6.68 (0.0012% of nisin); and TG: 6.67 (without HBS and nisin). Counts of the selected strains did not follow a normal distribution ($p < 0.005$ in Normality tests), leading to a nonparametric analysis based on Kruskal-Wallis and Dunn. Results indicate that *L. plantarum* ATCC 8014 and *L. lactis* subsp. *lactis* ATCC 19435, highlighted similar inhibitory profiles in both conditions tested; the same occurred for *L. lactis* subsp. *lactis* ATCC 13675 and *S. aureus* ATCC 43300 in treatments with the addition of JOHA® HBS (Figure 2). The inhibitory activity of JOHA® HBS at the adjusted pH (6.3) was revealed at a higher concentration when compared to treatments without pH adjustment; for *B. cereus* INV 10(3), *B. thuringiensis* CFBP 3476, *L. sakei*

ATCC 15521, *L. monocytogenes* ATCC 19112, Scott A, and *S. aureus* ATCC 14458. At high pH values, a higher dissociation of the phosphate molecules provides better ion exchange, corroborating the hypothesis that inhibitory effects may be caused by a combination of the pH value of the growth medium (by the tolerance of the tested targets to neutral pH) and the sequestration effect (Lorencová et al., 2012).

Moreover, data regarding the enumeration assay confirmed the results (Figure 2) obtained in the qualitative assay (Table 3) for bacterial targets that have shown greater resistance to JOHA® HBS; highlighting that these bacteria were mostly influenced by the pH adjustment. In particular, concentrations above 1% (w/v) of JOHA® HBS at pH 6.3 were not able to inhibit *E. faecalis* FAIR-E 77, FAIR-E 179, *E. coli* ATCC 25922, *L. innocua* ATCC 33090, *L. monocytogenes* ATCC 7644, *P. fluorescens* ATCC 13525, 07A, *S. enterica* subsp. *enterica* ATCC 13076, or *S. salivarium* 20P3. This result could be related to the fact that most bacteria grow better at neutral pH values and only a few species are able to grow at values below 4.0; where pH influences the microbial respiratory enzymes, disturbing the transport of nutrients into the cell (Buňková & Buňka, 2017). Treatments without pH adjustment showed high levels of inhibition. Therefore, for some targets, inhibition occurs in relation to pH. On the other hand, for some targets such *B. subtilis* ATCC 19659, *L. plantarum* ATCC 8014, *L. lactis* subsp. *lactis* ATCC 13675, *L. lactis* subsp. *lactis* ATCC 19435, *S. aureus* ATCC 6538 and *S. aureus* ATCC 43300, pH adjustment did not influence inhibitory activity, confirming the antimicrobial potential of JOHA® HBS. In addition, the results of quantitative protocol are similar to the results of qualitative agar protocols (streak assay and agar spot; Table 3). In addition to pH value of the environment (higher sensitivity at pH > 7.4), the antimicrobial effect is known to be influenced by temperature, initial microbial population or addition of metal ions (Lee, Hartman, Olson, & Williams, 1994; Zaika et al., 1997; Maier et al., 1999; Lorencová et al., 2012; Buňková & Buňka, 2017).

Simultaneously, it could be mentioned that the pH of processed cheese ranges between 5.6 and 6.0, and these values are required for protein configuration, solubility, and the extent to which emulsifying salts bind Ca^{2+} (Fox et al., 2016; Buňka et al., 2022). The effect of adding emulsifying salts is mainly related to operational conditions at which they can cause a change in pH or ionic strength of the solution (Buňková & Buňka, 2017). Therefore, further research will be carried out *in vitro*, using processed cheese at the corresponding technological conditions.

4. Conclusions

Based on the obtained results, it is possible to rapidly and effectively assess an *in vitro* protocol for screening the inhibitory activity of emulsifying salts with low solubility, such as JOHA® HBS. The obtained concentrations for bacterial inhibition are determinant for future research on *in situ* applications of polyphosphates in processed cheese manufacturing. Processed cheese formulation is critical to the product's stability during its shelf life, and the use of predictive microbiology methods is important for the development of shelf-stable formulations. In this sense, the success of formulations in processed cheese products could be obtained with the addition of emulsifying salts with antimicrobial activity. Accordingly, knowledge of the percentages of polyphosphates to access bacterial inhibition through *in vitro* protocols seems to be possible and promising. Therefore, the results of the present study will allow large-scale analyses on the application of emulsifying salts, which could become an indispensable food additive in the cheese industry or for other food sectors.

CRediT authorship contribution statement

Andressa Fusieger: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. **Raiane Rodrigues da Silva**: Validation, Formal analysis, Investigation, Visualization. **Sidney Rodrigues de Jesus Silva**: Validation, Formal analysis, Investigation, Visualization. **Jaqueline Aparecida Honorato**: Validation, Formal analysis, Investigation. **Camila Gonçalves Teixeira**: Validation, Formal analysis, Investigation, Visualization. **Luana Virgínia Souza**: Validation, Investigation. **Isabela Natali Silva Magalhães**: Validation, Investigation. **Nayara Aparecida da Silva Costa**: Validation, Investigation. **Alfredo Walter**: Conceptualization, Resources. **Luís Augusto Nero**: Conceptualization, Writing - Review & Editing, Visualization, Supervision. **Cinzia Caggia**: Conceptualization, Writing - Review & Editing, Visualization. **Antonio Fernandes de Carvalho**: Conceptualization, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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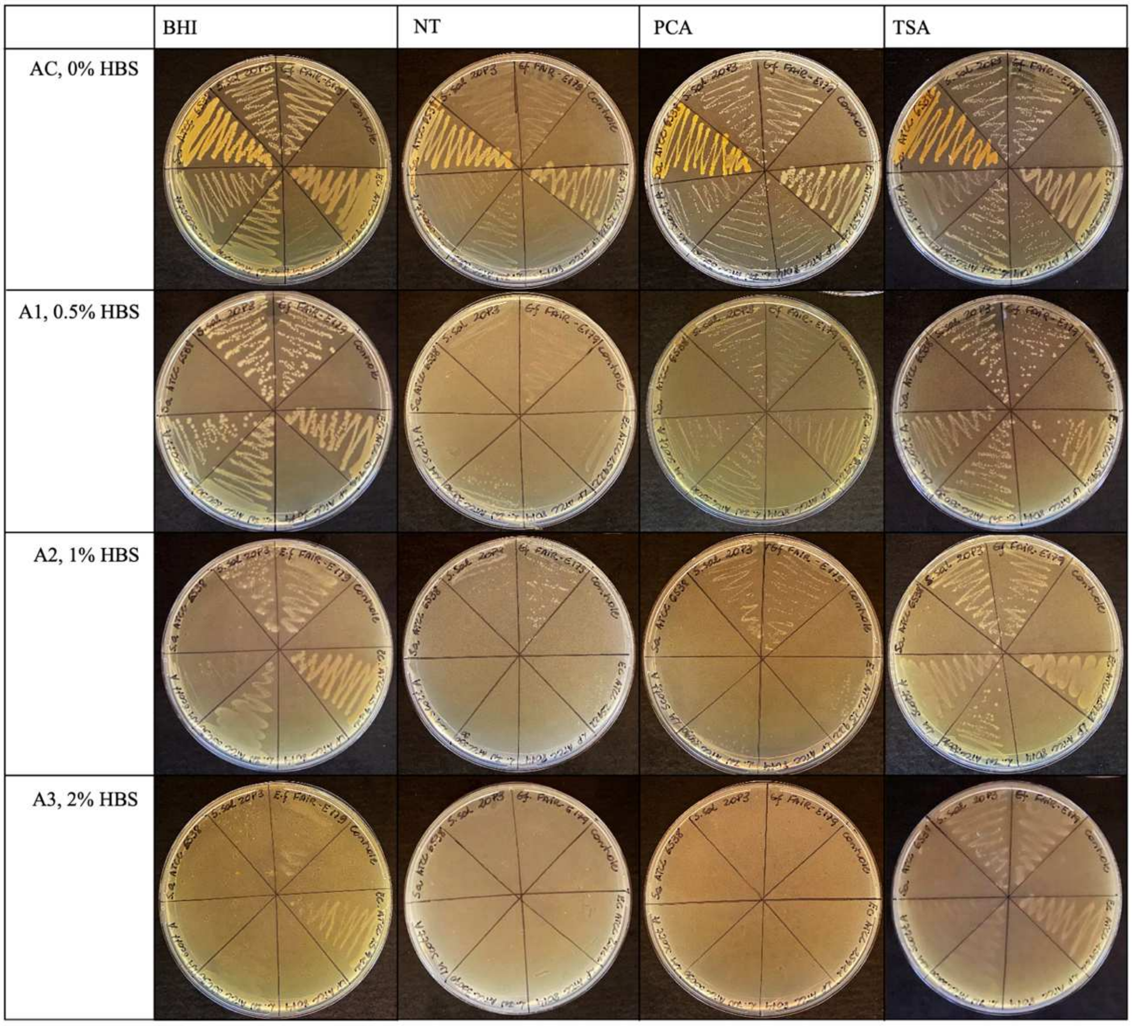
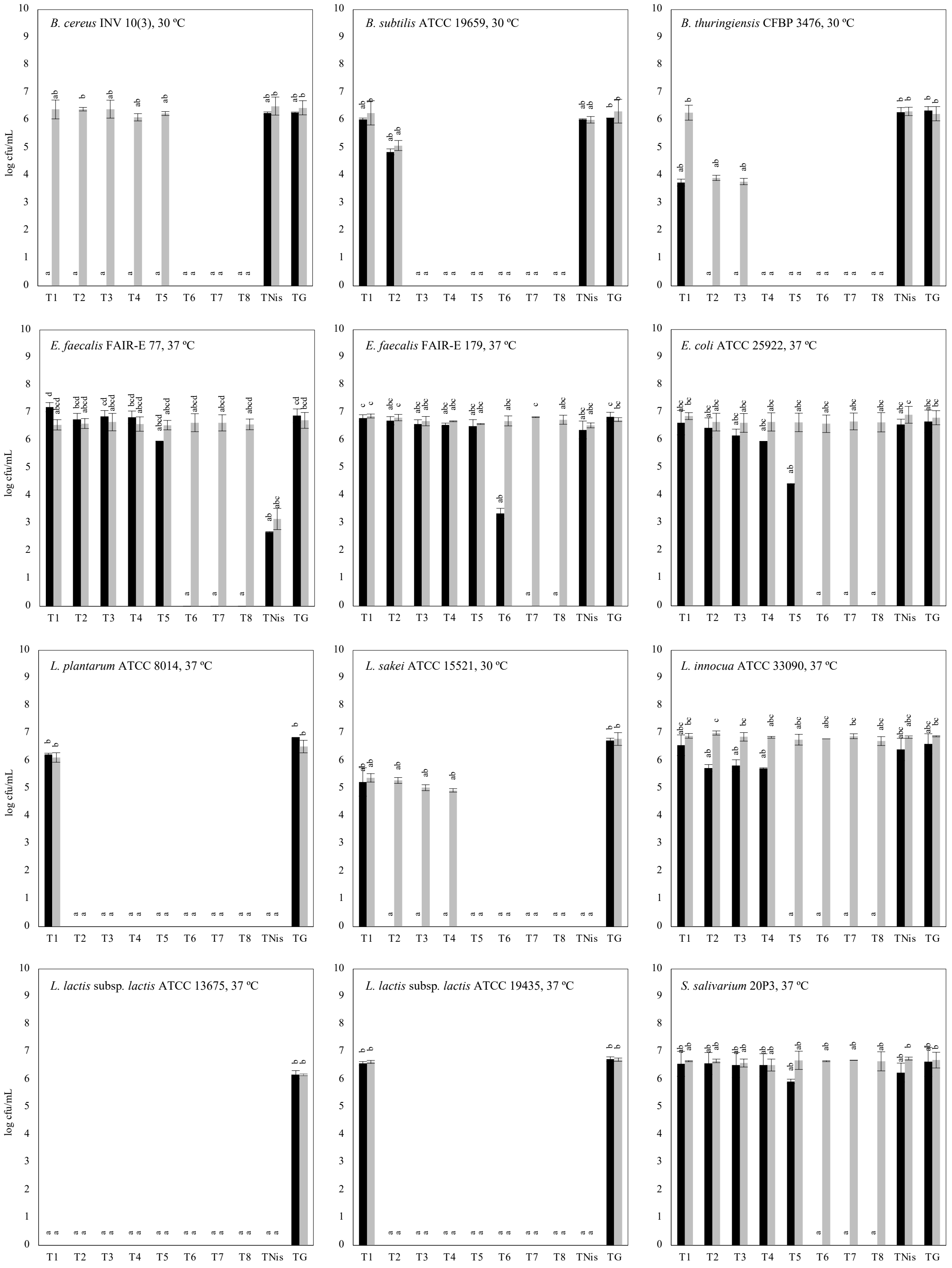


Figure 1. Comparison of the inhibitory activity of JOHA® HBS added to four different culture media through the streak assay against selected strains (n = 7).


B. cereus INV 10(3), 30 ºC
B. subtilis ATCC 19659, 30 ºC
B. thuringiensis CFBP 3476, 30 ºC
E. faecalis FAIR-E 77, 37 ºC
E. faecalis FAIR-E 179, 37 ºC
E. coli ATCC 25922, 37 ºC
L. plantarum ATCC 8014, 37 ºC
L. sakei ATCC 15521, 30 ºC
L. innocua ATCC 33090, 37 ºC
L. lactis subsp. lactis ATCC 13675, 37 ºC
L. lactis subsp. lactis ATCC 19435, 37 ºC
S. salivarium 20P3, 37 ºC
log cfu/mL
0
1
2
3
4
5
6
7
8
9
10
T1
T2
T3
T4
T5
T6
T7
T8
TNis
TG

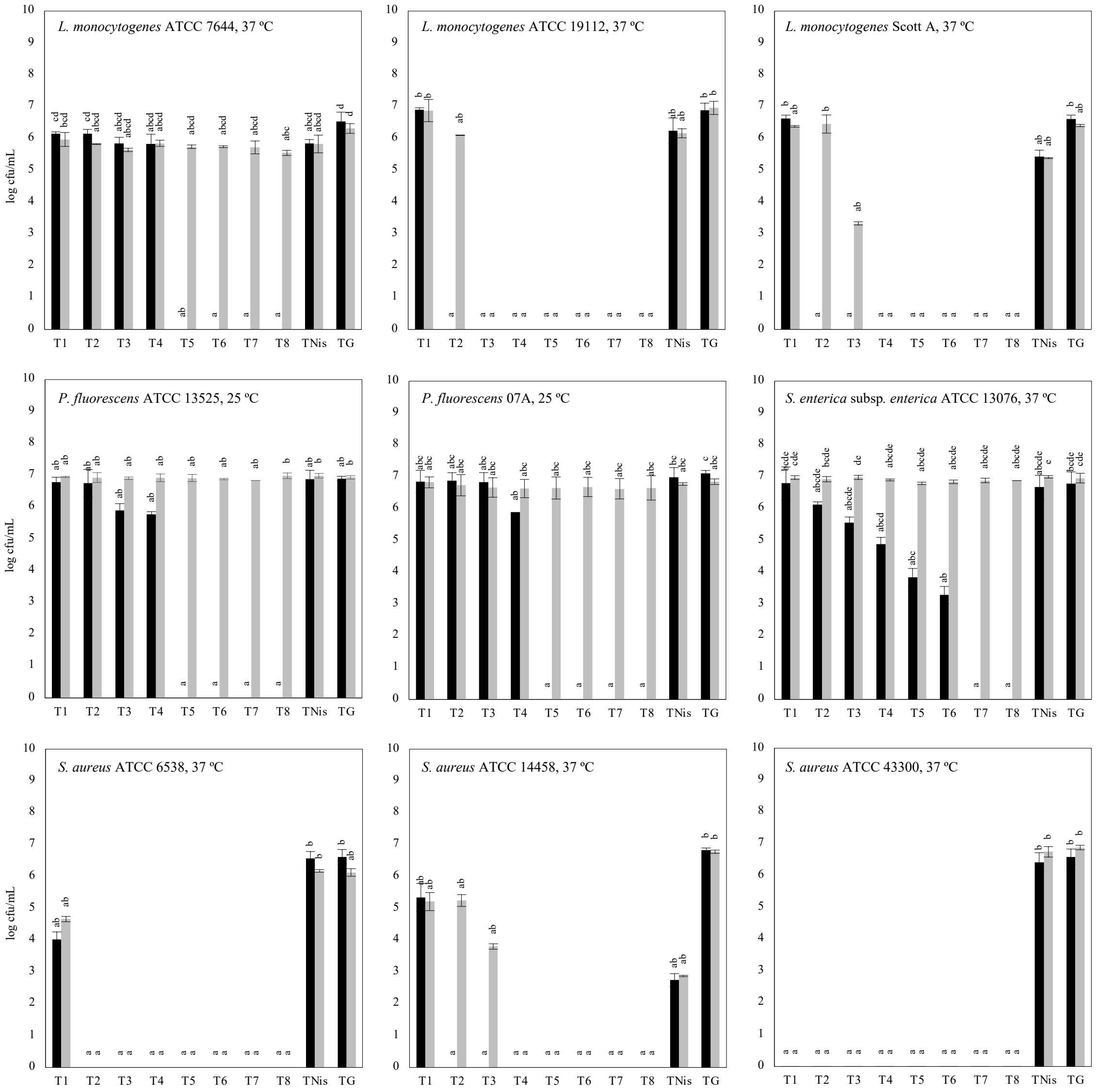


Figure 2. Mean counts (standard errors) of the selected target strains subjected to a broth assay to measure the effects of JOHA® HBS at specific conditions. Black bars: mean counts without pH correction; gray bars: mean counts with pH correction to 6.3. Treatments: (T1) 0.2% of HBS; (T2) 0.4% of HBS; (T3) 0.6% of HBS; (T4) 0.8% of HBS; (T5) 1% of HBS; (T6) 1.5% of HBS; (T7) 2% of HBS; (T8) 3% of HBS; (TNis) 0.0012% of nisin; and (TG) without HBS and nisin. Incubation temperatures are indicated in each graph, per selected strain. Different letters indicate significant differences of counts distribution per selected strain and treatment, based on Kruskal-Wallis and Dunn ($p < 0.05$); mean counts are presented only for illustrative purposes.

Table 1. Evaluation of the influence of the culture medium in the inhibitory activity of JOHA® HBS polyphosphate against selected target strains.

Agar medium treatment	*E. faecalis* FAIR-E 179	*E. coli* ATCC 25922	*L. plantarum* ATCC 8014	*L. innocua* ATCC 33090	*L. monocytogenes* Scott A	*S. aureus* ATCC 6538	*S. salivarium* 20P3
BHI							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	-	+	-
A3, 2% HBS	-	-	+	-	+	+	-
NT							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	+	+	-
A2, 1% HBS	-	-	+	+	+	+	-
A3, 2% HBS	+	+	+	+	+	+	+
PCA							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	+	+	-
A3, 2% HBS	+	+	+	+	+	+	+
TSA							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	-	+	-
A3, 2% HBS	-	-	+	-	-	+	-

Results: (+) presence of inhibitory activity without bacterial growth; (-) absence of inhibitory activity with bacterial growth.

Table 2. Evaluation of the inhibitory activity of JOHA® HBS added to different culture media against selected bacterial targets (n = 3).

Target strain and culture medium	Treatment			
	A1, 0.5% HBS	A2, 1% HBS	A3, 2% HBS	AC, 0% HBS
E. faecalis FAIR-E 179				
BHI	$8.73 \pm 0.014^{\text{bc, A}}$	$8.69 \pm 0.28^{\text{b, A}}$	$8.86 \pm 0.021^{\text{b, A}}$	$8.71 \pm 0.12^{\text{ab, A}}$
NT	$8.18 \pm 0.19^{\text{ab, A}}$	$6.77 \pm 0.10^{\text{a, B}}$	$5.46 \pm 0.042^{\text{a, C}}$	$8.11 \pm 0.035^{\text{a, A}}$
PCA	$7.96 \pm 0.24^{\text{a, A}}$	$6.48 \pm 0.011^{\text{a, B}}$	$5.03 \pm 0.05^{\text{a, C}}$	$8.13 \pm 0.25^{\text{a, A}}$
TSB	$8.89 \pm 0.12^{\text{c, A}}$	$9.07 \pm 0.28^{\text{b, A}}$	$8.97 \pm 0.12^{\text{b, A}}$	$8.97 \pm 0.28^{\text{b, A}}$
L. monocytogenes Scott A				
BHI	$9.05 \pm 0.020^{\text{c, A}}$	$9.02 \pm 0.065^{\text{c, A}}$	$4.71 \pm 0.12^{\text{c, B}}$	$9.39 \pm 0.22^{\text{b, A}}$
NT	$4.84 \pm 0.011^{\text{b, B}}$	N.D.	N.D.	$8.55 \pm 0.010^{\text{a, A}}$
PCA	$6.88 \pm 0.091^{\text{a, B}}$	$3.91 \pm 0.048^{\text{a, C}}$	$3.46 \pm 0.070^{\text{a, D}}$	$8.51 \pm 0.14^{\text{a, A}}$
TSB	$8.6 \pm 0.058^{\text{d, A}}$	$6.41 \pm 0.12^{\text{d, B}}$	$5.94 \pm 0.089^{\text{d, C}}$	$8.97 \pm 0.15^{\text{c, A}}$
S. aureus ATCC 6538				
BHI	$5.08 \pm 0.18^{\text{a, B}}$	$5.05 \pm 0.021^{\text{bc, B}}$	$5.13 \pm 0.085^{\text{b, B}}$	$9.37 \pm 0.043^{\text{a, A}}$
NT	$4.99 \pm 0.20^{\text{a, B}}$	$4.78 \pm 0.26^{\text{ab, B}}$	$4.02 \pm 0.14^{\text{a, C}}$	$8.96 \pm 0.12^{\text{a, A}}$
PCA	$5.06 \pm 0.11^{\text{a, B}}$	$5.4 \pm 0.028^{\text{c, B}}$	$4.05 \pm 0.22^{\text{a, C}}$	$9.25 \pm 0.072^{\text{a, A}}$
TSB	$4.89 \pm 0.06^{\text{a, B}}$	$4.42 \pm 0.053^{\text{a, BC}}$	$4.27 \pm 0.045^{\text{a, C}}$	$9.01 \pm 0.16^{\text{a, A}}$

N.D: data not definable, below the detection limit of the method. Capital letters: comparison between JOHA® HBS percentages. Lowercase: comparison between the culture medium tested.

Table 3. Evaluation of the inhibitory activity of JOHA® HBS against twenty-one target strains (n = 23) through four different qualitative protocols.

Target strains and protocols	Treatments									
	T1	T2	T3	T4	T5	T6	T7	T8	TNis	TG
B. cereus INV 10(3)										
streak assay	+	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	+	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
B. subtilis ATCC 19659										
streak assay	-	-	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
B. thuringiensis CFBP 3476										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
E. faecalis FAIR-E 77										
streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
E. faecalis FAIR-E 179										
streak assay	-	-	-	-	-	-	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	-	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
E. coli ATCC 25922										

streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
L. plantarum ATCC 8014										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
L. sakei ATCC 15521										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. innocua ATCC 33090										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	+	+	-	-
L. lactis subsp. *lactis* ATCC 13675										
streak assay	+	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	+	+	-
agar spot assay	+	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. lactis subsp. *lactis* ATCC 19435										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	+	+	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. monocytogenes ATCC 7644										

streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. monocytogenes ATCC 19112										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
L. monocytogenes Scott A										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
P. fluorescens ATCC 13525										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	+	+	-	-
P. fluorescens 07A										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
S. enterica subsp. *enterica* ATCC 13076										
streak assay	-	-	-	-	-	-	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	-	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
S. aureus ATCC 6538										

streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	+	+	+	+	+	+	-
S. aureus ATCC 14458										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	+	+	+	+	+	+	-
S. aureus ATCC 43300										
streak assay	+	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	+	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
S. salivarium 20P3										
streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-

Treatments: (T1) 0.2% of HBS; (T2) 0.4% of HBS; (T3) 0.6% of HBS; (T4) 0.8% of HBS; (T5) 1% of HBS; (T6) 1.5% of HBS; (T7) 2% of HBS; (T8) 3% of HBS; (TNis) 0.0012% of nisin, and (TG) without HBS and nisin. Streak assay and agar spot assay: (+) presence of inhibitory activity without bacterial growth; (-) absence of inhibitory activity with bacterial growth. Spot-on-the-lawn assay and well-diffusion assay: (+) presence of inhibitory activity with inhibition zones; (-) absence of inhibitory activity without inhibition zones.

CHAPTER III.

RESEARCH ARTICLE TWO

Influence of emulsifying salts on the growth of *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124 in processed cheese

Fusieger et al.

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Chapter presented according to the final format of the journal.

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Title page

Influence of emulsifying salts on the growth of *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124 in processed cheese

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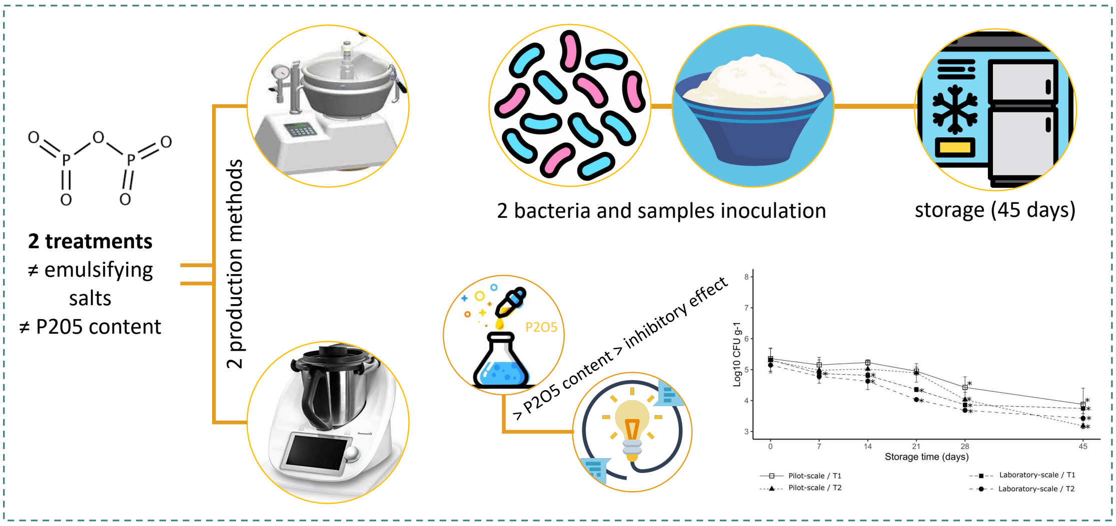
Abstract

Processed cheese is a dairy product with multiple end-use applications, where emulsifying salts play a fundamental role in physicochemical changes during production. Moreover, some of these salts may be a strategy to control spoilage and pathogenic microorganisms, contributing to safety and shelf life extension. This study aimed to evaluate the *in vitro* inhibitory activity of two emulsifying salts (ESSP = short polyP and BSLP = long polyP) against *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124, and to compare the *in situ* effects of two emulsifying salts treatments (T1 = 1.5% ESSP and T2 = 1.0% ESSP + 0.5% BSLP) in processed cheeses obtained by two different methods (laboratory- and pilot-scales), during 45-day storage at 6 °C. *C. perfringens* ATCC 13124 growth was not affected in vitro or in situ ($p > 0.05$), but both of the treatments reduced *B. thuringiensis* CFBP 4376 counts in the tested condition. Counts of the treatments with B. *thuringiensis* CFBP 3476 presented a higher and faster reduction in cheeses produced by the laboratory-scale method (1.6 log cfu/g) when compared to the pilot-scale method (1.8 log cfu/g) ($p < 0.05$). For the first time, the inhibitory effect of emulsifying salts in processed cheeses obtained by two different methods was confirmed, and changes promoted by laboratory-scale equipment influenced important interactions between the processed cheese matrix and emulsifying salts, resulting in *B. thuringiensis* CFBP 4376 growth reduction.

Keywords: dairy, food safety, melting salts, microbiological stability, polyphosphate, shelf life, spore-forming bacteria.

Graphical abstract



1. Introduction

Processed cheese is a relatively new category of dairy products, set up a little over 100 years ago independently in Europe and the United States. Driven by the need to increase the shelf life of natural cheese and with the possibility of recycling defective cheeses, processed cheeses have emerged with a distinct texture, flavor and/or functional properties. Through a technology able to standardize these properties, a versatile product was introduced to the market [1–4]. From a technological point of view, processed cheese is obtained by heating a mixture of cheeses, under partial vacuum conditions and constant stirring, in the presence of emulsifying salts which are able to chelate calcium, disrupting the casein structure and solubilizing it [1,5]. The process can be performed by using different equipment, in which various types of cookers with different designs and operating conditions can be employed. The equipment may differ mainly on the type of mixing or agitating systems, the type and mechanism of heating, and the mode of production, in batch or continuous, according to the industry needs [6,7]. The appropriate selection of the equipment and the type and quantity of emulsifying salts are among the most important variables to produce processed cheese with desired final properties [8,9].

During processed cheese production, monophosphates, polyphosphates (polyP) and citrates are emulsifying salts commonly used [5]. These salts consist of a monovalent cation, typically sodium, bound to a charged polyvalent anionic tail that acts as a calcium sequester, involved in the disruption of the calcium–phosphate-linked protein network present in natural cheese. Both functions have an effect in hydrating the caseins, allowing the interaction between water and fatty phases, thereby producing a homogeneous cheese emulsion [5,8].

The use of different emulsifying salts with different emulsification capabilities is a growing practice in the dairy industry [5,10,11]. Furthermore, based on the antimicrobial effect of some emulsifying salts, the addition of phosphates has recently been proposed as a strategy to control spoilage and pathogenic microorganisms, and ultimately, to reduce the sodium content and prolong the shelf life of processed cheese [5,12,13].

Most of the studies on emulsifying salts in processed cheese have been focused on mechanical, physicochemical, microstructure, and rheology properties, as well matrix stability, formation of casein fibrils and sensory traits [10,11,14–17]. However, the number of scientific studies on the antimicrobial potential of emulsifying salts is low and few studies have been published in the last five years [13,18,19]. Processed cheeses have been associated with certain microbiological safety concerns and despite the lower susceptibility to microbial spoilage, spore-forming bacteria, such as *Bacillus thuringiensis* and *Clostridium perfringens* have been associated with blowing and putrid odor development [20,21]. Although the spore-forming bacteria originated mainly from raw milk, the production environment can also act as a source of contamination, due to the ability of these microorganisms to survive cleaning and sanitation steps [22]. For this reason, the addition of emulsifying salts can be considered in many processed cheese productions as a control option.

Based on the above scenario, this is the first study aimed at comparing the inhibitory activity of emulsifying salts in processed cheese obtained by following different processes. In particular, the inhibitory activity of two emulsifying salts against *B. thuringiensis* CFBP 3476 and *C. perfringens* ATCC 13124 inoculated in processed cheese obtained on a laboratory-scale and a pilot-scale was explored.

2. Materials and Methods

2.1. Materials and Treatments

Two mixtures of emulsifying salts composed of polyphosphates (E452) and sodium phosphates (E339) were used in this study. These mixtures differ in the total phosphate content (P_2O_5), the average chain length, and pH (1% solution), as follows: (ESSP) emulsifying salt composed of short polyP, used as an emulsifier agent, 59.7 ± 1% P_2O_5, short-chain polyphosphate, pH 9.0 ± 0.3; (BSLP) bacteriostatic salt composed of long polyP, used as a bacteriostatic specialty, 69 ± 1% P_2O_5, long-chain polyphosphate, pH 6.0 ± 0.5.

To evaluate the inhibitory activity of emulsifying salts by in vitro and in situ approaches, two treatments were performed: Treatment 1 (T1) = 1.5% ESSP; and Treatment 2 (T2) = 1.0% ESSP + 0.5% BSLP.

The inhibitory activity of the emulsifying salts was tested against *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124. The strains were stored at −80 °C in a culture medium and 20% (*v/v*) glycerol. For strains cultivation, incubation was in a brain heart infusion broth (BHI; Kasvi, São José dos Pinhais, Brazil) for 22 ± 2 h at 30 °C was used for *B. thuringiensis* CFBP 3476 and trypticase soy broth (TSB; Kasvi) for 22 ± 2 h at 37 °C under anaerobiosis was used for *C. perfringens* ATCC 13124. Before use, the strains were transferred into a broth medium, incubated and checked for purity in BHI or TSA agar (Kasvi) after the incubation time. Then, a single colony was transferred to the appropriate broth medium, incubated, and diluted to turbidity similar to a 0.5 standard on the MacFarland scale, equivalent to 1.5×10^8 cfu/mL.

For the assessment of in vitro inhibitory activity, these cultures were diluted on a decimal scale to approximately 1.5 × 10^6 cfu/mL. For the assessment of in situ inhibitory activity, the fresh cultures were centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, Waltham, USA) at 3260× *g* for 15 min at 7 °C. After discarding the supernatant, the pellets were suspended in NaCl solution 0.85% (*w/v*) until turbidity similar to a 0.5 standard on the MacFarland scale, corresponding to approximately 1.5 × 10^8 cfu/mL. Cultures of *B. thuringiensis* CFBP 3476 and *C. perfringens* ATCC 13124 were separately used as primary inoculum in the processed cheese.

2.2. In Vitro Inhibitory Activity

To detect the in vitro inhibitory activity, a streak assay protocol was performed on nutrient agar (NT; Kasvi) [23]. Briefly, NT agar was prepared according to the manufacturer's instructions (Kasvi), autoclaved (121 °C for 15 min), and cooled to 45 °C, after which the emulsifying salts were added. To simulate the processed cheese processing, agar solutions were homogenized, heated for 5 min at 90 °C in a water bath (MA156/6, Marconi, Piracicaba, Brazil), and poured into Petri dishes. *B. thuringiensis* CFBP 3476 and *C. perfringens* ATCC 13124 at approximately 1.5 × 10^6 cfu/mL were streaked onto the plates and incubated under the appropriate conditions for each strain for 48 h. Sterile water was used as a negative control and agar plates without any addition of emulsifying salts were considered as a control treatment for bacterial growth. After incubation, the absence of bacterial growth was indicative of the inhibitory activity of the emulsifying salt treatments. This assay was conducted in duplicate and with three independent repetitions.

2.3. In Situ Inhibitory Activity

2.3.1. Production of Processed Cheese

To produce processed cheese, the following ingredients and concentrations were used: Mozzarella cheese aged for 2 weeks, 49% (*w/w*); butter, 17% (*w/w*); sterile mineral water, 39.5% (*w/w*), and emulsifying salts according to treatments T1 and T2. For each treatment, the production was carried out under aseptic conditions and the processing was performed using two different methods: (I) laboratory-scale, using a Thermomix TM-5 (Vorwerk & Co. Thermomix; GmbH, Wuppertal, Germany) with a 2 kg capacity, and (II) pilot-scale, using Stephan Geiger homogenizer-grinder GUMSK 12E NR12 (Geiger Indústria de Máquinas Ltda., Pinhais, PR, Brazil) with a 7 kg capacity. The ingredients were blended with subsequent processing by heating for 6 min until reaching a 90 °C melting temperature, and shearing with a constant increase in rotation until 1100 rpm for the laboratory-scale method, and until 1500 rpm for the pilot-scale method; the mixing speed was performed for 5 min at 90 °C.

Then, four 200 g portions of each processed cheese treatment were split into sterile bags (polyethylene bags; height 178 mm, width 76 mm, and thickness 0.07 mm; Kasvi) and cooled to 12 ± 2 °C. Two portions of each treatment were separately inoculated with each target strain (described above) at a final concentration of 1.5×10^5 cfu/g and homogenized for 5 min (Bagmixer 400VW; Interscience, Paris, France). The inoculated processed cheese was equally divided into nine sterile bags (25 g) and stored at 6 ± 2 °C for 45 days. In the next step, one portion of each processed cheese treatment was not inoculated and considered as the negative control samples (blank), and another portion was also not inoculated and used to monitor the pH and Aw during the storage time; each of them was divided equally into six sterile bags (25 g) and kept at the same storage conditions. Two independent repetitions of each processed cheese treatment were performed. For each repetition, different batches of ingredients (cheese, butter, sterile mineral water, and emulsifying salts) were used. The flow chart of the experimental design is reported in Figure 1.

2.3.2. Microbiological Analyses

Processed cheese samples were subjected to microbiological analyses during the 45-day storage at 6 ± 2 °C (at day 0, day 7, day 14, day 21, day 28, and day 45). In detail, a 25 g of sample was added to 225 mL of citrate solution 2% (*w/v*), homogenized, and 10-fold-diluted in NaCl solution 0.85% (*w/v*). For samples inoculated with *B. thuringiensis* CFBP 3476, aliquots of 100 μL of selected dilutions were plated onto mannitol egg yolk polymyxin (MYP; K25-1343, Kasvi) agar supplemented with egg yolk and polymyxin B (K25-6021, Kasvi) and incubated at 30 ± 1 °C for 18–24 h [24,25]. For samples inoculated with *C. perfringens* ATCC 13124, aliquots of 1 mL were pour-plated on tryptose sulfite cycloserine (TSC; K25-1029, Kasvi) agar supplemented with *Clostridium perfringens* selective supplement (K25-6020, Kasvi) and incubated under anaerobic conditions at 37 ± 1 °C for 24 h [26]. Control samples (blank samples) were analyzed following the same protocols described above and for total viable counting in plate count agar (PCA; Kasvi) incubated at 30 ± 1 °C for 72 h [27]. The results were expressed as colony-forming units per gram and counts were converted into Log10 (Log10 cfu/g). These analyses were conducted in triplicate and over two independent repetitions of each processed cheese treatment.

2.3.3. Water Activity and pH

The water activity (Aw) of blank processed cheese samples was measured using a water activity meter (AquaLab 3TE; Decagon Devices Inc., Pullman, WA, USA). The sample cup was filled to half its depth and placed in the sample chamber and the Aw was measured using the standard procedure. The pH of blank processed cheese samples was measured using a pH meter (Hanna Instruments Ltd., Leighton Buzzard, UK). These analyses were conducted in duplicate and over two independent repetitions of each processed cheese treatment.

2.4. Statistical Analysis

Statistical analyses were performed using SAS® Studio software (Release: 3.8; Enterprise Edition; SAS Institute, Cary, NC, USA). Data were checked for normality of residuals and homogeneity of variances using the Kolmogorov–Smirnov test and Bartlett's test, respectively, using the GLM and CAPABILITY procedures. Then, data were compared by ANOVA and Tukey tests ($p < 0.05$), using the GLM procedure. The R software, version 4.0.2 (The R Foundation, Boston, USA), and the RStudio, version 1.3.959 (Integrated Development for R. RStudio, Boston, USA), were used for graphical presentation with the ggplot2 package [28].

3. Results

3.1. In Vitro Inhibitory Activity

Results of the in vitro inhibitory activity, for both treatments T1 (1.5% ESSP) and T2 (1.0% ESSP + 0.5% BSLP), showed that *B. thuringiensis* CFBP 4376 growth was inhibited, whereas *C. perfringens* ATCC 13124 was not inhibited.

3.2. In Situ Inhibitory Activity

Plate counts for the processed cheese blank samples (without inoculation) did not present viable and cultivable cells during storage time. For the inoculated processed cheese samples, different profiles were found and the results are reported below.

All treatments showed a significant effect on the inactivation of *B. thuringiensis* CFBP 4376 in both production methods (laboratory and pilot-scale). In comparison with time zero, there was a significant difference ($p < 0.05$) from day 28 and 45 of storage in both production methods. In addition, for the laboratory-scale method a significant difference was observed after day 7 of storage for T1, and after day 14 of storage for T2 (Figure 2). Higher levels of inhibition were recorded for T2 after 45 days of storage, being 3.43 Log10 CFU/g for the laboratory-scale and 3.18 Log10 CFU/g for the pilot-scale.

According to Figure 3, none of the treatments showed an inhibitory effect on the multiplication of *C. perfringens* ATCC 13124. From the day 7 storage samples, the values of Log10 CFU/g increased and differed significantly from time zero ($p < 0.05$) and for both production methods. Between day 28 and day 45, a stationary phase was observed for all treatments.

3.3. Water Activity and pH

Blank samples produced at the pilot-scale showed significantly different pH values over time of storage for T2 (Table 1). For T1, only at day 21 of storage did the pH value not differ from time zero ($p > 0.05$). However, the pH of the blank samples was stable when produced at the laboratory-scale, since, in both treatments, there was no significant variation during storage ($p > 0.05$) compared to time zero.

According to the analysis of variance of the results obtained for water activity (Aw, Table 2), there was no statistical difference between the analyzed treatments ($p > 0.05$).

4. Discussion

The BSLP shows both bacteriostatic and fungistatic effects and prevents the outgrowth of aerobic and anaerobic spores, although the dosage rate depends on the microbial species and the matrix (according to the manufacturer's information). As BSLP is a long-chain polyP, its use is recommended simultaneously with other emulsifying salts, such as ESSP, mainly in spreadable and sliceable processed cheese production. In the present study, a standard formulation of ESSP, commonly applied in the dairy industry, and an additional formulation, with partial replacement by BSLP were investigated. While *C. perfringens* ATCC 13124 growth was not affected by any of the tested treatments, neither in vitro nor in situ, both treatments with PolyP emulsifying salts were able to control *B. thuringiensis* CFBP 4376 outgrowth, in both in vitro and in situ.

Buňková et al. [29] performed an in vitro study on the antimicrobial effects of three commercially available emulsifying salts on reference microorganisms and bacteria isolated from long-stored processed cheeses. The results of the present study agree with those reported by Buňková et al. [29], which described the positive inhibition of 0.5% emulsifying salts with 69.0% P_2O_5 treatment (the same P_2O_5 level as reported here for BSLP) against *B. cereus* CCM 3953, *Bacillus subtilis* CCM 2216, *Bacillus brevis* SPSM 4101, *Bacillus sphaericus* CCM 1615, *Bacillus stearothermophilus* SPSM 4103, *Bacillus* sp. NTS 01, NTS 02, NTS 03, NTS 05, and NTS 06. However, the same study reported that 0.5% emulsifying salts with a 59.7% P_2O_5 treatment (the same P_2O_5 level as reported here for ESSP) were not able to inhibit the growth of *B. subtilis* CCM 2216, *B. sphaericus* CCM 1615, *Bacillus* sp. NTS 01, NTS 03, NTS 05, and NTS 06. Lorencová et al. [30] carried out a study on the antibacterial effect of seven emulsifying salts at different chain lengths and different phosphate contents; the authors pointed out that the inhibitory effect can be affected by intrinsic factors, such as the number of phosphorus atoms and the acid–basic properties of emulsifying salts in aqueous solutions.

For processed cheese samples, the inactivation of *B. thuringiensis* CFBP 4376 growth by the emulsifying salt treatments was verified. Although the emulsifying salts had an effect on the inactivation of *B. thuringiensis* CFBP 4376 growth, a bacterial growth reduction was reached more rapidly along the storage time when the laboratory-scale method was applied; the means of T1 and T2 resulting in a 1.64 log unit reduction for laboratory-scale and in a 1.8 log unit reduction for pilot-scale samples at the end of storage time. The main differences between the two production methods were that on the laboratory-scale, no steam injection was performed and an 1100 rpm shearing was applied, while on the pilot-scale an indirect steam injection and a 1500 rpm shearing were performed. According to Guinee [8], a higher agitation speed during processing significantly increases the firmness and elasticity modulus of processed cheese and significantly reduces the level of flow and the fluidity of the melted product. In addition, the general bacteriostatic effect of phosphates may reflect interactions with bacterial proteins and the sequestration of calcium, which generally serves as an important cellular cation and cofactor for some microbial enzymes.

Species belonging to the genus *Bacillus* are commonly isolated from processed cheese [22,31] and a major focus is placed on the role of *B. cereus* in foodborne disease outbreaks. Furthermore, *B. thuringiensis* belongs to the phylogenetic group of *B. cereus* and it is biochemically identical to the *B. cereus* species [32]. Therefore, the occurrence of foodborne outbreaks of *B. thuringiensis* may be underestimated because routine microbial diagnostics of *B. cereus* and *B. thuringiensis* are not differentiated [33]. Therefore, the effects of the emulsifying salts observed here may be a useful strategy to control *Bacillus* spp. in processed cheese.

In addition, the *B. thuringiensis* CFBP 4376 growth inactivation can be explained by the mechanisms already described for *B. cereus*. According to Maier et al. [34], the antibacterial activity of polyphosphates with a high level of P2O5, such as BSLP, against *B. cereus* is related to the interaction of long-chain polyP in the exponential growth phase, affecting the cell morphology manifested by cell lysis and the inability of septum formation during division. Briefly, the authors proposed that polyP might influence the ubiquitous bacterial cell division protein FtsZ, whose GTPase activity is known to be strictly dependent on divalent metal ions.

For processed cheese samples inoculated with *C. perfringens* ATCC 13124, the bacterial growth was not inhibited under the tested conditions. Loessner et al. [19] evaluated the effect of long-chain polyP formulations on the growth of *Clostridium tyrobutyricum* ATCC 25755 in processed cheese spreads. Although the inoculation of spores was performed, the samples were processed with the addition of an emulsifying salt with 59.7% P2O5, and only at the end of the process was an aqueous solution of emulsifying salts with higher levels of P_2O_5 added to the samples. This is not a common procedure followed at an industrial scale. However, no viable cell was detected after 8 days of incubation by 0.5% emulsifying salts with higher levels of P2O5, and total inhibition was obtained until 50 days of storage in samples treated with 1.0% emulsifying salts with higher levels of P_2O_5 [19]. On the other hand, Akhtar et al. [35] performed a study to evaluate the effects of various polyPs (sodium polyP, tetrasodium pyrophosphate, sodium tripolyphosphate, and sodium acid pyrophosphate) on growth, sporulation, and spore germination of *C. perfringens*, and germination and outgrowth of *C. perfringens* spores. The authors pointed out that surprisingly, all 19 *C. perfringens* vegetative cells required higher polyP concentrations (1.0–1.4%) than those previously reported for other bacteria, such as *Staphylococcus aureus*, *B. cereus*, *Listeria monocytogenes*, *C. tyrobutyricum*, *C. pasteuranium*, and *C. butyricum*, where the minimum inhibitory concentration, independently of the type of polyP, an in vitro assay was at most 0.5%. According to Akhtar et al. [35], the high polyP resistance of *C. perfringens*, compared to other bacteria, could be attributed to the higher synthesis of phosphatases producing higher rates of polyP hydrolysis, and/or to differences in the cell wall structure.

In general, it is difficult to determine the polyP chain length and commonly only approximate estimations can be found in the literature. Therefore, polyP is characterized by the P_2O_5 content, which may reflect the average size of phosphate chains in a mixture, and the higher the P_2O_5 content, the greater the size of the chain; its concentration is given as a percentage rather than a molar [19,36]. BSLP is composed of 69.0 ± 1% P2O5, while ESSP of 59.7 ± 1% P2O5; consequently, the chain size of the BSLP is larger. The inhibitory effect of polyP is related to the length of the chain (condensation level), and polyP with a lower amount of phosphorus is generally less effective in the suppression of undesirable microorganism growth than long-chain phosphates [12,30]. Long-chain polyP are characterized by a high affinity for divalent metal ions (Ca^{2+} and Mg^{2+}), essential for the integrity of Gram-positive bacteria cell walls [5,37]. According to the above-mentioned results, the BSLP treatment was more effective against *C. perfringens* ATCC 13124 until 45 days of storage, even if no differences were observed for *B. thuringiensis* CFBP 4376.

Overall, the effect of polyP is mainly related to changes in environmental conditions where they induce changes in the pH and ionic strength of the solution, modifying protein configuration, solubility, and to the last extent, emulsifying salts bind Ca^{2+} [5,12]. PolyP are used to shift the pH of the cheese upwards, typically from 5.0–5.5 of natural cheese to 5.6–6.0 of pasteurized processed cheese, and the stabilization that occurs may be due to their high buffering capacity [1]. During processed cheese production, the pH change contributes to an enhanced dissociation and calcium-sequestering ability of the emulsifying salts, and an increased negative charge of the para-caseinate [1]. Thus, at a higher pH, more complete dissociation of the phosphate molecules provides better ion exchange.

Different emulsifying salts influence the final properties of processed cheeses due to their ability to exchange calcium ions for sodium ions and to stabilize pH values (buffering capacity) [38]. The ion exchange ability increases with the length of the polyphosphate chain [38,39]. In this way, processed cheese with the addition of short-chain emulsifying salts has a higher pH compared to the addition of long-chain emulsifying salts, as was the case for T1 and T2, respectively.

Regarding the Aw, one of the basic conditions for microbial growth, Awad et al. [40] evaluated the influence of different emulsifying salt mixtures on the Aw of block-processed cheese during storage at different temperatures for up to three months. The authors stated that neither the different combinations of emulsifying salts nor storage had a significant effect on the Aw of samples. In the present study, the detected Aw values are in accordance with those reported by Kim et al. [41] who, evaluating 800 processed cheese samples, found Aw values ranging from 0.8 to 1.0. In particular, the Aw values reported for cream cheese and cheese portions (0.97 ± 0.01 and 0.97 ± 0.02, respectively) were very close to the results reported in the present study (0.98 ± 0.004).

Finally, considering that the microbiological stability of processed cheese, as a low acidic complex food matrix, remains a challenge, the application of the hurdle theory represents the best strategy. In this scenario, the addition of polyP together with the microbiological quality of milk and other ingredients used in the manufacture of processed cheese becomes necessary. With the present study, for the first time, the inhibitory effect of emulsifying salts against *B. thuringiensis* in processed cheese obtained by two different methods was confirmed. Further studies on the use of different emulsifying salts are required to better establish microbiologically and technologically available formulations useful for the cheese industry, nevertheless, these preliminary findings must be considered as the first steps toward the application of multifunctional ingredients in processed cheeses.

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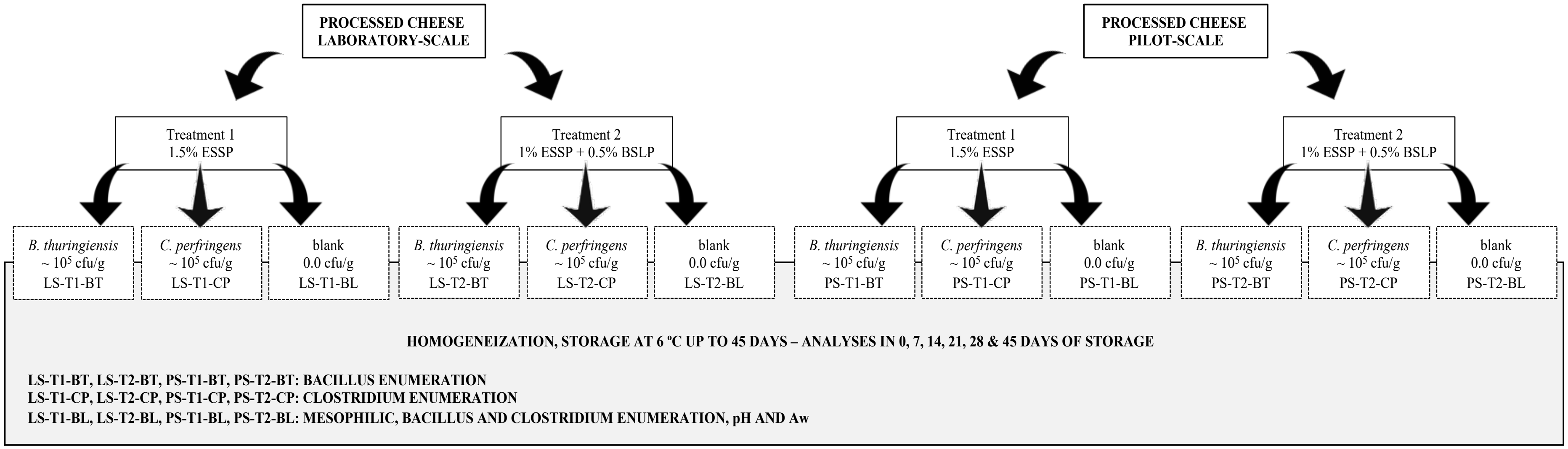

Figure 1. Flow chart of procedures employed in the production of processed cheese samples and the application of treatments to assess the inhibitory activity of emulsifying salts.

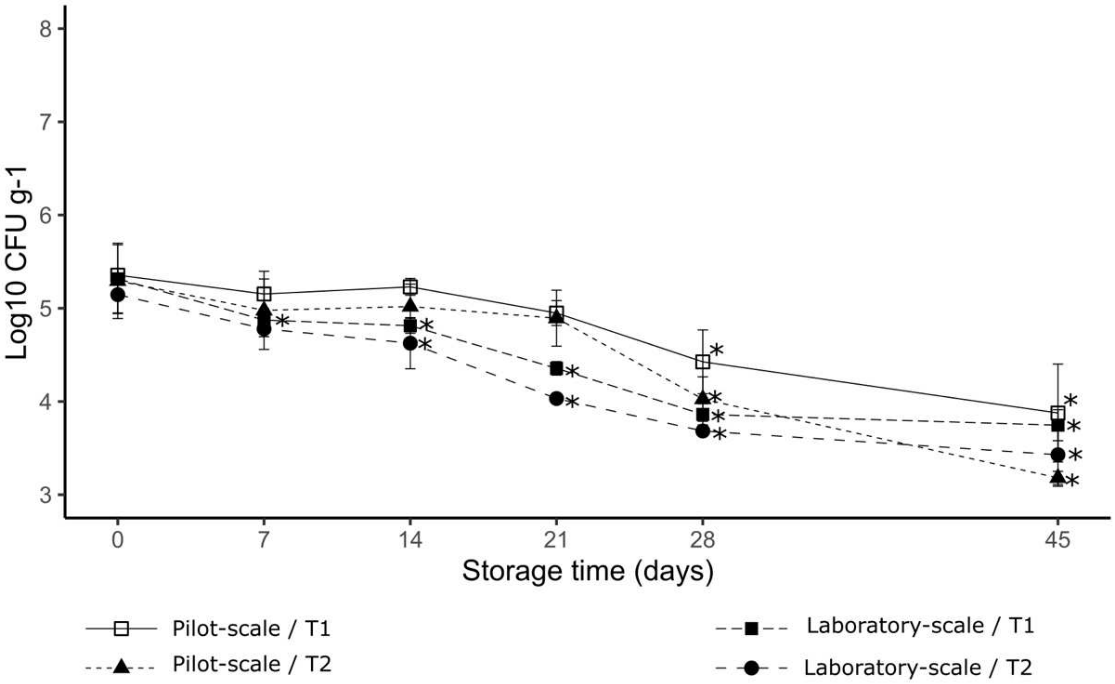

Figure 2. The effect of emulsifying salts on the inactivation of *B. thuringiensis* CFBP 4376, with different processing methods over storage time. (*) indicates a significant difference with the zero time of storage, according to Tukey's test ($p < 0.05$). Error bars show standard deviations of the mean.

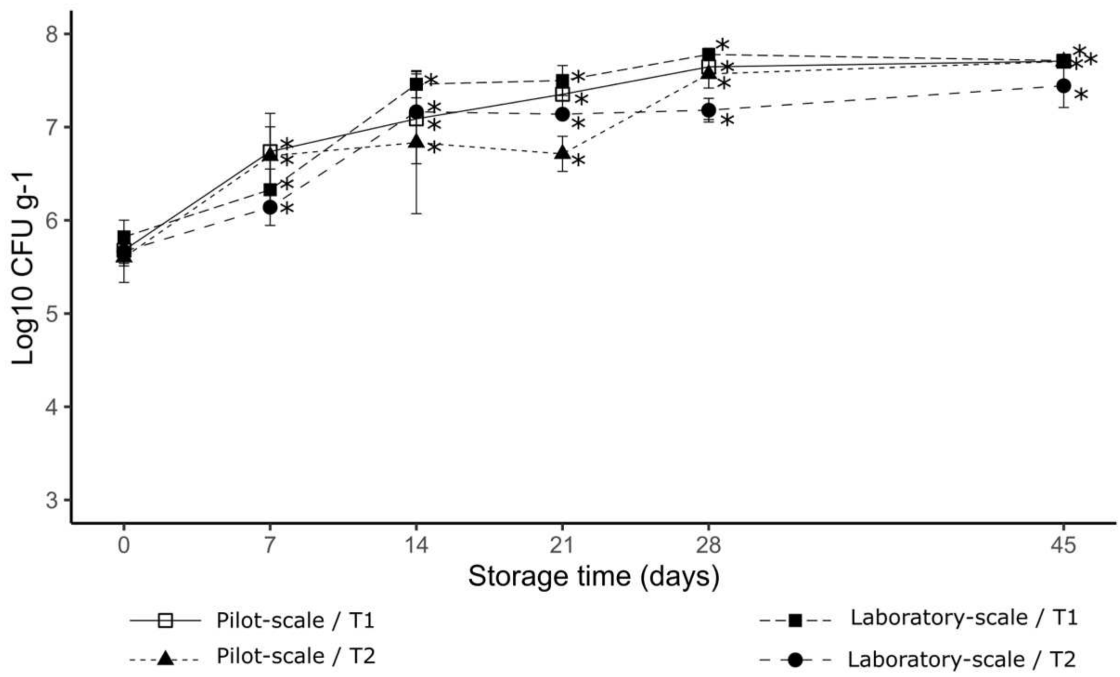

Figure 3. The effect of the emulsifying salts on *C. perfringens* ATCC 13124 development, with different processing methods over storage time. (*) indicates a significant difference with the zero time of storage, according to Tukey's test ($p < 0.05$). Error bars show standard deviations of the mean.

Table 1. The pH results were obtained according to the methods, treatments and storage time applied to blank processed cheese samples.

Storage Time (Days)	Pilot-Scale		Laboratory-Scale	
	T1	T2	T1	T2
0	5.88 ± 0.04 ab	5.77 ± 0.01 a	5.74 ± 0.01 a	5.52 ± 0.04 a
7	5.72 ± 0.04 d	5.49 ± 0.06 e	5.76 ± 0.12 a	5.52 ± 0.06 a
14	5.83 ± 0.07 bc	5.53 ± 0.01 de	5.87 ± 0.11 a	5.60 ± 0.04 a
21	5.92 ± 0.03 a	5.67 ± 0.04 b	5.75 ± 0.03 a	5.52 ± 0.04 a
28	5.78 ± 0.01 cd	5.59 ± 0.04 cd	5.80 ± 0.05 a	5.54 ± 0.07 a
45	5.85 ± 0.01 bc	5.63 ± 0.01 bc	5.78 ± 0.06 a	5.52 ± 0.03 a

Data are presented as mean ± standard deviation. Different letters within the column indicate a significant difference between storage times in each treatment, according to Tukey's test ($p < 0.05$).

Table 2. The Aw results were obtained according to the methods, treatments and storage time applied to blank processed cheese samples.

Storage Time (Days)	Pilot-Scale		Laboratory-Scale	
	T1	T2	T1	T2
0	0.983 ± 0.001	0.982 ± 0.001	0.985 ± 0.002	0.975 ± 0.010
7	0.985 ± 0.002	0.986 ± 0.001	0.983 ± 0.001	0.984 ± 0.001
14	0.991 ± 0.005	0.987 ± 0.005	0.990 ± 0.002	0.991 ± 0.007
21	0.983 ± 0.001	0.985 ± 0.002	0.979 ± 0.009	0.980 ± 0.012
28	0.984 ± 0.000	0.984 ± 0.002	0.986 ± 0.012	0.987 ± 0.010
45	0.984 ± 0.001	0.984 ± 0.001	0.983 ± 0.004	0.980 ± 0.004

Data are presented as mean ± standard deviation. No significant difference was observed according to Tukey’s test ($p > 0.05$).

CHAPTER IV.

RESEARCH ARTICLE THREE

Bactericidal and bacteriostatic effects of emulsifying salts of sodium polyphosphate on selected targets in processed cheese

Fusieger et al.

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Title page

Bactericidal and bacteriostatic effects of sodium polyphosphate emulsifying salts on selected targets in processed cheese

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Abstract

Processed cheeses (PC) are products with extended shelf-lives, they have in their composition such additives as emulsifying salts and preservatives. Emulsifying salts are used due their desirable emulsification properties; however, little is known about their inhibitory activity against microorganisms that can survive and multiply in these products; jeopardizing cheese quality or even posing as hazards to consumers. The aim is to determine how mixtures of emulsifying salts (ESSP: emulsifying salt composed of short polyP; BSLP: bacteriostatic salt composed of long polyP; and trisodium citrate) can influence the microbial populations in PC during 90 days of storage. A total of 14 treatments (T1 - T14) were evaluated against eight target strains (*Bacillus cereus* INV 10(3); *Bacillus subtilis* ATCC 19659; *Bacillus thuringiensis* CFBP 3476; *Clostridium perfringens* ATCC 13124; *Enterococcus faecalis* FAIR-E 179; *Listeria monocytogenes* Scott A; *Pseudomonas fluorescens* 07A; and *Staphylococcus aureus* ATCC 6538) inoculated into PC. A treatment composed of emulsifying salts without antimicrobial effect (ESSP 1.5%) was considered as the positive growth control, and another treatment composed of antimicrobial additives (nisin and potassium sorbate) was considered as the negative growth control. Bacterial growth was analyzed using ANOVA and Tukey ($p < 0.05$), based on selected treatment grouping. Most treatments resulted in some level of bactericidal or bacteriostatic effect against the target microorganisms. Bactericidal activity was evident against *Bacillus* spp., and bacteriostatic effect was clear against *C. perfringens, E. faecalis, L. monocytogenes* and *S. aureus* ($p < 0.05$) during the storage period. T13, composed of 1.5% BSLP, presented results comparable to those observed for the negative control for bacterial inhibition in PC samples during 90 days of storage. T13 was able to reduce the *B. cereus* INV 10(3) and *B. thuringiensis* CFBP 3476 population more than the negative control ($p < 0.001$), as well as present better results for the inhibition of *C. perfringens* ATCC 13124 and *L. monocytogenes* Scott A by the end of storage. The performance of the negative control was better than T13 only against *S. aureus* - ATCC 6538 ($p < 0.001$). Taken together, the results contribute to expanding the understanding of emulsifying salt application in PC, with a focus on microbiological safety and shelf-life.

Keywords: cheese model, dairy, melting salts, microbiological stability, polyP, shelf-life.

Highlights

- Replacement of emulsifying salts by bacteriostatic salts reduced bacterial growth.
- Higher concentrations of bacteriostatic salts lead to greater growth inhibition.
- The presence of emulsifying salts influences the effect of bacteriostatic salts.
- Phosphate interactions may influence bacteriostatic effects of emulsifying salts.

1. Introduction

Cheese is a widespread dairy product that has been produced and consumed since ancient times (Talbot-Walsh et al., 2018). Traditional cheese making procedures have regularly been adapted to develop a number of novel products, such as processed cheese (PC) (Fu et al., 2018). In Brazil, as well in The Southern Common Market (Mercosul), PC is defined as a dairy product obtained by grinding, mixing, melting and emulsification of natural cheeses by heating and emulsifying agents, added or not with dairy by products, spices and condiments, being cheese the main ingredient (Brasil, 1997; Mercosul, 1996). The use of phosphate or polyphosphate emulsifiers is approved as additive due to their functional properties, which can be used at a maximum concentration of 20 g/kg in the final product (expressed as phosphorus pentoxide, P_2O_5) (Brasil, 1997; Mercosul, 1996). The ability of emulsifying salts to promote protein hydration (calcium-sequestrating ability) and dispersion during cheese processing is ordered as: polyphosphates > pyrophosphates > monophosphates ~ citrates (Fox et al., 2017). The polyphosphates' ability to cause a protein hydrating effect is related to the greater calcium-sequestering capacity, with calcium-sequestering being pH-dependent. All these factors contribute to improved emulsification properties, given the structure of the PC (Fox et al., 2017; Salek et al., 2019; Talbot-Walsh & Selomulya, 2021).

From a microbiological point of view, the use of preservatives, such as nisin and potassium sorbate, inhibit the growth of some microorganisms that may be present; which can cause problems during storage of PC (Buňková & Buňka, 2017). Nisin is a preservative widely used by the food industry, especially in dairy products. This antimicrobial is produced by certain *Lactococcus lactis* strains and is effective for controlling bacterial growth, especially Gram-positive bacteria (Ibarra-Sánchez et al., 2020). Potassium sorbate is industrially produced via neutralizing the sorbic acid with potassium hydroxide and has been used as an efficient preservative in food; it is active against fungi, yeasts and some aerobic bacteria, due to its direct activity on the cell membrane, enhancing permeability to other acids (Dehghan et al., 2018; González-Fandos & Dominguez, 2007).

The antimicrobial activity of emulsifying salts was already suggested and noticeable through *in vitro* studies, demonstrating their inhibitory effects on the growth and in the increase of the length of the lag phase of several bacterial groups and species. The main bacterial targets of such studies were foodborne pathogens (*Clostridium* spp., pathogenic *Escherichia coli*, *Salmonella* spp., enterotoxigenic *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, among other) and dairy spoilage groups (*Bacillus* spp., *Clostridium* spp., *Corynebacterium* spp., *Micrococcus* spp., *Pseudomonas* spp., lactic acid bacteria, among other) (Akhtar et al., 2008; Borch & Lycken, 2007; Buňková et al., 2008; Frenzel et al., 2011; Fusieger et al., 2022; Lee et al., 1994a; Loessner et al., 1997; Lorencová et al., 2012; Maier et al., 1999; Moon et al., 2011; Obritsch et al., 2008; Zaika et al., 1997). These studies tested different emulsifying salts at various concentrations, and in general the highest concentrations were more effective against Gram-negative bacteria.

The microbiological application of emulsifying salts in PC was also explored in a few *in situ* studies. Martinez-Rios et al. (2019) developed a growth model and confirmed that increasing concentrations of phosphate salts reduced the growth of *L. monocytogenes* in PC, while Loessner et al. (1997) demonstrated that long-chain polyphosphate formulations were effective in controlling *C. tyrobutyricum* in PC spreads. Tanaka et al. (1979) and Ter Steeg et al. (1995) showed that polyphosphate appeared to be more inhibitory than citrate against *C. botulinum* in PC products. Glass et al. (2017) predicted the botulinum safety of processed cheese and pointed out that the addition of 0.8 and 1.6% disodium phosphate appears to be more effective than 1.2%. In this way, emulsifying salts may be important in controlling bacterial growth in PC, especially against bacteria that have already been isolated from this dairy matrix, as *B. cereus*, *B. subtilis*, *B. thuringiensis*, *Clostridium* spp., *Enterococcus spp.*, *L. monocytogenes*, *P. fluorescens*, *S. aureus* (Abbar & Mohammed, 1986; Catania et al., 2021; Hassanin, 1993; Javed et al., 2010; Kahraman et al., 2010; Lycken & Borch, 2006; Oliveira et al., 2018; Palmas et al., 1999). In many cases, the occurrence of pathogenic and nonpathogenic bacteria generally indicates secondary contamination when these microorganisms enter the products after the production (especially when the product is not packed at an appropriate temperature under 60-70 ºC) or worse hygienic conditions during the production (Buňková & Buňka, 2017; Linton & Harper, 2008).

Considering that emulsifying salts are ingredients required for PC production and little information is available about their inhibitory effect against bacteria that may potentially contaminate PC, this study aims to thoroughly evaluate how mixtures of emulsifying salts can influence different bacterial populations recognized for compromising cheese quality and as health risks to consumers.

2. Materials and methods

2.1. Additives and treatments

Emulsifying salt mixtures composed of polyphosphates (E452) and sodium phosphates (E339) were used in this study. These mixtures differ in the total P_2O_5 content, the chain length and pH (1% solution), and are as follows: (ESSP) emulsifying salt composed of short-chain polyphosphate, 59.7 ± 1% P_2O_5, pH 9.0 ± 0.3; (BSLP) emulsifying salt composed of long-chain polyphosphate, 69 ± 1% P_2O_5, pH 6.0 ± 0.5. Trisodium citrate (E331; Merck, Darmstadt, HE, Germany), pH 7.5 – 9.0 (5% solution), was also used as an emulsifier agent. Nisaplin® 2.5% w/w nisin A (E234; Danisco, Copenhagen, Denmark) and potassium sorbate (E202; Sigma-Aldrich Co., St. Louis, MO, USA) were used as commercial antimicrobial agents. Fourteen treatments (n = 14) were performed to evaluate the inhibitory activity of the additives (Table 1).

2.2. Target bacteria and inoculum preparation

Eight bacterial strains (n = 8) from the Laboratório de Pesquisa em Leites e Derivados (InovaLeite, Universidade Federal de Viçosa) were included in this study as targets: *B. cereus* INV 10(3) (UHT milk isolate); *B. subtilis* ATCC 19659 (maize isolate); *B. thuringiensis* CFBP 3476 (unknown source); *C. perfringens* ATCC 13124 (human gas gangrene isolate); *E. faecalis* FAIR-E 179 (mini pig feces isolate); *L. monocytogenes* Scott A (ATCC 49594, human isolate from an outbreak linked to drinking pasteurized milk); *P. fluorescens* 07A (raw cow's milk isolate; Martins et al., 2005); and *S. aureus* ATCC 6538 (human lesion isolate). These bacterial species were chosen because they have already been reported and identified in vegetative form in PC. The strains were stored at -80 ºC in brain heart infusion broth (BHI, Kasvi, São José dos Pinhais, PR, Brazil) or trypticase soy broth (TSB, Kasvi) in 20% (v/v) glycerol. Before use, strains were recovered from BHI or TSB broth, incubated at the specific growth temperature (25, 30 or 37 ºC) overnight, and checked for purity in BHI (Kasvi) or trypticase soy agar (TSA, Kasvi) after incubation.

For inoculum preparation, recovered strains were centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, Waltham, MA, USA) at 3,260 × *g* for 15 min at 7 °C, and the cell pellet was suspended in saline solution 0.85% (w/v) to a turbidity similar to the standard 0.5 of the MacFarland scale, equivalent to approximately 1.5 × 10^8 cfu/mL; these cultures were used as inoculum for PC samples.

2.3. Production of PC samples

For PC sample production the following ingredients were used: Mozzarella cheese (Sérvulo, Senador Firmino, Minas Gerais, Brazil), butter (Sérvulo), sterile mineral water, emulsifying salts and/or commercial antimicrobials agents according to the fourteen treatments (Table 1). PC production was carried out under aseptic conditions and processing was carried out using a Vorwerk Thermomix TM5 (Vorwerk & Co. Thermomix; GmbH, Wuppertal, Germany) with indirect heating and without a creaming process. In each production batch, 714 g of Mozzarella cheese and 289 g of butter were homogenized with 617.5 g of water, the emulsifying salts and/or commercial antimicrobial agents (amount was calculated for the total weight of the melt) were then added. Subsequent processing was achieved through heating and shearing, under the following conditions: (1) 6 min with a constant increase in rotation (from 100 to 750 rpm) until 90 °C (melting temperature); (2) 5 min at 1100 rpm at 90 ºC; and (3) 2 min with a constant decrease in rotation (from 1100 to 40 rpm) and constant temperature.

Then, portions of PC (300 g) were divided into sterile bags (Kasvi) and cooled to 12 ± 2 ºC. Portions of each treatment were inoculated separately with each prepared inoculum (as described above) at a final concentration of 3 × 10^5 cfu/g, and homogenized for 5 min (Bagmixer 400VW; Interscience, Paris, France). The inoculated PC portions were divided equally into 9 sterile bags (25 g) and stored at 6 ± 2 ºC for 90 days, following the storage temperature recommendations of the current Brazilian legislation (Brasil, 1997). Two portions of each treatment were not inoculated and used as negative control (without inoculation) samples and for pH and Aw analysis; each were also equally divided into 9 sterile bags (25 g) and keep under the same storage conditions. In total, 2,520 samples of PC were produced. Two independent repetitions for each PC treatment were performed, and a flow chat of the experiment procedure is presented in Figure 1.

2.4. Microbial analysis

PC samples were subjected to microbial analysis during the 90-day storage (d_0, d_7, d_{14}, d_{21}, d_{28}, d_{45}, d_{60}, d_{90}) at 6 ± 2 ºC. For the microbiological plate counts, 25 g of sample was added to 225 mL of citrate solution 2% (w/v), homogenized, and 10-fold diluted in saline solution 0.85% (w/v). Selected dilutions of each inoculated sample were plated, according to the following enumeration protocols: (1) for samples inoculated with *B. cereus* INV 10(3) and *B. thuringiensis* CFBP 3476, aliquots of 100 µl were plated onto Mannitol Egg Yolk Polymyxin (MYP; K25-1343, Kasvi) agar supplemented with egg yolk and Polymyxin B (K25-6021, Kasvi), and incubated at 30 ± 1 °C for 18 to 24 h (ISO 7932:2004; Zhao et al., 2021); (2) for samples inoculated with *B. subtilis* ATCC 19659, aliquots of 100 µl were plated onto Plate Count Agar (PCA; K25-1056, Kasvi) and incubated at 30 ± 1 °C for 24 h; (3) for samples inoculated with *C. perfringens* ATCC 13124, aliquots of 1 mL were plate-poured onto Tryptose Sulfite Cycloserine (TSC; K25-1029, Kasvi) agar supplemented with Clostridium Perfringens Selective Supplement (K25-6020, Kasvi) and incubated under anaerobic conditions at 37 ± 1 °C for 24 h (ISO 7937:2004); (4) for samples inoculated with *E. faecalis* FAIR-E 179, aliquots of 1 mL were plate-poured onto de Man, Rogosa and Sharpe (MRS; K25-1043, Kasvi) agar and incubated at 32 ± 1 °C for 48 ± 3 h (ISO 15214:1998); (5) for samples inoculated with *L. monocytogenes* Scott A, aliquots of 100 µl were plated onto Listeria Chromogenic Agar Base, according to Ottaviani and Agosti (ALOA; K25-1345, Kasvi), supplemented with Listeria Chromogenic Lipase C Supplement (K25-6031, Kasvi) and Listeria

Chromogenic Selective Supplement (K25-6040, Kasvi), and incubated at 37 ± 1 °C for 48 h (ISO 11290-2:2017); (6) for samples inoculated with *P. fluorescens* 07A, aliquots of 100 µl were plated onto Pseudomonas Agar Base (PAB; CM0559, Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with Pseudomonas CFC selective agar supplement (SR0103, Oxoid) and incubated at 25 ± 1 °C for 48 h (ISO 11059:2009); and (7) for samples inoculated with *S. aureus* ATCC 6538, aliquots of 100 µl were plated onto Baird-Parker (BP; K25-610004, Kasvi) agar supplemented with egg yolk and potassium tellurite (Merck), and incubated at 37 ± 1 °C for 48 h (ISO 6888-1:1999).

Control samples (without inoculation) were analyzed using all the protocols mentioned above, and for total viable count in PCA (Kasvi) agar and incubated at 35 ± 1 °C for 48 h (ISO 4833:2003). After incubation, growth colonies were enumerated, and results were expressed as log cfu/g.

2.5. Water activity and pH analysis of PC control samples

Water activity (Aw) of PC control samples (without inoculation) were estimated using a water activity meter (AquaLab Series 3TE; Decagon Devices Inc., Pullman, WA, USA). Briefly, the sample cup was filled to half its depth and placed in the sample chamber and Aw measured using standard instrument procedure. The pH of the control samples were measured directly (at a temperature of 25 ± 1 ºC) by inserting a glass tip electrode of a calibrated pHmeter (Hanna Instruments Ltd., Leighton Buzzard, UK) directly into the sample. Water activity and pH analysis were measured in triplicate on the control samples during the storage time.

2.6. Statistical analysis

The 14 treatments were divided into 5 groups, according to the agents that were added to the PC samples, and are as follows: (I) composed of antimicrobial additives (nisin and potassium sorbate), T1, T2 and T3, used as negative control for bacterial growth; (II) composed of trisodium citrate and emulsifying salt ESSP, T4 and T5, and used as positive control for bacterial growth; (III) produced with the partial replacement of the emulsifying salt ESSP with BSLP, T6, T7, T8, T9 and T10 + a positive and negative control; (IV) composed of different concentrations of BSLP, T11, T12, T13 and T14 + a positive and negative control; and (V) composed of selected treatments from each of the 4 groups. Counts obtained during storage time were checked for normality (Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera, $p < 0.05$) and were compared based on treatments through ANOVA and Tukey's honest significant difference (HSD) test ($p < 0.05$). Aw and pH results were compared based on mean values during storage time of each treatment through ANOVA and Tukey's honest significant difference (HSD) test ($p < 0.05$). All analyses were conducted using the XLSTAT 19.01 software (Addinsoft, New York, NY, USA).

3. Results

Plate counts for the PC control samples (without inoculation) did not present viable, cultivatable cells during storage (data not shown). This indicated that no microbial contamination occurred during the preparation of the samples, and that they were stable over the 90 days of storage. To describe the results of the 14 treatments (Table 1) on the inhibition of eight target strains in inoculated PC samples we create different topics based on the groups presented in the Table 1. Thus, the results are presented below in the same sequence which the groups/treatments were presented in Table 1. In addition, the group V was created and is comprised by a treatment choose from each of previous groups, based on their inhibition effect.

3.1. Group I: commercial antimicrobial additives

Group I considered the treatments that had the addition of antimicrobial additives (nisin and potassium sorbate) commonly applied in PC (T1, T2 and T3). Treatments T2 and T3 showed bactericidal or bacteriostatic effects against all the target microorganisms (except *P. fluorescens* 07A) (Figure 2). Treatment T1 also showed such behavior against *Bacillus* spp. and *S. aureus* ATCC 6538; however, the bacteriostatic effects against *E. faecalis* FAIR-E 179 was maintained until 45 days of storage, and no inhibition was observed against *C. perfringens* ATCC 13124, *L. monocytogenes* Scott A, and *P. fluorescens* 07A. For samples inoculated with *P. fluorescens* 07A, high levels of bacterial growth were recorded up to day 21 of storage, followed by a stationary phase up to 90 days of storage. Significant differences in microbial population were also observed during PC storage and the antimicrobial effect of the treatments varied according to the target (Figure 2). *Bacillus* spp. were amongst the microorganisms that were most sensitive to the treatments, followed by *S. aureus*. In general, T1 and T3 exhibited significantly better bactericidal action against *Bacillus* spp. ($p < 0.0001$), while T3 stood out against *S. aureus* ATCC 6538 ($p < 0.0001$). On the other hand, the bacteriostatic effect of T2 and T3 against *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, and *L. monocytogenes* Scott A was clearly better ($p < 0.0001$) than T1 (Figure 2). Higher levels of inhibition were recorded with T3 against the bacterial targets and, therefore, this treatment was selected as the negative growth control to compare with the other groups.

3.2. Group II: trisodium citrate and emulsifying salt ESSP

Group II included the treatments with trisodium citrate and emulsifying salt ESSP, commonly used for melting PC ingredients (Figure 3). Treatments T4 and T5 were used as positive control for bacterial growth. During the 90 days of storage, lower counts were obtained in T5 in relation to T4 against all the bacteria tested, and this result was also shown amongst the inoculated PC samples, with *B. subtilis* ATCC 19659, *C. perfringens* ATCC 13124, *L. monocytogenes* Scott A and *S. aureus* ATCC 6538 (Figure 3). Considering these findings, T5 was selected as the positive growth control to compare with the other groups.

3.3. Group III: partial replacement of ESSP by BSLP

PC samples with the partial replacement of ESSP emulsifying salt by BSLP (T6, T7, T8, T9 and T10) were included in group III. The observed trends were similar to those previously reported for group II; however, bactericidal action was more restricted to *Bacillus* spp. (Figure 4). For PC samples inoculated with *B. cereus* INV 10(3), the most effective treatment for microbial inhibition was T10, where at day 7 of storage the presence of viable cultivable cells was not verified ($p < 0.0001$). The same profile was found for T3 and T9, however at day 21 of storage ($p < 0.0001$). Comparing to the others target strains, *B. cereus* INV 10(3) was the most sensitive to the treatments. Against *B. subtilis* ATCC 19659 and *B. thuringiensis* CFBP 3476, treatments T8, T9 and T10 showed the higher inhibitory activity among the treatments with partial replacement of ESSP by BSLP. In addition, bacteriostatic effects (at different levels) were detected against the other target microorganisms through this replacement (except *P. fluorescens* 07A) (Figure 4); against *C. perfringens* ATCC 13124, bacteriostatic activity was verified in T9 up to 28 days of storage and in T10 throughout the entire storage period; against *E.*

faecalis FAIR-E 179, bacteriostatic activity was verified between days 21 and 90 of storage in T10; against *L. monocytogenes* Scott A, bacteriostatic activity was verified in T8, T9, and T10 up to 45 days of storage followed by a bactericidal behavior until the end of storage time; and against *S. aureus* ATCC 6538, bacteriostatic activity was observed in T7, T8, T9 and T10 until 60 days of storage followed by a bactericidal behavior until the end of storage time. Interestingly, in the treatments where the replacement of ESSP by BSLP was greater (T9 and T10), a higher level of bacterial inhibition was observed (Figure 4). This trend highlights the better performance of T9 and T10 (with higher concentrations of BSLP) when compared to T6, T7 and T8 for the inhibition of *Bacillus* spp., *C. perfringens* ATCC 13124*, E. faecalis* FAIR-E 179, and *L. monocytogenes* Scott A in PC samples. As T10 presented the higher inhibitory activity, this treatment was chosen as the comparative treatment for the final analysis.

3.4. Group IV: different concentrations of BSLP

Group IV was composed of PC samples containing different concentrations of BSLP (T11, T12, T13 and T14). In general, all treatments had a similar inhibitory profile, but the performance of T13 and T14 (with higher concentrations of BSLP) stood out against *Bacillus* spp., *E. faecalis* FAIR-E 179, and *L. monocytogenes* Scott A when compared to other treatments (Figure 5). Amongst all evaluated formulations, these treatments were the only ones which presented some level of inhibition against *P. fluorescens* 07A. Comparing BSLP treatments (T13 and T14) with T3 (negative growth control), only the PC samples inoculated with *S. aureus* ATCC 6538 (T3) showed the best antimicrobial activity during the storage period. T13 and T14 were able to reduce the *B. cereus* INV 10(3) and *B. thuringiensis* CFBP 3476 populations in less time than T3 ($p < 0.001$), as well as produce better results in the inhibition of *C. perfringens* ATCC 13124 and *L. monocytogenes* Scott A by the end of storage. In general, lower counts were obtained for T13 and T14 against the target strains, however T13 was selected to compare the group in the final analysis, due to the concentration of BSLP.

3.5. Group V: selected treatments

All the treatments in group V contain the same final concentration of emulsifying salts (1.5% w/w) and was composed of the four selected treatments from the other groups based on their inhibition effect: T3 (0.00125% nisin + 0.1% PS + 1.5% ESSP) from group I, T5 (1.5% ESSP) from group II, T10 (0.25% ESSP + 1.25% BSLP) from group III, and T13 (1.5% BSLP) from group IV. As previously mentioned in this study, T5 (composed of emulsifying salts without antimicrobial effect) was considered the positive control for bacterial growth; and the formulation that included antimicrobial additives already applied in PC production (nisin and potassium sorbate – T3) was considered as the negative control for bacterial growth. Similar results were observed for T10 (0.25% ESSP + 1.25% BSLP) and T13 (1.5% BSLP). However, T13 presented a significantly ($p < 0.001$) better bacteriostatic effect against *Enterococcus faecalis* FAIR-E 179 and *Pseudomonas fluorescens* 07A at most of the evaluated time points, and reduced the *B. thuringiensis* CFBP 3476 population faster when compared with T10. T13 was able to reduce the *B. cereus* INV 10(3) and *B. thuringiensis* CFBP 3476 populations in less time than T3 ($p < 0.001$); as well as present better results in the inhibition of *C. perfringens* ATCC 13124 and *L. monocytogenes* Scott A by the end of storage. The performance of T3 was better than T13 only against *S. aureus* ATCC 6538 ($p < 0.001$).

3.6. The influence of ESSP on BSLP inhibitory activity

Although it was not the main objective of the study, the results of the treatments that contained the same concentration of BSLP (T7 × T11 and T9 × T12) showed different behaviors. The treatments in which BSLP was applied alone showed greater inhibitory activity; that is, equal concentrations of BSLP result in less bacterial inhibition when in the presence of ESSP. These results were more evident when treatments were applied against *Bacillus* spp., as the microbial population reduced in less time (Supplementary Table 1) or by the end of storage, when the inhibition of *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179 and *L. monocytogenes* Scott A was at least 1 log when compared to treatments with ESSP (Supplementary Table 1).

3.7. Water activity and pH

The raw pH and Aw data obtained over the storage time for the PC control samples are shown in Supplementary Table 2. After the production of PC control samples (day 0), the pH values ranged between 5.01 to 5.93, being higher for control samples produced only with 1.5% citrate and lower for control samples produced only with BSLP (Supplementary Table 2). At the end of the storage time (day 90), the pH values ranged between 4.95 (T14) to 6.45 (T4) and, as reported for the initial pH (day 0), the treatment containing 1.5% citrate showed a higher pH and treatments containing only BSLP showed a lower pH. During the storage time, significant differences ($p < 0.001$) were observed for T4 after 60 and 90 days (Supplementary Table 2). Regarding the Aw, treatments T1, T2, T3, T4 and T11 did not show statistical differences during the storage time (Supplementary Table 2).

4. Discussion

The microbial stability and safety of most traditional and novel foods depend on a combination of several preservative factors (hurdles) which, when acting together, the microorganisms present in the food are unable to overcome (Leistner and Rahman, 2020). In this study, potassium sorbate, nisin and emulsifying salts, even at high concentration, were not able to prevent the growth of *P. fluorescens* 07A in PC stored for 90 days. However, significant effects on growth control of *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *S. aureus* ATCC 6538 were obtained. The use of emulsifying salts attracts attention due to the possibility to inhibit microbial growth and, consequently, can be an applicable preservation factor to assure the quality and safety of PC. Thus, the obtained data may be useful for industries to assess and design PC formulations aiming to extend shelf-life through functional ingredients that can perform technological and preservative functions in combination.

Nisin and potassium sorbate are well established antimicrobial ingredients in the food industry, but without technological functions in PC, and the use of an emulsifying salt is required. The results of the present study show that nisin at 12.5 mg/kg plus 1.5% ESSP (T1) did not prevent the growth of *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *P. fluorescens* 07A in PC samples; however, it prevented the growth of *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, and *S. aureus* ATCC 6538 (Figure 2). On the other hand, potassium sorbate plus 1.5% ESSP (T2) showed bacteriostatic effects against *E. faecalis* FAIR-E 179 and *L. monocytogenes* Scott A (Figure 2). When compared to the use of nisin or potassium sorbate plus 1.5% ESSP, the association of nisin and potassium sorbate plus 1.5% ESSP

(T3) had significant effects in controlling the growth of target strains (except for *P. fluorescens* 07A; Figure 2). This result corroborates with the study of Khanipour et al. (2016), who demonstrated the combined effect of salt, potassium sorbate and nisin on the probability of growth of *C. sporogenes* in a high moisture PC analogue. The authors showed that nisin had a significant inhibitory effect against *C. sporogenes*; however, a high concentration of nisin (240 ppm) did not prevent the growth of *C. sporogenes* in the absence of salt and/or potassium sorbate.

On the other hand, trisodium citrate and ESSP are used as emulsifier agents and are not commonly used to play a bioconservative role in PC. This was confirmed in our results, treatments supplemented with 1.5% trisodium citrate (T4) or 1.5% ESSP (T5) did not prevent the growth of *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, *P. fluorescens* 07A, and *S. aureus* ATCC 6538 in PC samples during 90 days of storage (Figure 3), and higher counts were obtained compared to the other treatments tested against *Bacillus* spp. (Figures 3 and 6). Ter Steeg et al. (1995) assessed the effects of combinations of stability factors on growth of proteolytic *C. botulinum* in PC and the results showed that replacement of phosphate by trisodium citrate has a negative influence on *C. botulinum* stability. Similar results were reported by Tanaka et al. (1979), where sodium citrate did not have the preservative effectiveness of either disodium phosphate or a mixture of disodium and trisodium phosphate against *C. botulinum* spores in PC. Although *C. botulinum* was not included in this study, because the probability of occurrence of *C. perfringens* in PC is higher than that of *C. botulinum* (Buňková & Buňka, 2017), our results showed similar behavior to the above mentioned studies on bacterial survival and growth in PC supplemented with trisodium citrate or ESSP. For all targets tested, 1.5% ESSP (T5) showed lower counts and significant differences in at least two days of analyzed storage time (Figure 3).

Other ways to prevent bacterial growth may be effective. Long-chain polyphosphates, as BSLP, have been shown in previous *in vitro* studies to inhibit Gram-positive bacteria (Buňková et al., 2008; Loessner et al., 1997; Maier et al., 1999). The technological role performed by ESSP can be added to the preservative role of BSLP, and thus, be an alternative blend for the cheese industry. In general, the results of the treatments with partial replacement of ESSP by BSLP showed that there is a reduction in Gram-positive bacterial growth with increasing BSLP concentrations (Figure 4). These findings are probably related to the difference in the number of phosphorus atoms in a molecule of ESSP and BSLP, <10 phosphorus atoms in a molecule of short polyphosphates and >10 phosphorus atoms in a molecule of long polyphosphates, respectively (Nagyová et al., 2014). On the other hand, these findings may also be related to pH values, where lower values were obtained in treatments with higher BSLP content (Supplementary Table 2). In a US patent, Kohl & Ellinger (1972) suggested that the number of phosphorus units in a polyphosphate may affect its antimicrobial activity, with greater inhibitory effect associated with long-chain polyphosphates. Subsequently, studies confirmed that long-chain phosphates have a higher suppression of undesirable microorganism growth compared to short-chain phosphates (Lee et al., 1994a; Lee et al., 1994b). It is important to mention that it is complex to determine the exact phosphates chain length and commonly only approximate estimations can be found in the literature. Therefore, phosphates are usually characterized by the P_2O_5 content, which may reflect the average size of phosphate chains in a mixture, that is, the higher the P_2O_5 content, the greater the size of the chain, and their concentration is given in percent rather than molarity (Christ & Blank, 2018; Loessner et al., 1997); BSLP presents 69 ± 1% P_2O_5, while ESSP presents 59.7 ± 1% P_2O_5, consequently, the chain size of BSLP is larger.

Furthermore, the reduction in Gram-positive bacteria growth with increasing BSLP concentrations (Group III, Figure 3) or only in the presence of BSLP (Group IV, Figure 4), may also be related to the high affinity (cleavage) of long-chain polyphosphates for divalent metal ions (Ca^{2+} and Mg^{2+}), which they bind readily, resulting in bactericidal and bacteriolytic effects (Buňková & Buňka, 2017; Lee et al., 1994b). These ions form cross bridges between the teichoic acid chains in the cell walls of Gram-positive bacteria and are essential for maintaining the integrity of the cell, consequently, bacterial metabolism (Lee et al., 1994b; Maier et al., 1999). Gram-positive targets (*B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *S. aureus* ATCC 6538) were always more sensitive than Gram-negative targets (*P. fluorescens* 07A) due to the absence of teichoic acid in the cell wall of Gram-negative bacteria (Swoboda et al., 2010). This aligns with previous studies demonstrating that polyphosphates are particularly interesting thanks to the inhibitory effect on the growth and/or spore germination of many Gram-positive bacteria and the inhibitory effects of polyphosphates on Gram-negative bacteria are described mainly in laboratory conditions (Buňková & Buňka, 2017; Fusieger et al., 2022; Lorencová et al., 2012).

When different concentrations of BSLP were used alone, the inhibition profile was similar even in different concentrations (Figure 5). A different behavior was reported by Glass et al. (2017) in the prediction of the safety of open shelf-stable PC using the parameters of moisture, pH, NaCl, and disodium phosphate to inhibit toxin production by *C. botulinum*. Even if different bacterial targets and emulsifying salts were used to those in our study, the authors reported that the addition of 0.8 and 1.6% disodium phosphate appears to be more effective than 1.2% disodium phosphate, yielding a horseshoe-shaped curve rather than a dose-dependent curve. Also, it is unclear why the 0.8% phosphate treatments were more inhibitory than the 1.2% treatments (Glass et al., 2017).

In the meantime, the same concentration of BSLP resulted in different inhibition profiles when in the presence or absence of ESSP (Supplementary Table 1), a behavior that may influence PC shelf-life, especially against *Bacillus* spp. This allows us to hypothesize that BSLP in presence of ESSP leads to a synergistic interaction and, as a result, reduced the inhibitory potential of BSLP. Other studies have reported similar behavior (Glass et al., 2017; Loessner et al., 1997). Loessner et al. (1997) evaluated the effect of long-chain polyphosphates on the growth of *C. tyrobutyricum* ATCC 25755 spores in PC spreads. Two different PC models were produced: (A) 2.0% of an emulsifying salt with 59.7 ± 1% P_2O_5 (same P_2O_5 concentration of ESSP used in this study) plus 0.1, 0.3, 0.5, and 1.0% of an long-chain polyphosphate with 69 ± 1% P_2O_5 (same P_2O_5 concentration of BSLP used in this study), and inoculated with 5×10^5 spore/g and; (B) 1.5% of an emulsifying salt with 59.7 ± 1% P_2O_5 plus 0.5 and 1.0% of an long-chain polyphosphate with 69 ± 1% P_2O_5, and inoculated with 2.5×10^6 spore/g. In model A, only the treatment supplemented with 1.0% long-chain polyphosphate was able to inhibit *C. tyrobutyricum* during 49 days of storage. On the other hand, in model B both treatments (supplemented with 0.5 and 1.0% long-chain polyphosphate) presented the same behavior (Loessner et al., 1997). We believe that the bacterial behavior reported by Loessner et al. (1997) and in our results occurs due to the reduction of the emulsifying salt with 59.7 ± 1% P_2O_5 in the formulation (Supplementary Table 1). There is no information available to explain the difference between the inhibitory levels in this case, but these findings will be verified in our further research.

Regarding the pH values, treatments with higher content of ESSP showed a higher pH, such as T1, T2, T3 and T5, and lower pH values were obtained according the BSLP content increased in the treatments, such as T10, T13 and T14 (Supplementary Table 2). Phosphates are used to shift the pH of the cheese upwards, typically from 5.0-5.5 of natural cheese to 5.6-6.0 of pasteurized processed cheese, and the stabilization that occurs may be due to their high buffering capacity (Fox et al., 2017). However, treatments with higher BSLP content did not show the recommended pH for PC; future studies with standardization of pH values will be considered. These treatments also showed the lowest bacterial counts, leading us to believe that pH may be one of the factors that interfered with the inhibition of bacterial targets. As we did not measure the pH of the inoculated samples, we do not know their real pH in order to allow the association. According to Buňka et al. (2014), different emulsifying salts influence the final properties of PC due to their ability to exchange calcium ions for sodium ions and to stabilize pH values (buffering capacity). The ion exchange ability increases with the length of the polyphosphate chain (Buňka et al., 2014; Nagyová et al., 2014), and this explain the pH increase or decrease according to the amount of short-chain emulsifying salts and/or long-chain emulsifying salts, respectively (Supplementary Table 2). In relation to the Aw results (Supplementary Table 2), the obtained Aw values are in accordance with those reported by Kim et al. (2018), that evaluated 800 processed cheese samples and found Aw values ranging from 0.8 to 1.0. Awad et al. (2004) evaluated the influence of different mixtures of emulsifying salt in block PC during storage at different temperatures for up to three months and the storage has a slight effect on the Aw of PC samples, but not significant.

In view of the experimental parameters chosen (relatively high starting inoculum concentration and low storage temperature), T10 with 0.25% ESSP and 1.25% BSLP, and T13 with 1.5% BSLP (Figure 6), may be suitable tools to control the growth of Gram-positive bacteria under more realistic conditions, where initial contamination are lower

(Buňková & Buňka, 2017). However, it could tum out that the total BSLP content (or other available polyphosphates with preservative potential) necessary to control bacterial growth could be lower once more detailed studies are available to determine the specific polyphosphate and P_2O_5 content responsible for antimicrobial action and, more important, the synergistic interaction between different emulsifying salts. Further studies will be carried out to evaluated bacterial spores and the differences between the influence of PC composition, mainly considering the Ca^{2+} and Mg^{2+} content, to better understand their relationship in bacterial inhibition and to determine the ideal concentration of phosphates. Furthermore, Glass et al. (2017) considered PC after the package is opened in their predictive models and this is another factor to be considered. In fact, we have a broad area of knowledge that can be explored.

Finally, here we have provided relevant insights into the antimicrobial effect of emulsifying salts in PC. It has been shown that the composition of emulsifying salts significantly influences bacterial inhibition and the phosphate interactions that may infer such activity. PC samples manufactured with BSLP alone or where the replacement of ESSP by BSLP was greater, a better bactericidal or bacteriostatic effect was identified against *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *C. perfringens* ATCC 13124, *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *S. aureus* ATCC 6538. The T13 results were comparable to those observed for T3 (negative control for bacterial growth), but each one had a specific performance according to the target microorganism. Based on the results, we have been able to expand our understanding of the application of emulsifying salts in PC, with a focus on microbiological safety and shelf-life to provide more quality for cheese industry

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CRediT authorship contribution statement

Andressa Fusieger: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. **Sidney Rodrigues de Jesus Silva**: Methodology, Validation, Investigation. **Raiane Rodrigues da Silva**: Methodology, Validation, Investigation. **Anderson Carlos Camargo:** Validation, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Jaqueline Aparecida Honorato**: Methodology, Validation, Investigation. **Camila Gonçalves Teixeira**: Validation, Investigation. **Luana Virgínia Souza**: Validation, Investigation. **Cinzia Caggia**: Validation, Writing - Review & Editing, Visualization. **Luís Augusto Nero**: Conceptualization, Validation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision. **Antonio Fernandes de Carvalho**: Conceptualization, Validation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109580.

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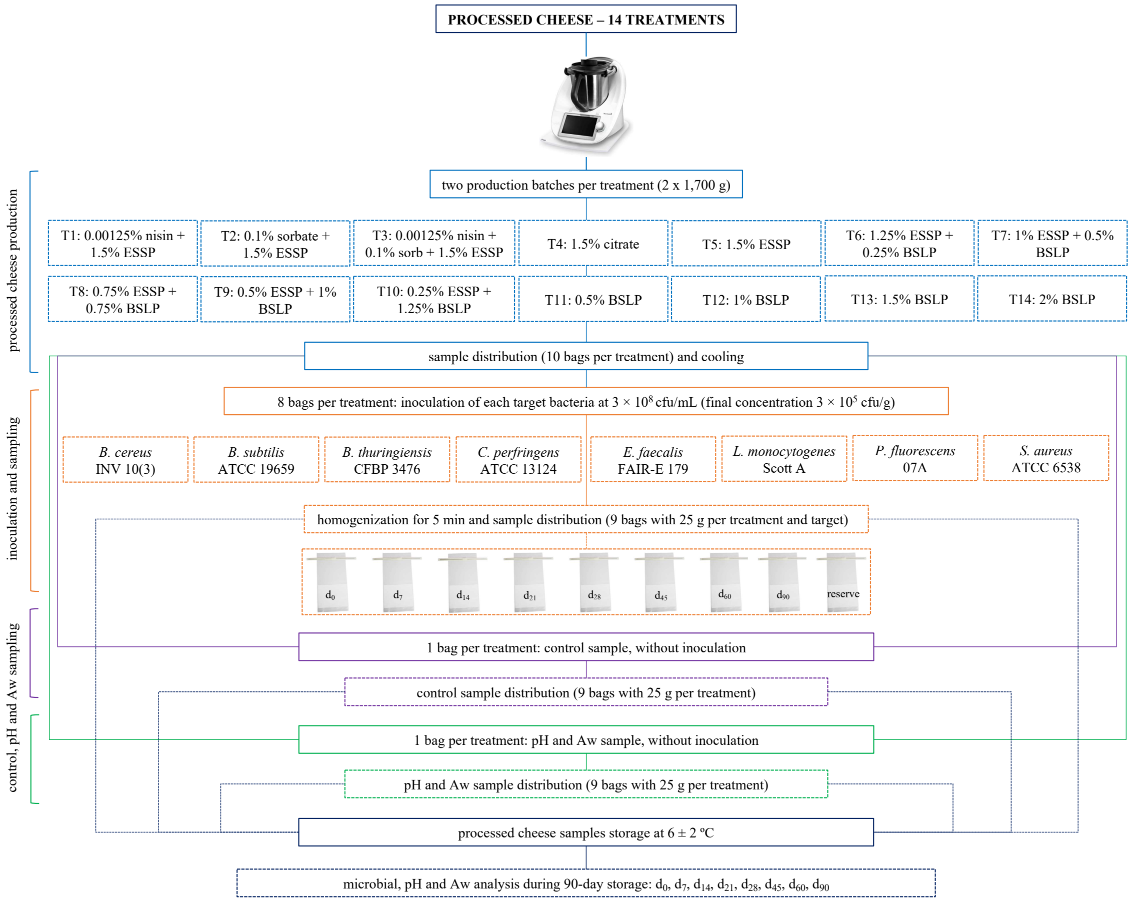

Figure 1. Flow chart of procedures employed in the production of PC samples.

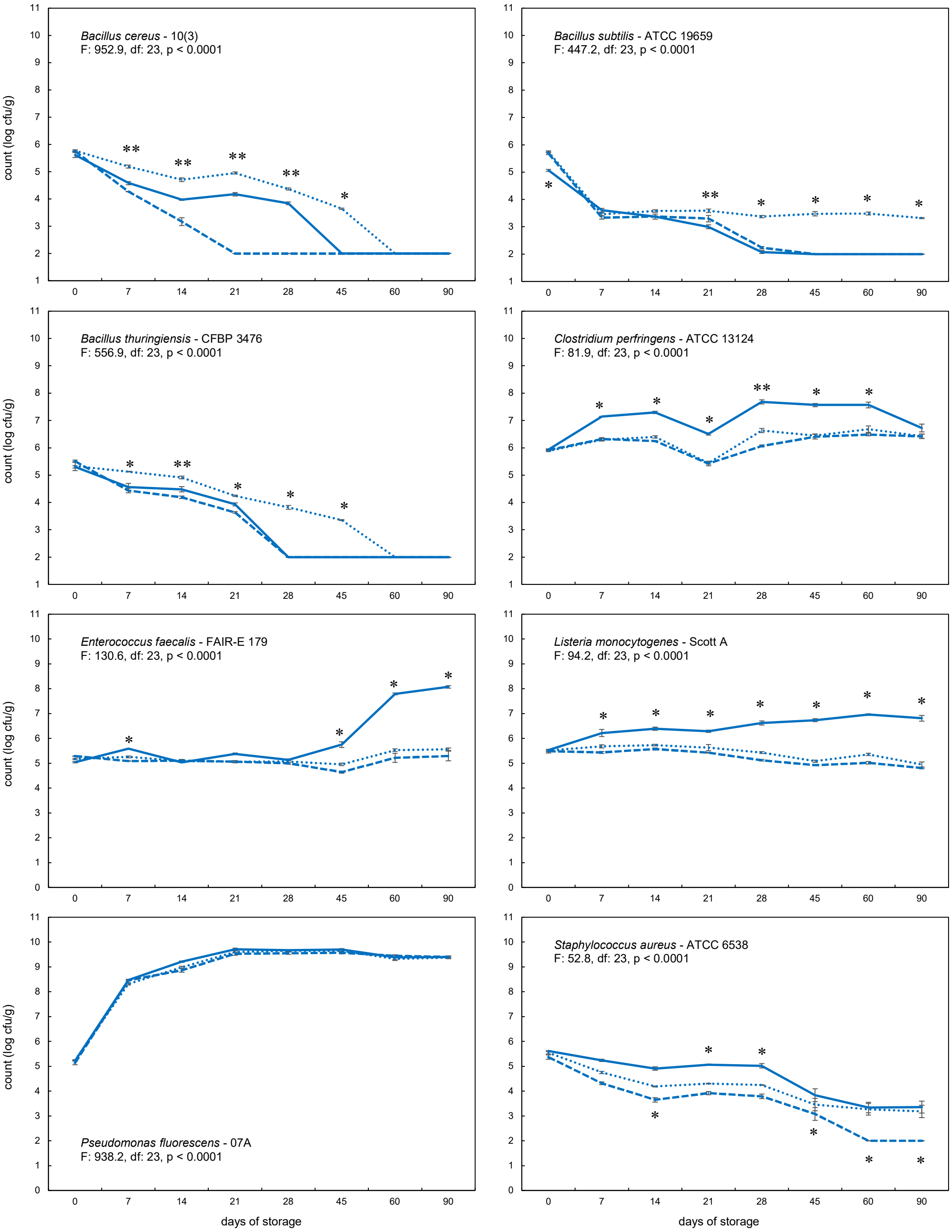

Figure 2. Growth of distinct bacteria strains in processed cheese supplemented with nisin and ESSP (0.00125% and 1.5%, w/v, respectively, full lines – T1), potassium sorbate and ESSP (0.1% and 1.5%, w/v, respectively, dotted lines – T2) and nisin and potassium sorbate and ESSP (0.00125%, 0.1% and 1.5%, w/w, respectively, dashed lines – T3). Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), double asterisks indicate significant differences among mean values for the same day of storage; single asterisks indicate significant difference of the closest mean value. In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

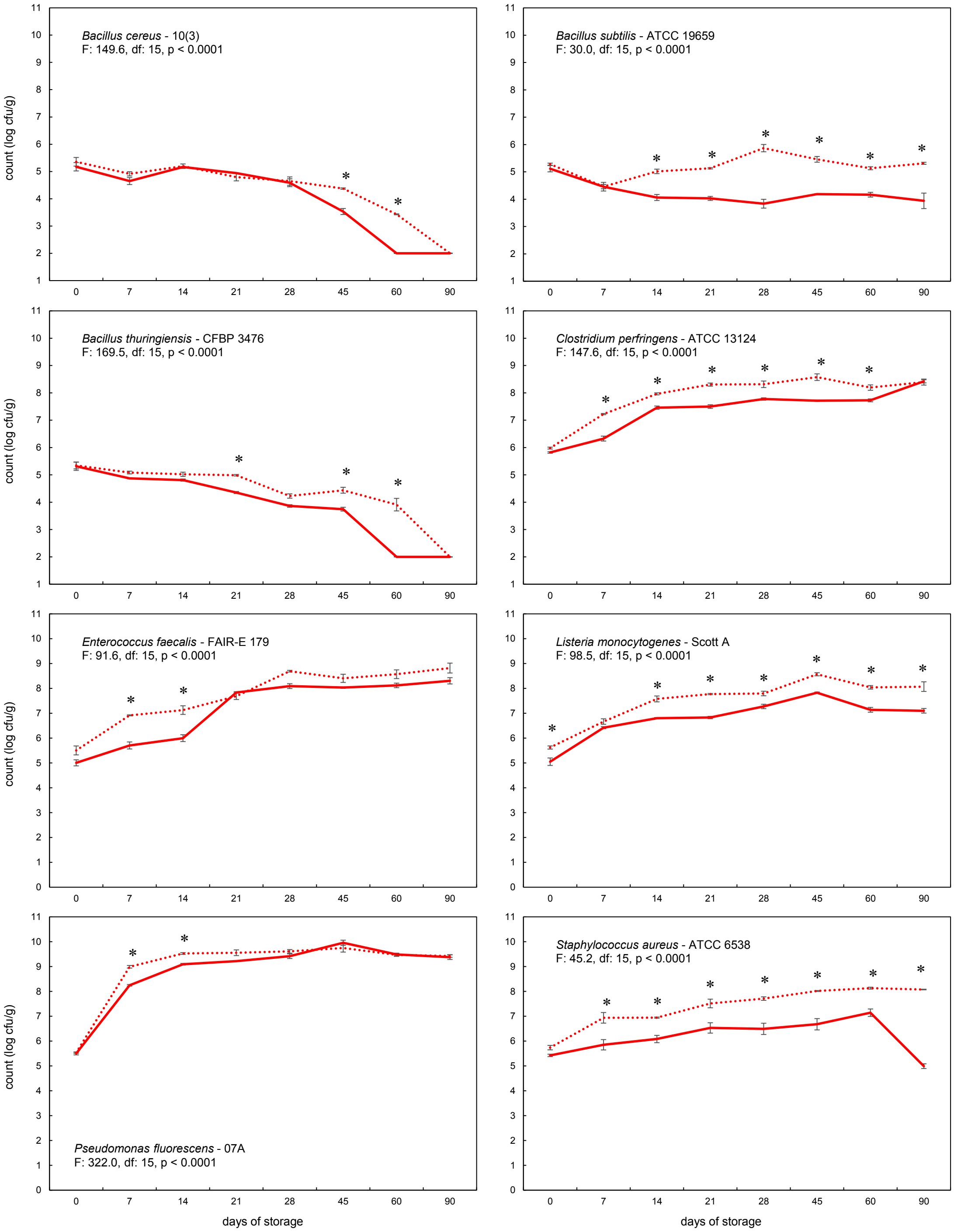

Figure 3. Growth of distinct bacteria strains in processed cheese supplemented with trisodium citrate (1.5%, w/w, dotted lines – T4) and ESSP (1.5%, w/w, full lines – T5). Mean values compared by ANOVA and Tukey ($p < 0.05$), asterisks indicate significant differences between mean values for the same day of storage. In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

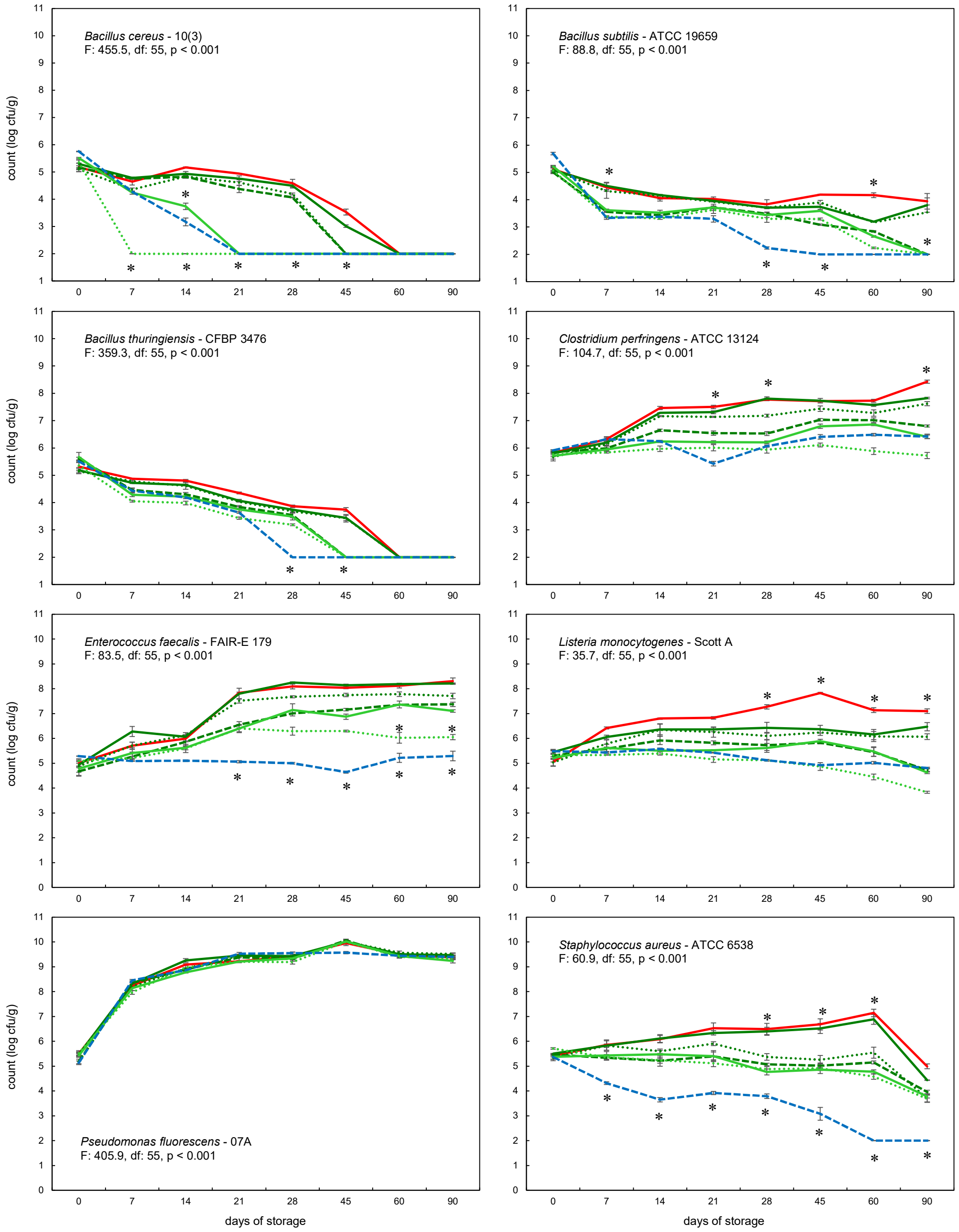

Figure 4. Growth of distinct bacteria strains in processed cheese supplemented with nisin and potassium sorbate and ESSP (0.00125% and 0.1%, w/w and 1.5%, respectively, blue dashed lines – T3), ESSP (1.5%, w/w, red lines – T5), and ESSP and BSLP at different concentrations (1.25% ESSP + 0.25% BSLP, dark green lines – T6; 1% ESSP + 0.5% BSLP, dark green dotted lines – T7; 0.75% ESSP + 0.75% BSLP, dark green dashed lines – T8; 0.5% ESSP + 1% BSLP, light green lines – T9; 0.25% ESSP + 1.25 BSLP, light green dotted lines – T10; all concentrations in w/w). Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), asterisks indicate significant differences of the closest mean values (single or groups). In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

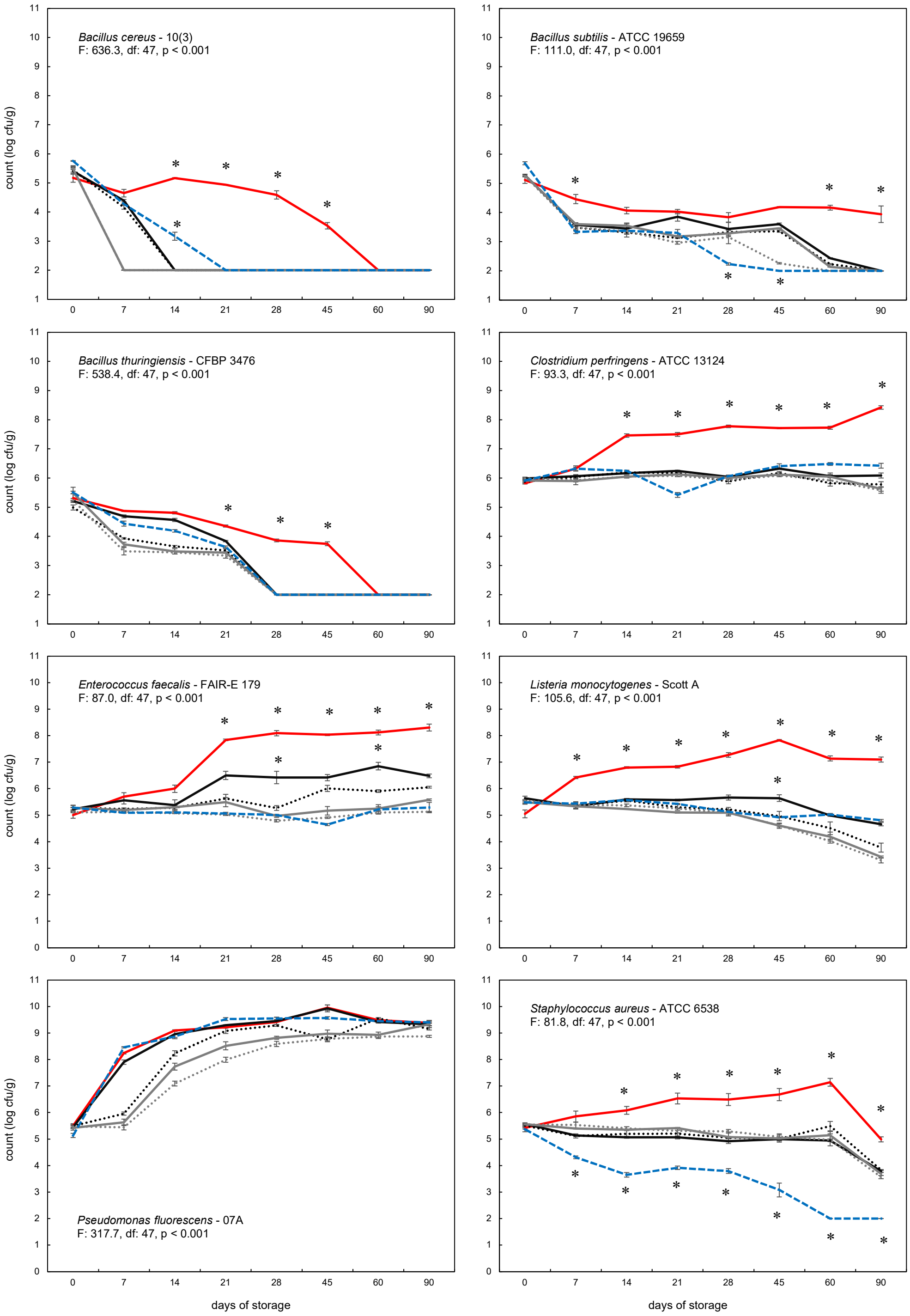

Figure 5. Growth of distinct bacteria strains in processed cheese supplemented with nisin and potassium sorbate and ESSP (0.00125% and 0.1%, w/w and 1.5%, respectively, blue dashed lines – T3), ESSP (1.5%, w/w, red lines – T5), and BSLP at different concentrations (0.5%, black lines – T11, 1%, black dotted lines – T12, 1.5%, grey lines – T13, 2%, grey dotted lines – T14; all concentrations in w/w). Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), asterisks indicate significant differences of the closest mean values (single or groups). In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

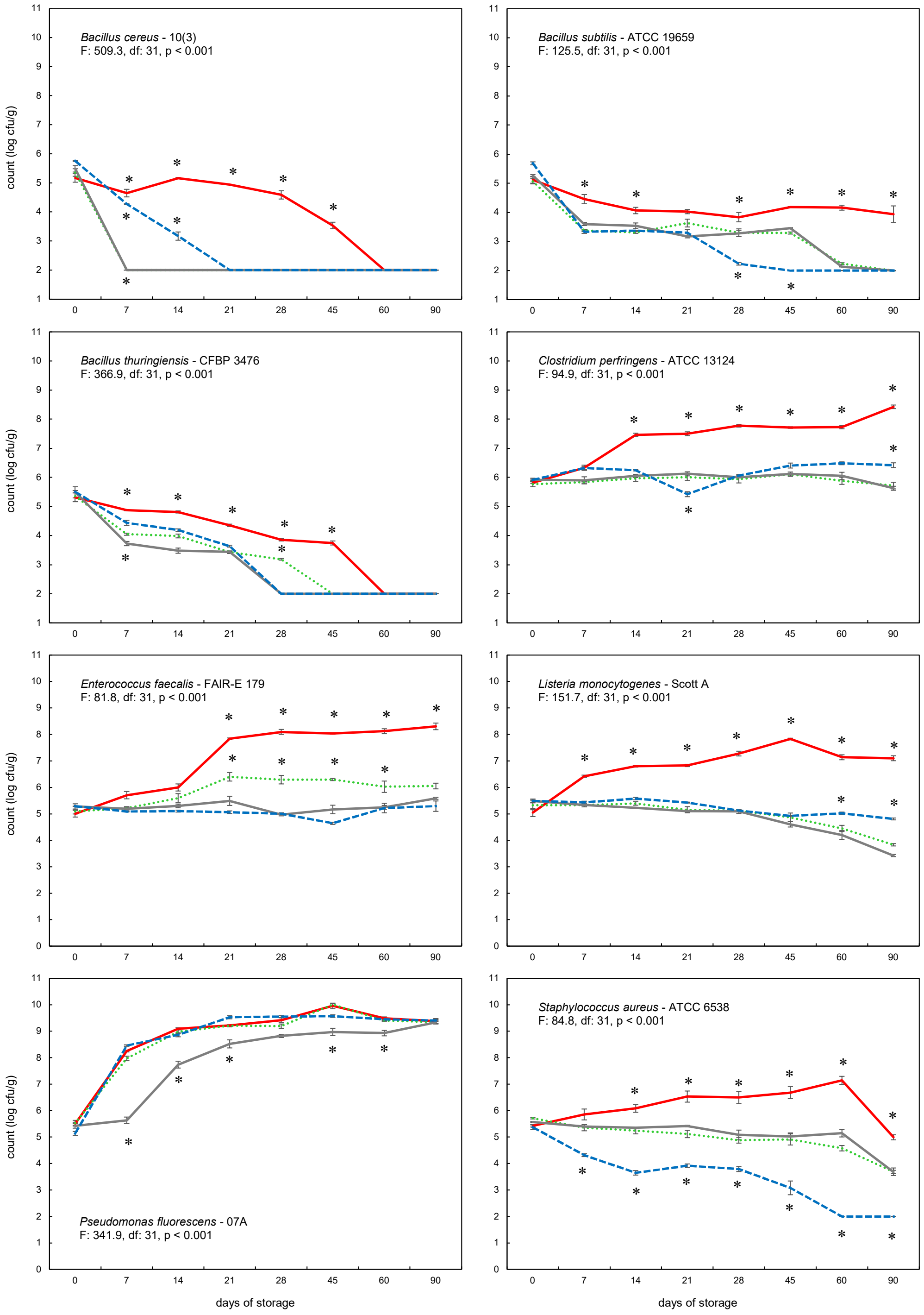

Figure 6. Growth of distinct bacteria strains in processed cheese supplemented with nisin and potassium sorbate and ESSP (0.00125% and 0.1%, w/w and 1.5%, respectively, blue dashed lines – T3), ESSP (1.5%, w/w, red lines – T5), ESSP and BSLP (0.25% and 1.25%, w/w, respectively, light green dotted lines – T10) and BSLP (1.5%, w/w, grey lines – T13). Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), asterisks indicate significant differences of the closest mean values (single or groups). In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

Table 1. Additives and treatments used in PC production.

Agents	Groups	Additive	Treatment	Combinations*
Antimicrobials	Group I	Nisin**	T1	0.00125% nisin + 1.5% ESSP
		Potassium sorbate	T2	0.1% sorbate + 1.5% ESSP
		Nisin + potassium sorbate	T3	0.00125% nisin + 0.1% sorbate + 1.5% ESSP
Emulsifying salts	Group II	Trisodium citrate	T4	1.5% trisodium citrate
		Short-chain polyphosphate	T5	1.5% ESSP
	Group III	Short-chain polyphosphate + long-chain polyphosphate	T6	1.25% ESSP+ 0.25% BSLP
			T7	1% ESSP+ 0.5% BSLP
			T8	0.75% ESSP+ 0.75% BSLP
			T9	0.5% ESSP+ 1% BSLP
			T10	0.25% ESSP + 1.25% BSLP
	Group IV	Long-chain polyphosphate	T11	0.5% BSLP
			T12	1% BSLP
			T13	1.5% BSLP
			T14	2% BSLP

*The amount (g) of additives was calculated for the total weight of the melt; for each PC production batch, 714 g of Mozzarella cheese, 289 g of butter, and 617.5 g of water were used.

**0.00125% nisin = 12.5 mg pure nisin per kilogram product.

Supplementary Table 1. Comparison between treatments with the same concentration of BSLP in the presence or absence of ESSP.

Target strain	Storage (days)	T7: 1% ESSP + 0.5% BSLP	T11: 0.5% BSLP	T9: 0.5% ESSP + 1% BSLP	T12: 1% BSLP
B. cereus 10(3)	0	5.10 ± 0.03	5.41 ± 0.21	5.51 ± 0.06	5.44 ± 0.26
	7	4.36 ± 0.10	4.39 ± 0.08	4.25 ± 0.11	4.16 ± 0.13
	14	4.84 ± 0.13	2.00 ± 0.00	3.74 ± 0.23	2.00 ± 0.00
	21	4.61 ± 0.20	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	28	4.20 ± 0.06	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	45	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	60	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	90	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
B. subtilis ATCC 19659	0	5.09 ± 0.25	5.27 ± 0.08	5.22 ± 0.08	5.28 ± 0.05
	7	4.32 ± 0.08	3.58 ± 0.14	3.61 ± 0.09	3.48 ± 0.09
	14	4.15 ± 0.05	3.45 ± 0.32	3.53 ± 0.18	3.32 ± 0.13
	21	3.93 ± 0.10	3.85 ± 0.29	3.71 ± 0.10	3.13 ± 0.04
	28	3.71 ± 0.04	3.43 ± 0.45	3.44 ± 0.18	3.33 ± 0.14
	45	3.90 ± 0.15	3.61 ± 0.07	3.59 ± 0.04	3.36 ± 0.07
	60	3.17 ± 0.02	2.43 ± 0.03	2.66 ± 0.06	2.23 ± 0.05
	90	3.54 ± 0.54	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
B. thuringiensis CFBP 3476	0	5.14 ± 0.17	5.21 ± 0.05	5.67 ± 0.34	4.99 ± 0.17
	7	4.78 ± 0.07	4.69 ± 0.10	4.29 ± 0.14	3.93 ± 0.05
	14	4.61 ± 0.25	4.57 ± 0.11	4.22 ± 0.02	3.65 ± 0.07
	21	4.03 ± 0.03	3.84 ± 0.04	3.75 ± 0.19	3.52 ± 0.11
	28	3.68 ± 0.05	2.00 ± 0.00	3.49 ± 0.25	2.00 ± 0.00
	45	3.43 ± 0.28	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	60	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	90	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
C. perfringens ATCC 13124	0	5.67 ± 0.27	5.99 ± 0.09	5.71 ± 0.22	5.88 ± 0.14
	7	6.14 ± 0.16	6.07 ± 0.06	5.95 ± 0.11	6.04 ± 0.16
	14	7.16 ± 0.04	6.17 ± 0.06	6.24 ± 0.08	6.19 ± 0.05

	21	7.14 ± 0.04	6.24 ± 0.06	6.21 ± 0.10	6.17 ± 0.11
	28	7.18 ± 0.11	6.04 ± 0.10	6.20 ± 0.10	5.88 ± 0.16
	45	7.44 ± 0.20	6.33 ± 0.04	6.79 ± 0.16	6.18 ± 0.11
	60	7.28 ± 0.23	6.06 ± 0.11	6.86 ± 0.08	5.83 ± 0.20
	90	7.62 ± 0.15	6.09 ± 0.18	6.41 ± 0.13	5.78 ± 0.19
E. faecalis FAIR-E 179	0	4.88 ± 0.49	5.22 ± 0.12	4.78 ± 0.55	5.28 ± 0.16
	7	5.71 ± 0.28	5.56 ± 0.27	5.41 ± 0.43	5.24 ± 0.09
	14	6.13 ± 0.04	5.38 ± 0.41	5.63 ± 0.17	5.28 ± 0.12
	21	7.52 ± 0.18	6.50 ± 0.31	6.40 ± 0.26	5.63 ± 0.31
	28	7.68 ± 0.08	6.42 ± 0.47	7.15 ± 0.49	5.27 ± 0.18
	45	7.75 ± 0.13	6.42 ± 0.24	6.87 ± 0.21	6.01 ± 0.24
	60	7.78 ± 0.18	6.85 ± 0.29	7.37 ± 0.28	5.91 ± 0.11
	90	7.71 ± 0.22	6.48 ± 0.14	7.11 ± 0.10	6.05 ± 0.07
L. monocytogenes Scott A	0	5.04 ± 0.31	5.64 ± 0.16	5.21 ± 0.33	5.51 ± 0.10
	7	5.79 ± 0.29	5.33 ± 0.20	5.61 ± 0.32	5.36 ± 0.22
	14	6.34 ± 0.51	5.60 ± 0.06	5.50 ± 0.06	5.55 ± 0.10
	21	6.26 ± 0.41	5.57 ± 0.06	5.52 ± 0.06	5.29 ± 0.21
	28	6.10 ± 0.29	5.66 ± 0.19	5.62 ± 0.36	5.22 ± 0.14
	45	6.24 ± 0.25	5.64 ± 0.25	5.89 ± 0.15	4.97 ± 0.36
	60	6.08 ± 0.41	4.99 ± 0.06	5.47 ± 0.38	4.51 ± 0.48
	90	6.07 ± 0.23	4.66 ± 0.13	4.63 ± 0.09	3.78 ± 0.35
P. fluorescens 07A	0	5.49 ± 0.18	5.45 ± 0.10	5.43 ± 0.15	5.49 ± 0.17
	7	8.20 ± 0.02	7.90 ± 0.16	8.13 ± 0.19	5.96 ± 0.13
	14	8.93 ± 0.07	8.95 ± 0.23	8.78 ± 0.07	8.23 ± 0.20
	21	9.44 ± 0.13	9.29 ± 0.04	9.21 ± 0.04	9.08 ± 0.20
	28	9.38 ± 0.16	9.46 ± 0.27	9.34 ± 0.11	9.29 ± 0.07
	45	10.01 ± 0.21	9.93 ± 0.26	10.02 ± 0.12	8.76 ± 0.19
	60	9.57 ± 0.13	9.42 ± 0.10	9.43 ± 0.06	9.55 ± 0.06
	90	9.51 ± 0.09	9.33 ± 0.24	9.24 ± 0.17	9.16 ± 0.08
S. aureus ATCC 6538	0	5.34 ± 0.24	5.57 ± 0.10	5.37 ± 0.19	5.51 ± 0.16

7	5.83 ± 0.14	5.13 ± 0.07	5.43 ± 0.32	5.12 ± 0.15
14	5.60 ± 0.17	5.07 ± 0.06	5.47 ± 0.32	5.20 ± 0.24
21	5.91 ± 0.15	5.06 ± 0.12	5.40 ± 0.44	5.20 ± 0.27
28	5.37 ± 0.27	4.92 ± 0.19	4.77 ± 0.24	5.05 ± 0.08
45	5.26 ± 0.35	4.99 ± 0.13	4.86 ± 0.09	5.01 ± 0.14
60	5.54 ± 0.44	4.94 ± 0.09	4.77 ± 0.16	5.49 ± 0.36
90	3.71 ± 0.29	3.79 ± 0.08	3.78 ± 0.12	3.83 ± 0.03

Values reported are the means ± standard deviations. Data presented with a result of 2.00 log cfu/g are below the detection limit of the method.

Supplementary Table 2. pH and Aw values during processed cheese storage.

		d_0	d_7	d_{14}	d_{21}	d_{28}	d_{45}	d_{60}	d_{90}
pH									
	T1	5.79 ± 0.09^{a}	5.74 ± 0.09^{a}	5.76 ± 0.04^{a}	5.58 ± 0.03^{a}	5.65 ± 0.02^{a}	5.63 ± 0.00^{a}	5.62 ± 0.02^{a}	5.61 ± 0.03^{a}
	T2	5.76 ± 0.01^{a}	5.71 ± 0.09^{a}	5.78 ± 0.05^{a}	5.61 ± 0.01^{a}	5.71 ± 0.01^{a}	5.68 ± 0.02^{a}	5.67 ± 0.03^{a}	5.66 ± 0.04^{a}
	T3	5.73 ± 0.05^{a}	5.74 ± 0.00^{a}	5.84 ± 0.01^{a}	5.62 ± 0.03^{a}	5.72 ± 0.01^{a}	5.68 ± 0.01^{a}	5.66 ± 0.01^{a}	5.65 ± 0.01^{a}
	T4	5.93 ± 0.04^{a}	5.93 ± 0.10^{a}	5.91 ± 0.13^{a}	6.00 ± 0.00^{a}	5.95 ± 0.11^{a}	6.02 ± 0.02^{a}	6.16 ± 0.13^{ab}	6.45 ± 0.06^{b}
	T5	5.74 ± 0.01^{a}	5.76 ± 0.12^{a}	5.87 ± 0.11^{a}	5.75 ± 0.03^{a}	5.80 ± 0.05^{a}	5.78 ± 0.06^{a}	5.79 ± 0.09^{a}	5.83 ± 0.04^{a}
	T6	5.67 ± 0.01^{a}	5.63 ± 0.07^{a}	5.77 ± 0.09^{a}	5.66 ± 0.08^{a}	5.65 ± 0.02^{a}	5.67 ± 0.05^{a}	5.74 ± 0.13^{a}	5.66 ± 0.01^{a}
	T7	5.52 ± 0.04^{a}	5.52 ± 0.06^{a}	5.60 ± 0.04^{a}	5.52 ± 0.04^{a}	5.54 ± 0.07^{a}	5.52 ± 0.03^{a}	5.65 ± 0.21^{a}	5.60 ± 0.04^{a}
	T8	5.42 ± 0.02^{a}	5.43 ± 0.11^{a}	5.48 ± 0.04^{a}	5.42 ± 0.04^{a}	5.41 ± 0.01^{a}	5.41 ± 0.05^{a}	5.50 ± 0.18^{a}	5.43 ± 0.06^{a}
	T9	5.34 ± 0.09^{a}	5.36 ± 0.13^{a}	5.43 ± 0.01^{a}	5.38 ± 0.04^{a}	5.33 ± 0.03^{a}	5.31 ± 0.06^{a}	5.41 ± 0.17^{a}	5.38 ± 0.16^{a}
	T10	5.15 ± 0.11^{a}	5.17 ± 0.22^{a}	5.22 ± 0.05^{a}	5.16 ± 0.16^{a}	5.18 ± 0.07^{a}	5.16 ± 0.09^{a}	5.26 ± 0.23^{a}	5.24 ± 0.16^{a}
	T11	5.15 ± 0.04^{a}	5.16 ± 0.19^{a}	5.15 ± 0.10^{a}	5.18 ± 0.07^{a}	5.18 ± 0.05^{a}	5.19 ± 0.08^{a}	5.28 ± 0.18^{a}	5.19 ± 0.06^{a}
	T12	5.16 ± 0.01^{a}	5.11 ± 0.06^{a}	5.20 ± 0.02^{a}	5.16 ± 0.05^{a}	5.14 ± 0.01^{a}	5.19 ± 0.02^{a}	5.24 ± 0.11^{a}	5.18 ± 0.04^{a}
	T13	5.03 ± 0.04^{a}	5.06 ± 0.15^{a}	5.23 ± 0.23^{a}	5.03 ± 0.09^{a}	5.05 ± 0.00^{a}	5.00 ± 0.05^{a}	5.14 ± 0.18^{a}	5.02 ± 0.02^{a}
	T14	5.01 ± 0.08^{a}	5.05 ± 0.21^{a}	5.25 ± 0.20^{a}	5.02 ± 0.13^{a}	5.04 ± 0.06^{a}	5.02 ± 0.06^{a}	5.11 ± 0.20^{a}	4.95 ± 0.06^{a}
Aw		d_0	d_7	d_{14}	d_{21}	d_{28}	d_{45}	d_{60}	d_{90}
	T1	0.990 ± 0.001^{a}	0.986 ± 0.002^{a}	0.990 ± 0.003^{a}	0.990 ± 0.002^{a}	0.985 ± 0.004^{a}	0.990 ± 0.003^{a}	0.988 ± 0.002^{a}	0.986 ± 0.001^{a}

T2	0.987 ± 0.001^{a}	0.986 ± 0.002^{a}	0.986 ± 0.003^{a}	0.987 ± 0.001^{a}	0.986 ± 0.002^{a}	0.987 ± 0.002^{a}	0.987 ± 0.002^{a}	0.986 ± 0.003^{a}
T3	0.988 ± 0.002^{a}	0.989 ± 0.002^{a}	0.989 ± 0.001^{a}	0.989 ± 0.002^{a}	0.985 ± 0.002^{a}	0.988 ± 0.003^{a}	0.988 ± 0.002^{a}	0.988 ± 0.001^{a}
T4	0.980 ± 0.007^{a}	0.985 ± 0.001^{a}	0.987 ± 0.004^{a}	0.985 ± 0.002^{a}	0.979 ± 0.008^{a}	0.985 ± 0.001^{a}	0.981 ± 0.001^{a}	0.982 ± 0.012^{a}
T5	0.985 ± 0.004^{ab}	0.983 ± 0.020^{abc}	0.990 ± 0.002^{a}	0.979 ± 0.008^{bc}	0.986 ± 0.012^{ab}	0.983 ± 0.006^{abc}	0.973 ± 0.012^{c}	0.983 ± 0.010^{abc}
T6	0.978 ± 0.007^{b}	0.984 ± 0.004^{ab}	0.992 ± 0.006^{a}	0.986 ± 0.001^{ab}	0.986 ± 0.003^{ab}	0.982 ± 0.007^{ab}	0.979 ± 0.009^{b}	0.982 ± 0.009^{ab}
T7	0.975 ± 0.009^{b}	0.984 ± 0.001^{ab}	0.991 ± 0.006^{a}	0.980 ± 0.010^{b}	0.987 ± 0.010^{ab}	0.980 ± 0.008^{ab}	0.970 ± 0.005^{b}	0.983 ± 0.007^{ab}
T8	0.974 ± 0.010^{b}	0.985 ± 0.003^{ab}	0.993 ± 0.007^{a}	0.984 ± 0.003^{a}	0.987 ± 0.003^{a}	0.984 ± 0.007^{ab}	0.977 ± 0.008^{ab}	0.982 ± 0.013^{ab}
T9	0.967 ± 0.027^{b}	0.985 ± 0.006^{b}	0.998 ± 0.010^{a}	0.977 ± 0.012^{b}	0.978 ± 0.014^{b}	0.979 ± 0.009^{b}	0.983 ± 0.006^{b}	0.979 ± 0.013^{b}
T10	0.977 ± 0.009^{b}	0.986 ± 0.002^{abc}	0.992 ± 0.004^{a}	0.979 ± 0.012^{a}	0.985 ± 0.006^{abc}	0.981 ± 0.006^{bc}	0.981 ± 0.007^{bc}	0.984 ± 0.012^{abc}
T11	0.981 ± 0.007^{a}	0.987 ± 0.001^{a}	0.990 ± 0.001^{a}	0.983 ± 0.004^{a}	0.981 ± 0.009^{a}	0.982 ± 0.008^{a}	0.983 ± 0.006^{a}	0.985 ± 0.007^{a}
T12	0.980 ± 0.011^{ab}	0.986 ± 0.002^{ab}	0.989 ± 0.002^{a}	0.985 ± 0.003^{a}	0.981 ± 0.005^{ab}	0.978 ± 0.011^{ab}	0.974 ± 0.007^{b}	0.980 ± 0.010^{ab}
T13	0.981 ± 0.006^{a}	0.983 ± 0.003^{a}	0.985 ± 0.004^{a}	0.977 ± 0.015^{ab}	0.979 ± 0.003^{ab}	0.982 ± 0.003^{a}	0.970 ± 0.007^{b}	0.979 ± 0.010^{ab}
T14	0.977 ± 0.006^{ab}	0.984 ± 0.003^{a}	0.986 ± 0.006^{a}	0.984 ± 0.009^{a}	0.984 ± 0.004^{a}	0.976 ± 0.011^{ab}	0.972 ± 0.006^{b}	0.975 ± 0.012^{ab}

Mean values compared by ANOVA and Tukey ($p < 0.001$), different lowercase letter indicates significant differences among mean values during storage time of each treatment. Treatments: (T1) 0.0012% of nisin + 1.5% ESSP; (T2) 0.1% of potassium sorbate + 1.5% ESSP; (T3) 0.0012% of nisin + 0.1% of potassium sorbate + 1.5% ESSP; (T4) 1.5% trisodium citrate; (T5) 1.5% ESSP; (T6) 1.25% ESSP + 0.25% BSLP; (T7) 1% ESSP + 0.5% BSLP; (T8) 0.75% ESSP + 0.75% BSLP; (T9) 0.5% ESSP + 1% BSLP; (T10) 0.25% ESSP + 1.25% BSLP; (T11) 0.5% BSLP; (T12) 1% BSLP; (T13) 1.5% BSLP; (T14) 2% BSLP.

CHAPTER V.

RESEARCH ARTICLE FOUR

Melting salts and P_2O_5 content combined with processing method as promising strategies to control *Bacillus cereus* growth during storage of processed cheese

Fusieger et al.

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Chapter presented according to the final format of the journal.

All analyzes described in this chapter were conducted at Department of Agriculture, Food and Environment, University of Catania, Catania, CT, Italy and at ICL Group/BK Giulini GmbH, Ladenburg, BW, Germany.

Title page

Melting salts and P_2O_5 content combined with processing method as promising strategies to control *Bacillus cereus* growth during storage of processed cheese

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Abstract

The prevention of spore-forming bacteria growth represents a challenge for the dairy industry, since processed cheeses (PC) have been associated with microbiological safety concerns related to *Bacillus cereus* presence. Melting salts have been proposed as strategy to control food spoilage and to prolong PC shelf-life. In this study, the bacteriostatic effect of six melting salt blends, containing phosphorus pentoxide (P_2O_5), was investigated in PC samples deliberately contaminated with *B. cereus* spores. Moreover, the influence of PC processing methods (pasteurization and creaming), storage temperature (6 and 30 ºC) and storage time (until 120 days) were evaluated. Results showed that lower storage temperature and melting salt significantly influenced bacterial growth, being the sodium phosphate the major antibacterial agent. In addition, the creaming process and the P_2O_5 high content enhanced the inhibitory effect of melting salts in PC. In PC treated with the blends containing the highest concentration of P_2O_5, the lowest average value of both vegetative cells and spore was detected. Moreover, the same treatment did not visually modify the PC color up to 120 days of storage. The present study, for the first time, demonstrated that appropriate melting salts represent a promising approach for controlling *B. cereus* in PC.

Keywords: dairy, emulsifiers, microbiological stability, spore-forming bacteria.

Highlights

- Strategy to control the growth of aerobic spore-forming bacteria in processed cheese.
- Composition of melting salts and storage temperature significantly influences bacterial growth.
- The creaming process may improve the inhibitory effect of melting salts in processed cheeses.
- Melting salts can extend the shelf life of processed cheese.

1. Introduction

The use of sodium phosphates, commonly called melting salts, in processed cheese (PC) was introduced, in a patent, by George Herbert Garstin, at the Phoenix Cheese Company, few years later the start of PC production (Garstin, 1921). This ingredient allowed to prolonging storage of PC, which in its natural form would undergo excessive proteolysis, lipolysis, and other detrimental changes (Fox et al., 2017). The addition of phosphates as melting salts resulted in a marked increase in the production of PC (Caric et al., 1985) and currently monophosphates, polyphosphates and citrates are the emulsifying salts commonly used in cheese industry (Buňka et al., 2022).

PC can be described as a viscoelastic matrix produced by heat-treated cheese with the addition of emulsifying salts (El-Bakry and Mehta, 2022). The production process consists in blending shredded natural cheeses of various types and maturity degrees with emulsifying agents and by heating the blend under reduced pressure, with constant stirring, to produce a smooth and homogeneous mass (Caric et al., 1985; El-Bakry and Mehta, 2022). The list of ingredients is based on natural cheese/cheeses, at different stages of maturity, and on manufacturing and by a wide range of dairy and non-dairy ingredients and additives which are added in order to modify physicochemical or functional properties of the end-product (Fox et al., 2017; Hill and Ferrer, 2022; Kapoor and Metzger, 2008). The formulations depend on the type of PC being manufactured, texture, taste, final application, local regulations and economic considerations (Kapoor and Metzger, 2008). Nevertheless, after natural cheese, emulsifying salts are the most relevant constituents of the formulation in terms of texture, and the first in terms of product stability.

According to Caric et al. (1985), the emulsifiers agents are used to provide a uniform structure during the melting process and to supplement the emulsifying capability of cheese proteins, being accomplished by: (i) removing calcium from the protein system; (ii) peptizing, solubilizing, and dispersing proteins; (iii) hydrating and swelling proteins; (iv) emulsifying fat and stabilizing the emulsions; (v) controlling/stabilizing pH, and (vi) forming an appropriate structure after cooling. On the other hand, some melting salts are also related to bacterial inhibition and have been proposed such a strategy to control spoilage and pathogenic microorganisms to prolong shelf-life of PC (Fusieger et al., 2022a; Glass et al., 2017; Loessner et al., 1997; Martinez-Rios et al., 2019; Steeg et al., 1995; Tanaka et al., 1979). One of the first studies to understand the mechanism of polyphosphate in the bacterial inhibition was carried out by Maier et al. (1999). Through *in vitro* analyses, using *Bacillus cereus* WSBC 10030 as target bacteria, the application of polyphosphates on the cells in the exponential growth phase led to changes in the cell morphology revealed by cell lysis and inability to form the septum during cell division (Maier et al., 1999).

Through PC processing, the thermal treatment allows the inactivation of most vegetative forms, while bacterial spores can survive (Catania et al., 2021; Oliveira et al., 2016). In particular, *B. cereus* can negatively affect dairy products causing serious issues by production heat-stable extracellular enzymes, such as proteases responsible for the bitter taste and for the gelling of milk, and lipases associated with a rancid flavor (Arslan et al., 2014; Berthold-Pluta et al., 2019; Tirloni et al., 2022). *B. cereus* is of great concern to the food industry also for producing toxins responsible for two different types of foodborne illness, the emetic and the diarrheic syndrome (Carroll et al., 2019; Messelhäußer and Ehling-Schulz, 2018; Webb et al., 2019). Indeed, PC have been associated with microbiological safety concerning with spore-forming bacteria, such as

B. cereus (Catania et al., 2021; Hassanin, 1993; Oliveira et al., 2018; Palmas et al., 1999). For this reason, the addition of an appropriate melting salt as a multifunctional ingredient and not just an emulsifying ingredient in PC production can be seen as a control option.

In previous studies (Fusieger et al., 2022a, 2022b), the addition of melting salts in processed cheese was evaluated by *in vitro* and *in situ* approaches against non-spore-forming bacteria and spore-forming bacteria. Based on the preliminary findings, in this study we aimed to focus on the bacteriostatic effect of six melting salts, based on the phosphorus pentoxide (P_2O_5) content, against *B. cereus* spores in PC samples. The influence of PC processing methods (pasteurization and creaming), storage temperature (6 and 30 ºC) and storage time(until 120 days) was evaluated to assess the potential use of melting salts as multifunctional ingredients.

2. Materials and methods

2.1. Bacillus cereus strains and spore preparation

B. cereus INV 10(3) (isolated from UHT milk) and *B. cereus* DSM 626 (extensively used in sporulation and germination studies) were used as target strains. The strains were stored at -80 ºC in brain heart infusion (Oxoid, Basingstoke, United Kingdom) with 20% (v/v) of glycerol. Aliquots of each stock strain were inoculated into BHI broth and incubated at 30 ± 2 ºC. After overnight incubation, strains were checked for purity in mannitol egg yolk polymyxin agar (MYP; Oxoid) and one typical colony of each strain was transferred into BHI broth and incubated at 30 ± 2 ºC for 20 h. In order to obtain a blend culture of *B. cereus* INV 10(3) and *B. cereus* DSM 626, aliquots (1% v/v) of each strain were transferred together into BHI broth (in a 1:1 ratio of each strain), incubated at 30 °C for 20 h, and spread plated onto BBL™ Sporulating Agar #2 (AK; BD

Bioscience, Le Pont de Claix, France) and plates incubated at 30 ± 2 ºC for 10 days. When the sporulation was achieved, AK plates were flooded with 5 mL of cold sterile deionized (DI) water and spores were dislodged by a sterile bent rod. The pooled suspensions were placed into sterile 50 mL Falcons, centrifuged (SL 40R Centrifuge, Thermo Fisher Scientific, Waltham, USA) at 7,825 × *g* for 15 min at 4 °C and the cell pellets washed five times with cold sterile DI water. In order to kill the remaining vegetative cells, the spore suspension was heated at 80 ± 1 ºC for 12 min and cooled in ice bath. Then, the spore suspension was washed once more, re-suspended in the original volume, and stored at 4 ± 2 ºC until use (Tirloni et al., 2017). The spore population was determined by spread plating onto plate count agar (PCA; Oxoid), incubated at 30 ± 2 °C for 20 h and colonies enumerated (typical yields were 10^8 to 10^9 spores/mL).

2.2. PC materials

Six melting salts with different P_2O_5 contents were kindly provided by ICL Foods Specialties, BK Giulini GmbH (Ladenburg, Germany) and used for formulation recipes, as reported in Supplementary Table 1. All formulations were added at the total amount of 2% (wt/wt) of melting salts (the amount was calculated based on the total weight of the melt). The final concentration of P_2O_5 of each formulation was calculated according to the combinations of melting salts added to the processed cheese (Table 1), according to the following formula:

$$\text{Final } P_2O_5\ (\%) = \frac{\text{AMS} \times \text{POMS}}{100}$$

where AMS refers to % of the melting salt added in relation to the total amount (2% wt/wt) and POMS refers to P_2O_5 presence (as %) in the melting salt.

Cheddar type natural cheese (38.60%; 12 weeks aged), butter (10.70%), skimmed milk powder (5.50%; Lactoland Trockenmilchwerk GmbH, Dülmen, Germany), rennet casein (1.00%; Lactalis Ingredients, Bourgbarré, France), NaCl (0.30%), citrate acid (0.18%, used as buffer; Merck, Darmstadt, Germany), trisodium citrate (0.12%, used as buffer; BK Giulini GmbH), and water (25.60%) were used for the PC preparation. About 16.00% of each batch weight was incorporated as condensate by the steam injection system. Rework (5.00%; Adler Edelcreme® Sahne, Bel Brands Deutschland GmbH, Grasbrunn, Germany) was added to the creaming process, as described below. Composition of the ingredients to produce PC was designed to reach the target values of dry matter (DM) content of 40% (wt/wt) and pH from 5.50 to 5.70.

2.3. PC production

To produce PC samples a Stephan Universal Machine (Stephan VM/CR44; A. Stephan u. Söhne GmbH & Co., Hameln, Germany) was used; 12 kg were produced in each batch. Cheddar cheese, butter, rennet casein, NaCl, citrate acid, trisodium citrate, melting salts (according to Table 1) and half of required water was placed in the Stephan machine. Under standard processing conditions, the blend was agitated for 1 min, heated using indirect steam injection until 40 ± 1 ºC whilst continuously shearing at 1,200 rpm. Skimmed milk powder and the remaining water were added and homogenized for 1 min at 1,200 rpm, then the heating was continued until 90 ± 1 ºC (this temperature increase was achieved in ≈ 5 min). A PC sample was collected and DM content (measured by microwave moisture analyzer; SMART 6™ Analyzer, CEM Corporation, Matthews, USA) and pH (measured by direct insertion of a spear electrode into the samples; Portavo 902 pH, Knick Elektronische Messgeräte GmbH & Co., Berlin, Germany) were determined. Subsequently, when required, DM and pH were adjusted to reach the target values (based on calibration equation model). The calculated amount of citrate acid, or trisodium citrate or water were added to the Stephan machine, homogenized for 1 min at

1,200 rpm at 90 ± 1 ºC and a PC sample was collected to check DM and pH. Afterwards, the blend was pasteurized at 90 ± 1 ºC for 5 min by continuously mixing.

After processing, 4 kg of PC were immediately packaged into 200-mL polypropylene containers, sealed and stored at 6 ± 2 ºC, the obtained samples were called PC-PST. Then, other 4 kg were submitted to creaming process, using Stephan UMC-5 equipment (Stephan u. Söhne GmbH & Co). Creaming PC was performed by the addition of rework (50 g/kg), followed by homogenization at 500 bar and by heating using indirect steam injection up to 90 ± 1 ºC when the homogenization was stopped, and the creaming process occurred in 5 min. The PC was packed into 150-mL polypropylene containers, sealed and stored at 6 ± 2 ºC, the obtained samples were called PC-CRE. Two independent trials, each with six different PC formulations, were undertaken over a 2-week period.

2.4. Inoculation of PC samples

PC-PST and PC-CRE samples were inoculated with the obtained spore inoculum (as described above) at a final concentration of approximately 1×10^6 spore/g and homogenized for 5 min (Bagmixer 400W; Interscience, Paris, France). The inoculated samples were equally split into sterile bags (25 g) and stored at 6 ± 2 ºC and at 30 ± 2 ºC. Samples without inoculum were used as controls (blank) and stored at the same conditions described above. A flow chart of the experiment process is presented in Figure 1.

2.5. Microbial analysis

PC samples were subjected to *B. cereus* enumeration and mesophilic spore counting throughout 120-days of storage (day-0, day-15, day-30, day-45, day-60, day-90, day-120) at 6 ± 2 ºC and 30 ± 2 ºC. In details, 25 g of sample were added to 225 mL of citrate solution 2% (wt/v), homogenized, and 10-fold diluted in saline solution 0.85%

(wt/v). For *B. cereus* enumeration, aliquots of 100 µL of selected dilutions were spread plated onto MYP agar and incubated at 30 ± 1 °C for 20 h (ISO 7932, 2004). For mesophilic spore count, 10 mL of the 1:10 dilution were heated at 80 ± 1 ºC for 12 min to inactivate vegetative cells, rapidly cooled in ice bath, 10-fold diluted in saline solution 0.85% (wt/v), spread plated onto PCA medium and incubated at 30 ± 1 °C for 24 h (Wehr and Frank, 2004). After incubation, growth colonies were enumerated and expressed as CFU/g for *B. cereus* and as spore/g for mesophilic spore. Control samples were analyzed for total viable count (ISO 4833-1, 2003) and mesophilic spore count (Wehr and Frank, 2004).

2.6. Physico-chemical analysis

Chemical analyses of PC blank samples obtained with different formulations were performed at initial time. The pH values were determined at ambient temperature by direct insertion of a spear electrode (Portavo 902 pH, Knick Elektronische Messgeräte GmbH & Co.) into samples into two randomly chosen locations. The moisture content was determined through direct drying of 3 g of samples at 102 ± 2 ºC to constant mass (ISO 5534, 2004) and DM content was calculated as a difference (ISO 5534, 2004). Total protein (TN) was detected by conventional Kjeldahl method (ISO 17837, 2008). Ash content was determined by sample incineration at 550 ± 1 ºC (IDF 027, 1964). Gravimetric method was used for fat determination (ISO 1735, 2004). Fat in DM (F/DM) was calculated as a difference.

2.7. Statistical analysis

B. cereus and mesophilic spore counts obtained from PC-PST and PC-CRE samples were transformed to Log base 10, checked for normality (Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera, $p < 0.05$) and were compared based on treatments and storage time through ANOVA and Tukey's honest significant difference (HSD) test by using XLSTAT 19.01 (Addinsoft, New York, NY, USA). Bacterial growth (decrease or increase) during the storage time was calculated based on the initial inoculum (day-0), as follow:

$$\text{Bacterial behavior} = \frac{\bar{x}\,(\text{Log day15 :day120})}{\text{Log day0}}$$

where values < 1.00 Log indicate bacterial decrease, and > 1.00 Log indicate bacterial increase in the cell population during the storage period. Physico-chemical results were compared based on treatments through ANOVA and Tukey's honest significant difference (HSD) test ($p < 0.05$) by using the XLSTAT software.

3. Results

3.1. Microbiological counting

Overall in PC-PST and PC-CRE samples, stored at 6 ºC, obtained by F2, F3, F4, F5 and F6 treatments, a bacteriostatic effect was observed against *B. cereus* during the 120 days of storage (Figure 2-A and 3-A). In particular, the F1 treatment showed a bacteriostatic effect up to 60 days of storage in samples obtained with both processing methods. Between 60 and 120 days of storage, a significant increase ($p < 0.0001$) in microbial population was observed both in PC-PST and PC-CRE samples. In details, in PC-PST samples, in 90-60 days and in 120-90 days of storage, an increase of 2.54 and 1.78 Log CFU/g, respectively, were detected. A similar trend was observed in for PC-CRE samples, where an increase of 2.07 and 0.58 Log CFU/g, respectively were observed (Figure 2-A and 3-A). Regarding the mesophilic spore count, all treatments were effective in inhibiting their growth in samples stored at 6 ºC and at the endo of storage time even a decrease was observed (Figure 2-C and 3-C). Among the applied treatments, the F3 exhibited the lowest decrease in relation to the initial spore counts (storage time 0), being for PC-PST and PC-CRE samples, as 0.35 and 0.39 Log spore/g, respectively.

For samples stored at 30 ºC, only the F6 treatment was able to inhibit *B. cereus* growth with an overall decrease, during the 120 days, of 0.65 and 0.80 Log CFU/g, for PC-PST and PC-CRE samples, respectively (Figure 2-B and 3-B). In the first 15 days, the highest increase in microbial population was observed for F1 treated PC-PST and PC-CRE samples, that from 5.96 reached 8.48 Log CFU/g and from 6.17 reached to 8.45 Log CFU/g ($p < 0.0001$), respectively. Similar effects were found for F3 and F4 treatments, for which an increase in the microbial population in the first 15 days was observed, although a higher increase was detected after 30 days of storage ($p < 0.0001$). Furthermore, a bacteriostatic effect was observed until the 30 days of storage for F2 treatment in PC-CRE samples, while a significant increase, already after the first 15 days of storage, was detected in F2 treated PC-PST samples. Moreover F5 treatment, showed a bacteriostatic pattern until 45 days in PC-PST samples and until day 60 in PC-CRE samples (Figure 2-B and 3-B). Furthermore, between the tested conditions, the lowest microbial populations were observed in the mesophilic spore count for samples stored at 30 ºC (Figure 2-D and 3-D). As for the samples storage at 6 ºC, all treatments reduced the spore population during the storage time.

Table 2 is focused on bacterial count decrease (< 1.00 Log) or increase (> 1.00 Log), observed during the storage time, in relation to the initial inoculum (day 0). In samples stored at 6 ºC, the F1 treatment showed the greatest increase of *B. cereus*; increases of 1.14 and 1.10 Log CFU/g, for PC-PST and PC-CRE, respectively were found (Table 2). Amongst evaluated formulations, exclusively the F6 produced a *B. cereus* decrease in samples stored at 30 ºC; as 0.93 and 0.95 Log CFU/g, for PC-PST and PC-

CRE samples, respectively (Table 2). At the same storage conditions, the highest levels of bacterial increase were found in PC-PST samples: as 1.18 Log CFU/g for F1; 1.12 Log CFU/g for F2; 1.14 Log CFU/g for F3; 1.12 Log CFU/g for F4; and 1.05 Log CFU/g for F5 (Table 2). In samples stored at 6 ºC, all treatments decreased spore densities, with a higher effect detected for F4 treatment (0.90 Log spore/g) and F6 (0.91 Log spore/g) in PC-CRE samples (Table 2). The same effect was revealed in samples stored at 30 ºC, where the spore decrease was of 0.84 Log spore/g for F4 and 0.82 Log spore/g for F6 (Table 2). In addition, the inoculated samples, during storage at 30 ºC, exhibited a color change, according to the *B. cereus* growth except when treated with the F6 (Supplementary Figure 1).

For blank PC-PST and PC-CRE samples stored at 6 and 30 ºC, until 120 days of storage, both total viable count (< 2 Log CFU/g) and mesophilic spore count (< 2 Log spore/g) were always below the detectable limit. This indicated that no microbial contamination occurred during samples preparation, and that they were stable over the 120 days of storage at 6 and 30 ºC.

3.2. Physico-chemical analysis

The mean values of samples differently treated are reported in Table 3. In details, the DM content in control samples ranged from 37.3 to 39.52% and from 36.82 to 40.5% in PC-PST and PC-CRE, respectively. The pH values ranged from 5.45 to 5.71.

4. Discussion

Although vegetative cells of *B. cereus* are not able to survive to pasteurization, spores resist to heat treatments, highlighting the possibility to be present in pasteurized dairy products (Tirloni et al., 2022, 2017). Certain conditions may stimulate the spore germination with a return to a vegetative form and a restart of exponential cell division (Buňková and Buňka, 2017). Viable cells may persist in PC for a quite long time, especially when stored at low temperatures. Results of the present study indicated that most samples stored at lower temperature (6 ºC) did not show bacterial growth. Moreover, a bacteriostatic effect against vegetative cells and a decrease of bacterial spores, during the 120 days of storage, were observed. As recently reported, low temperatures lead to interruption of substance transport across cytoplasmic membrane, with effects on metabolic activity that results considerably decelerated or reduced to a minimum, due to an inappropriate reaction of enzymes (Yousef and Abdelhamid, 2019). Although PC-PST and PC-CRE samples, stored at 6 ºC, showed a bacteriostatic effect on mesophilic spores, the F1 treatment showed growth of *B. cereus* vegetative cells after 90 days of storage in both processing methods. Different results were reported by Spanu et al. (2016) in commercial refrigerated *ricotta salata* cheese during shelf-life, where the level of *B. cereus* vegetative cells decreased from initial value of 4.65 ± 0.74 to 1.99 ± 0.55 Log CFU/g after 90 days. However, as found in the present study, the prolonged refrigerated storage was not favorable to sporulation (Spanu et al., 2016).

According to Schär and Bosset (2002), the shelf-life of PC, prepared at temperatures from 90 to 100 ºC and stored at refrigerated conditions (4-8 ºC) is estimated in several months. The results suggested that the shelf-life of the PC stored at low temperature can be increased by adding appropriate melting salt as a relevant strategy to control *B. cereus*, mainly in refrigerated processed foods of extended durability (Tirloni et al., 2017). The storage temperature of PC has a significant influence on the development or slowdown in the growth of the present bacteria (Buňková and Buňka, 2017). In the present study, only the F6 treatment was able to inhibit the multiplication of

B. cereus. Although no spore growth was observed at both 6 and 30 ºC, bacterial spores are resistant to various environmental stresses and can also survive for a long time in nutrient pour matrix waiting to return to the vegetative state and resume multiplication and normal metabolism (McClure, 2006). This behavior has been previously reported in food inoculated with *B. cereus* spores where the spore counts were always lower than vegetative cell counts (Rajkovic et al., 2013).

Spore-forming bacteria are a problem for the dairy industry due to spore formation and to their subsequent returning to vegetative state during dairy storage, where cells (> 10^3 CFU/g) can produce two types of toxins: the emetic toxin (in food) and diarrheal toxins (in the small intestine) if ingested (Carroll et al., 2019; McClure, 2006; Messelhäußer and Ehling-Schulz, 2018). However, the germination does not ensure complete outgrowth, as was the case for F6 where vegetative cells were inhibited. Indeed, with F6 treatment no vegetative or spore growth under all tested conditions (storage time, storage temperature and processing method), were detected, suggesting a probable inhibitory effect of polyphosphate on bacterial growth. In the present study, the tested F6 treatment contains polyphosphates (E452), with the highest P_2O_5 content (66.0%). Interestingly although the F5 showed the same P_2O_5 content (65.7%) it did not show the same inhibitory effect and this could be because F5 does not contain exclusively polyphosphates, as sodium phosphates (E339) is also present. In a previous study, treatments with the same concentration of polyphosphates resulted in a different inhibition profiles based on the presence/absence of sodium phosphates, in PC inoculated with vegetative cells of *B. thuringiensis* CFBP 3476 (Fusieger et al., 2022a). Loessner et al. (1997) evaluated the effect of polyphosphates on the growth of *Clostridium tyrobutyricum* ATCC 25755 spores in sodium phosphates and polyphosphates PC treated, and found that when the amount of sodium phosphates was lower, the inhibitory effect against *C. tyrobutyricum,* during 49 days of storage, was greater (Loessner et al., 1997).

Here bacterial growth occurred faster in F1, F2 and F3 treatments, with a lower P_2O_5 content, compared to F5. In previous studies, polyphosphates has been found to inhibit Gram-positive bacteria (Buňková et al., 2008; Loessner et al., 1997; Maier et al., 1999) more efficiently than sodium phosphates (Lee et al., 1994a, 1994b), Moreover, according to Tanaka et al. (1979) and Steeg et al. (1995), which reported that in PC polyphosphate appeared to be more inhibitory than citrate against *C. botulinum,* in the present study the F1 treatment, where the citrate was the main constituent, showed the lowest effect. Finally, it is important to note, that polyphosphates formulation also affect the antibacterial potential. Indeed, after 30 days of storage at 30 ºC, the F4 treatment showed an increase of *B. cereus* densities in both processing methods, while F6 showed a bacteriostatic effect. This behavior may be related to phosphorus units of the polyphosphates present as sodium tetrapolyphosphate ($Na_6P_40_{13}$) and sodium hexametaphosphate [$(NaPO_3)_n$ ($n = 10$ to 15)] in turn relatable with the P_2O_5 content, of 60% and 70% of P_2O_5 (Guinee et al., 2004). In a USA patent, Kohl and Ellinger (1972) suggested that the number of phosphorus units in a polyphosphate may affect its antimicrobial activity, with greater inhibitory effect associated with long-chain polyphosphates.

Furthermore, according to the desired final product (as formulation, texture, and shelf-life), different processing methods can be employed in PC processing, as low mixing speeds, ranging from 50 to 150 rpm, or high mixing speed, ranging from 1500 to 3000 rpm, and the binomial time x temperature can range from 70 to 90 ºC for 3 to 7 min, from 95 to > 100 ºC for 2 to 5 min, or from 130 to 145 ºC for 2 or 3 s (Kapoor and Metzger, 2008). In the present study, samples were processed with the same mixing speeds and binomial time x temperature, with the only difference in the addition of the creaming step. Creaming process is important for the homogeneous texture, stability and to improve firmness of PC, thanks the uniform fat droplet dispersion and, consequently, for the stability of the final product (Li et al., 2020; Weiserová et al., 2011). In this process melting salts can play an important role, they removes calcium caseinate bound to caseins, allowing casein to emulsify the fat in PC matrix (Hougaard et al., 2015). At the same time, the melting salts can inhibit the multiplication of undesirable microorganisms, as observed here against *B. cereus* (Figure 2 and 3). The relationship of creaming process to microbial inhibition has never been explored for PC. Our results showed that the growth of *B. cereus* vegetative cells occurred faster in PC-PST samples stored at 30 ºC; e.g., F2-PC-PST showed growth after 15 days, while F2-PC-CRE only after 45 days, F5-PC-PST after 60 days, while F5-PC-CRE after 90 days, and in F3, for both processing methods, the growth occurred after 15 days, but higher counts were recorded for F3-PC-PST (Figure 2-B and 3-B). Such an effect was confirmed by bacterial decrease/increase, during the storage time, in PC-CRE samples a higher decrease of vegetative and spore cells of *B. cereus* was observed.

Moreover, during the PC production, the extension of creaming process a reduce water and oil separation and enhances the exchange of calcium of the para-casein micelles by sodium ions (introduced by sodium phosphates) (Glenn III et al., 2003). The calcium lower availability could be related to the inhibitory mechanism of melting salts, therefore the PC-CRE samples were more stable. Lee et al., (1994b) evaluated the antibacterial mechanism of long-chain polyphosphates in *Staphylococcus aureus*, and levels of free Ca^{2+} and Mg^{2+} in polyphosphate treated cells were significantly lower than in cells without polyphosphate, in liquid culture medium. The polyphosphates chelate structurally Ca^{2+} and Mg^{2+} of cell wall, and these metal ions probably form cross bridges between the teichoic acid chains in the cell walls of Gram-positive bacteria, resulting in bactericidal and bacteriostatic effects (Lee et al., 1994b). Later, Mayer et al. (1999) investigated the cellular mechanisms of growth inhibition, morphological changes, and lysis of *B. cereus* WSBC 10030 treated with a long-chain polyphosphate in liquid culture medium. Supplemental divalent metal ions (Ca^{2+} and Mg^{2+}) could almost completely block and reverse the antimicrobial activity of long-chain polyphosphate and the authors proposed that the salts might have an effect on the ubiquitous cell division protein FtsZ, whose GTPase activity is strictly dependent on divalent metal ions. In addition, the authors hypothesized that long-chain polyphosphate indirectly blocks the dynamic formation (polymerization) of the Z ring, which would explain the aseptate phenotype observed (Maier et al., 1999). In this way, the creaming process may improve the effect of emulsifying salts on bacterial inhibition by reducing the availability of metal ions, mainly calcium. These findings will be considered in future studies through the supplementation of metal ions in creaming and not creaming PC.

Hence, in PC the contamination by spore-forming bacteria might be detected by abnormal odors and/or colors, as spoilage microorganisms are responsible for various sensory defects, but these sensory descriptors are not easily associated with enzymatic functions or metabolic pathways (Oliveira et al., 2016). For samples stored at 30 ºC, we observed that the F6 treatment did not apparently change the sample color (Supplementary Figure 1).

Regarding physicochemical results, the pH values of blank PC-CRE samples were lower than blank PC-PST samples, from 5.45 to 5.66 and from 5.46 to 5.71, respectively. The optimal pH for suitable structural and sensory properties of PC should range between 5.50 and 6.00 (Guinee et al., 2004). Weiserová et al., (2011) described pH values within the interval between 5.48 and 6.59 for PC spreads produced with different combinations of melting salts and treatments with polyphosphates exhibited the lowest pH values. Salek et al. (2019) evaluated melting salts on selected properties of PC sauce and the pH of the samples, after pH adjustment, ranged from 5.62 to 5.87; and a possible explanation for the “close” variability observed in the pH could be found in the melting salts buffering capacity. The buffering capacity of sodium salts in the pH of PC normally decreases with an increase in chain size (Fox et al., 2017). The DM similarity between the samples allows their comparison, as this factor could affect their properties, and values ranging from 40.84 to 41.91% were reported by Weiserová et al. (2011). Fat values are lower than those reported by Chen and Liu (2012), 22.90% in PC made from Mozzarella. The changes of protein content of PC treatments can be attributed mainly to the hydrolysis of polyphosphate in emulsifying salts, which determined higher solubilization of proteins (Fox et al., 2017).

In view of the experimental parameters chosen, treatment F6 can be a suitable tool to control the growth of *B. cereus* under more realistic conditions, where initial contamination are lower (Buňková and Buňka, 2017). According to the food matrix, the inoculum may die off initially before adapting to the environment. If too low of an inoculum concentration is used, the incorrect assumption could be made that the food is stable when it is not (Komitopoulou, 2011). Khanipour et al. (2016) performed a study on the application of hurdle technology to inhibit the growth of *C. sporogenes* in high moisture, low salt, ambient shelf-stable PC analogue and also inoculated 10^6 spore/g to confirm the effect of a preservation system in food, and demonstrated that growth did not occur in practice. Still, a relatively high inoculum level is chosen to ensure that preservative levels that are found to be necessary to prevent growth are conservatively high (Khanipour et al., 2016).

5. Conclusion

The present study highlighted that the creaming process and the P_2O_5 content can improve the inhibitory effect of melting salts in PC, during the storage time, on *B. cereus* vegetative and spore growth. In the present study, the storage temperature and the composition of melting salts significantly influences bacterial growth and the presence of sodium phosphate may infer such activity. However, more detailed studies are needed to determine the specific melting salt composition and the relation of P_2O_5 content responsible for bacterial inhibition. Food spoilage and safety are challenges in the dairy industry and, based on the results here described, we show the potential of melting salts with a focus on *B. cereus* inhibition in PC.

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Declaration of competing interest

The authors have no competing interests to declare.

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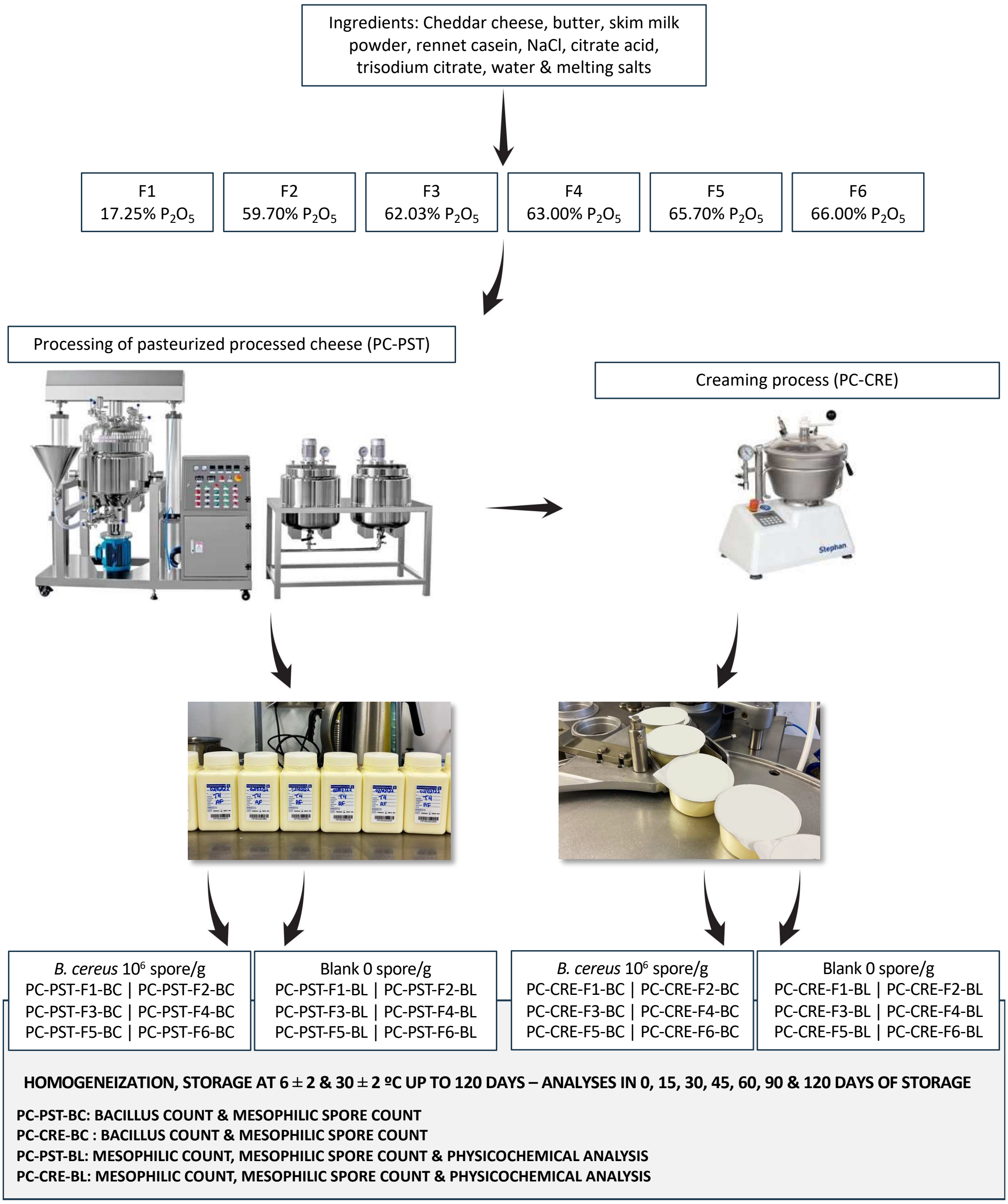


Figure 1. Flow chart of applied procedures for obtaining PC samples.

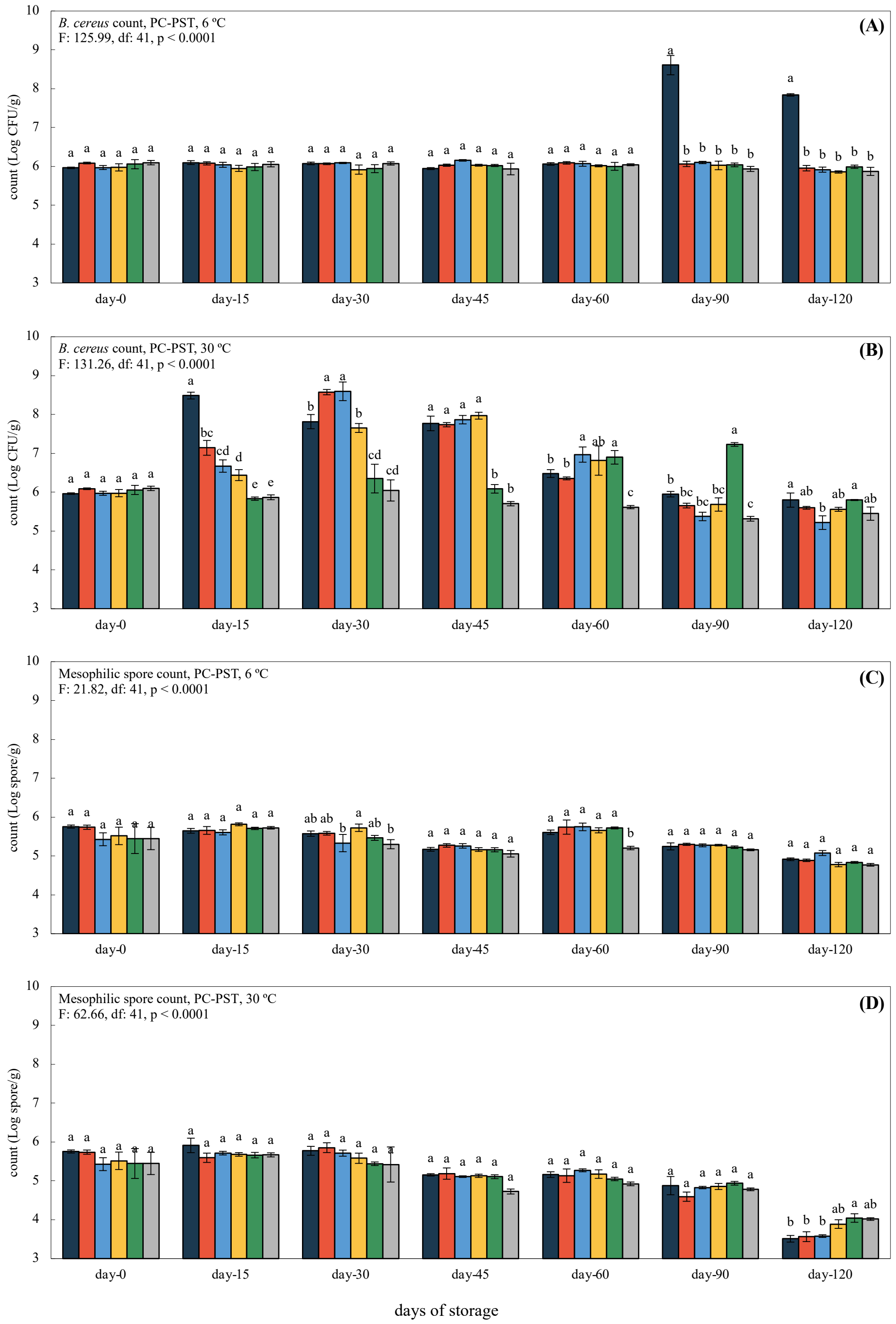


Figure 2. Growth of vegetative (A and B) and spore cells (C and D) of *B. cereus* in PC-PST samples stored at 6 and 30 ºC. (■) Dark blue bars – F1; (🟥) Red bars – F2; (🟦) Light blue bars – F3; (🟨) Gold bars – F4; (🟩) Green bars – F5; (⬜) Light gray bars – F6. Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), different letter indicates significant differences among mean values during storage time of each treatment. In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance.

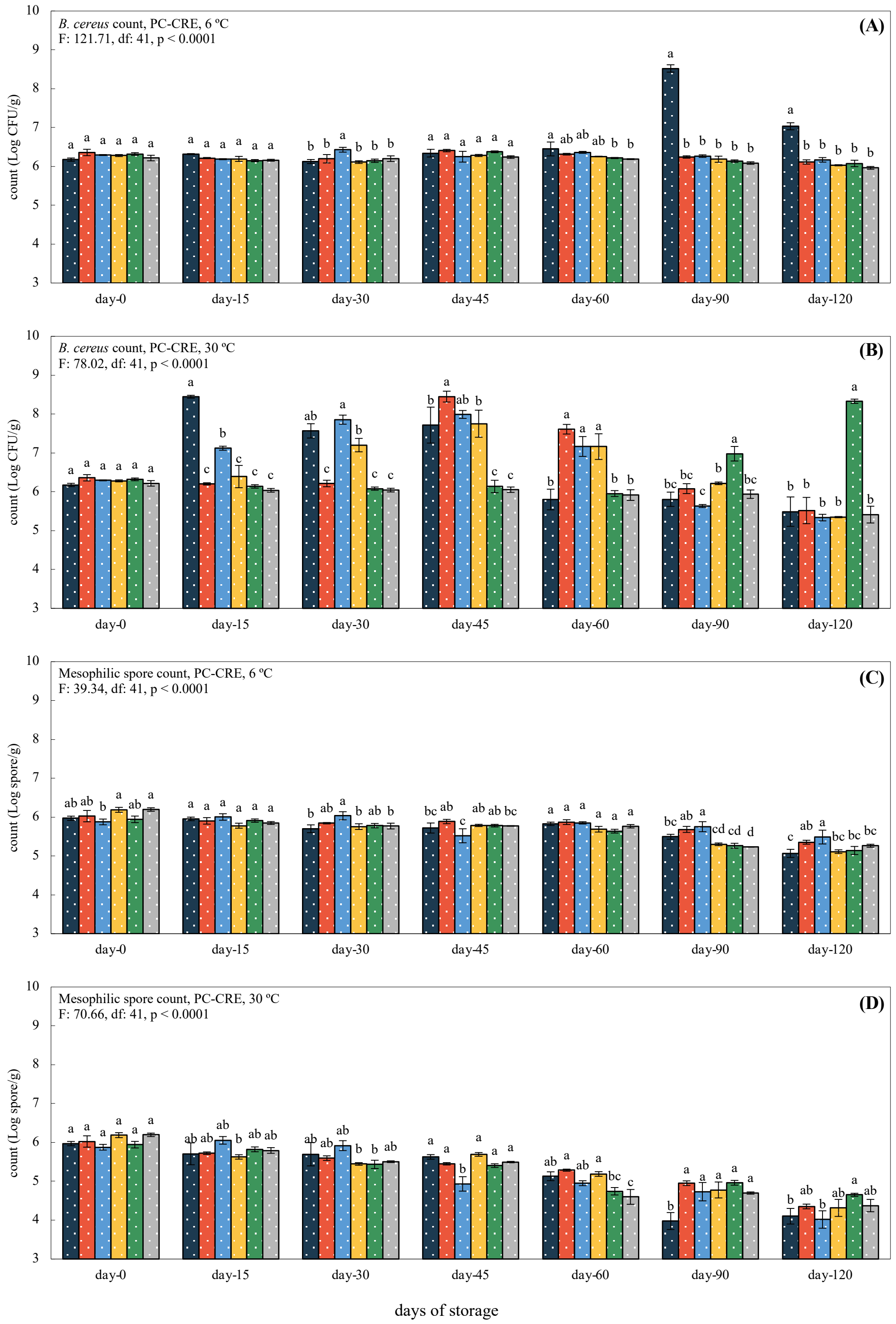


Figure 3. Growth of vegetative (A and B) and spore cells (C and D) of *B. cereus* in PC-CRE samples stored at 6 and 30 ºC. (⬛) Dark blue bars – F1; (🟥) Red bars – F2; (🟦) Light blue bars – F3; (🟨) Gold bars – F4; (🟩) Green bars – F5; (⬜) Light gray bars – F6. Mean values (standard errors) compared by ANOVA and Tukey ($p < 0.05$), different letter indicates significant differences among mean values during storage time of each treatment. In each graph: F: ANOVA reference value, df: degrees of freedom, p: level of significance..

Table 1. Treatments applied in the production of PC samples.

Treatment	Melting salt	Final P_2O_5 (%)
F1	1.5% M-I + 0.5% M-II	17.25 ± 1.0
F2	2.0% M-III	59.7 ± 1.0
F3	1.5% M-III + 0.5% M-II	62.03 ± 1.0
F4	2.0% M-IV	63.0 ± 1.0
F5	2.0% M-V	65.7 ± 1.0
F6	2.0% M-VI	66.0 ± 1.0

Table 2. Bacterial behavior, during the storage time, in relation to the initial inoculum (day 0) in PC samples.

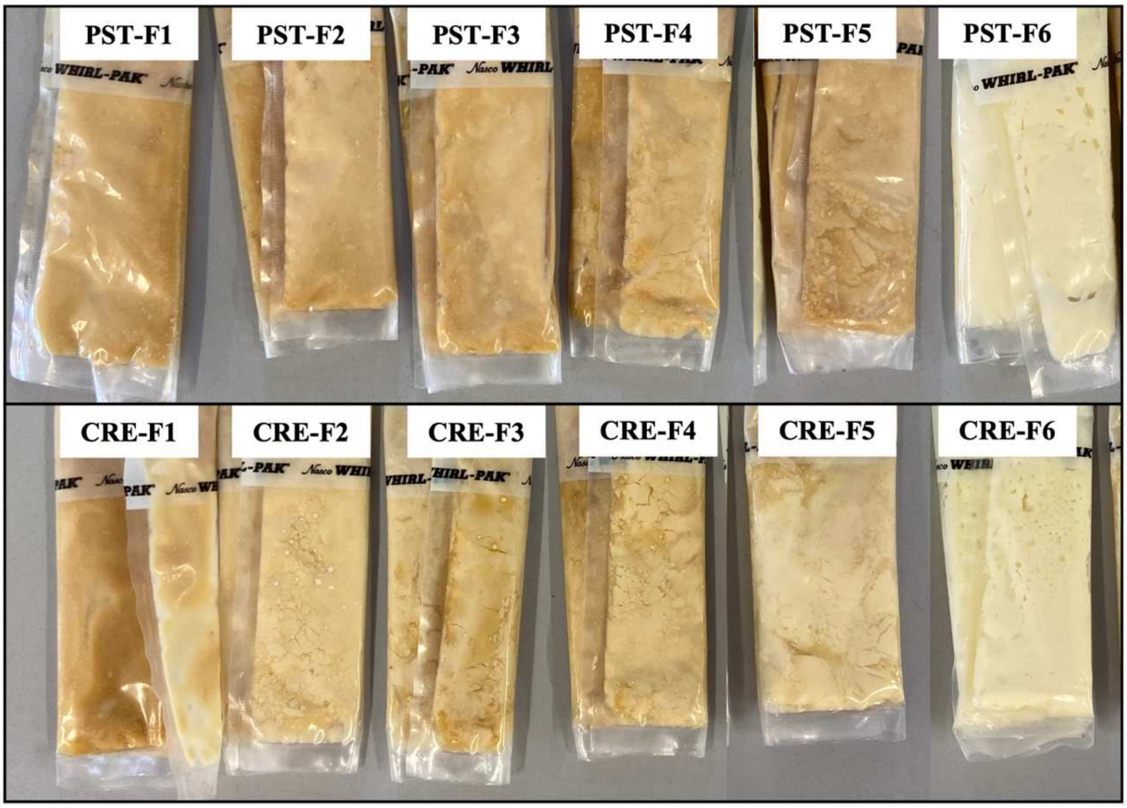
Count	Condition	F1	F2	F3	F4	F5	F6
B. cereus count	PC-PST 4 ºC	1.14 ± 0.18	0.99 ± 0.01	1.02 ± 0.02	1.00 ± 0.03	0.99 ± 0.03	0.98 ± 0.02
(log cfu/g)	PC-CRE 4 ºC	1.10 ± 0.14	0.98 ± 0.02	1.00 ± 0.02	0.98 ± 0.02	0.98 ± 0.02	0.99 ± 0.02
	PC-PST 30 ºC	1.18 ± 0.18	1.12 ± 0.18	1.14 ± 0.21	1.12 ± 0.16	1.05 ± 0.10	0.93 ± 0.05
	PC-CRE 30 ºC	1.10 ± 0.20	1.05 ± 0.17	1.09 ± 0.17	1.06 ± 0.13	1.04 ± 0.14	0.95 ± 0.04
Spores count	PC-PST 4 ºC	0.93 ± 0.05	0.94 ± 0.05	0.99 ± 0.06	0.98 ± 0.08	0.99 ± 0.09	0.96 ± 0.08
(log spore/g)	PC-CRE 4 ºC	0.94 ± 0.05	0.96 ± 0.04	0.98 ± 0.05	0.90 ± 0.05	0.94 ± 0.05	0.91 ± 0.04
	PC-PST 30 ºC	0.88 ± 0.14	0.87 ± 0.14	0.93 ± 0.14	0.92 ± 0.12	0.93 ± 0.12	0.91 ± 0.11
	PC-CRE 30 ºC	0.84 ± 0.13	0.87 ± 0.08	0.87 ± 0.13	0.84 ± 0.08	0.87 ± 0.07	0.82 ± 0.09

Values reported are the means ± standard deviations. Values < 1.00 indicate bacterial decrease, and > 1.00 indicate bacterial increase in the cell population during the storage time.

Table 3. Physico-chemical traits detected in PC-PST and PC-CRE blank samples.

Treatment	pH	DM %	moisture %	crude fat %	fat/dm %	total protein %	ash %
PC-PST-F1	5.69 ± 0.01^{ab}	38.99 ± 0.56^{bc}	61.02 ± 0.56^{cd}	20.80 ± 0.10^{b}	53.37 ± 1.03^{cd}	9.95 ± 0.05^{abcd}	5.69 ± 0.01^{ab}
PC-PST-F2	5.71 ± 0.01^{a}	39.52 ± 0.47^{ab}	60.48 ± 0.47^{de}	20.90 ± 0.09^{b}	52.89 ± 0.63^{cde}	10.07 ± 0.02^{ab}	5.71 ± 0.01^{a}
PC-PST-F3	5.60 ± 0.01^{de}	38.44 ± 0.44^{bcd}	61.56 ± 0.44^{bcd}	20.20 ± 0.09^{c}	52.56 ± 0.60^{de}	9.75 ± 0.25^{bcde}	5.60 ± 0.01^{de}
PC-PST-F4	5.60 ± 0.01^{de}	37.30 ± 0.30^{de}	62.70 ± 0.30^{ab}	20.50 ± 0.09^{bc}	54.96 ± 0.44^{ab}	9.87 ± 0.05^{abcd}	5.60 ± 0.01^{de}
PC-PST-F5	5.49 ± 0.03^{f}	37.89 ± 0.20^{cde}	62.11 ± 0.20^{abc}	19.65 ± 0.05^{d}	51.86 ± 0.14^{e}	9.53 ± 0.03^{ef}	5.49 ± 0.03^{f}
PC-PST-F6	5.46 ± 0.03^{f}	38.80 ± 0.25^{bc}	61.20 ± 0.25^{cd}	20.25 ± 0.05^{c}	52.19 ± 0.21^{de}	9.64 ± 0.05^{de}	5.46 ± 0.03^{f}
PC-CRE-F1	5.62 ± 0.02^{cd}	36.85 ± 0.95^{e}	63.15 ± 0.95^{a}	19.65 ± 0.25^{d}	53.34 ± 0.70^{cd}	9.25 ± 0.25^{f}	5.62 ± 0.02^{cd}
PC-CRE-F2	5.66 ± 0.01^{bc}	39.70 ± 0.50^{ab}	60.30 ± 0.50^{de}	21.55 ± 0.15^{a}	54.29 ± 0.31^{bc}	10.10 ± 0.10^{a}	5.66 ± 0.01^{bc}
PC-CRE-F3	5.56 ± 0.01^{e}	40.50 ± 0.40^{a}	59.50 ± 0.40^{e}	21.60 ± 0.10^{a}	53.34 ± 0.28^{cd}	10.10 ± 0.10^{a}	5.56 ± 0.01^{e}
PC-CRE-F4	5.59 ± 0.01^{de}	38.80 ± 0.27^{bc}	61.20 ± 0.27^{cd}	20.55 ± 0.05^{bc}	52.96 ± 0.13^{cde}	9.70 ± 0.01^{cde}	5.59 ± 0.01^{de}
PC-CRE-F5	5.49 ± 0.02^{f}	40.50 ± 0.60^{a}	59.50 ± 0.60^{e}	20.35 ± 0.25^{c}	50.25 ± 0.13^{f}	9.45 ± 0.05^{ef}	5.49 ± 0.02^{f}
PC-CRE-F6	5.45 ± 0.03^{f}	38.40 ± 0.20^{bcd}	61.60 ± 0.20^{bcd}	21.50 ± 0.30^{a}	55.99 ± 0.49^{a}	10.00 ± 0.10^{abc}	5.45 ± 0.03^{f}

Values (means ± error) followed by different superscript letters indicate significant difference among each treatment by Tukey ($p < 0.05$). ANOVA: pH, F = 90.06, df = 11, p = < 0.0001; DM, F = 17.75, df = 11, p = < 0.0001; moisture, F = 17.75, df = 11, p = < 0.0001; crude fat, F = 61.72, df = 11, p = < 0.0001; fat/dm, F = 26.33, df = 11, p = < 0.0001; total protein, F = 16.79, df = 11, p = < 0.0001; ash, F = 118.59, df = 11, p = < 0.0001.



Supplementary Figure 1. Inoculated PC-PST and PC-CRE samples after 120 days of storage at 30 ºC.

Supplementary Table 1. General data of melting salts, produced by BK Giulini GmbH, used in PC samples production.

Melting salt	Compound name	P_2O_5 (%)	pH (1% solution)	E-number	Product number
I	trisodium citrate	-	8.4 ± 0.3	E331	2-9263
II	poly- and sodium phosphates	69.0 ± 1.0	6.0 ± 0.3	E452, E339	NA*
III	poly- and sodium phosphates	59.7 ± 1.0	9.0 ± 0.3	E452, E339	NA
IV	polyphosphates	63.0 ± 1.0	8.0 ± 0.3	E452	NA
V	poly- and sodium phosphates	65.7 ± 1.0	7.3 ± 0.3	E452, E339	NA
VI	polyphosphates	66.0 ± 1.0	6.7 ± 0.3	E452	NA

*NA: information not available.

CHAPTER VI.

GENERAL CONCLUSIONS AND FUTURE STUDY

General comments and conclusions

The overall objective of this thesis was to analyze the *in vitro* and *in situ* inhibitory effect of emulsifying salts and to expand the knowledge on the effect in processed cheese. The initial hypothesis was confirmed and it was possible to build a “story” during the execution of this thesis.

Preliminary work was done at the beginning of this study to evaluate the *in vitro* inhibitory activity of an emulsifying salt polyphosphate against a panel of twenty-one target bacteria, as described in *Chapter II*. JOHA HBS$^{®}$ is known for its bacteriostatic effect against Gram-positive bacteria and fungistatic effect against yeasts and molds, which was confirmed in our results against bacterial targets. The bacterial inhibition of JOHA$^{®}$ HBS (at different concentrations, from 0.2% to 3.0%) was evaluated by streak assay, agar-spot method, and by spot-on-the-lawn and well- diffusion method. One of our initial problems was in the methodology adopted for the inhibitory activity, as high concentrations of the polyphosphate were necessary to inhibit bacterial targets in the previous project. However, we were able to solve this issue through the use of different culture medium (Brain Heart Infusion Broth, BHI; Nutrient, NT; Plate Count, PCA; Trypticase Soy, TSA/B), either in liquid or solid form. Among the culture media, both NT and PCA showed satisfactory results; but we chose to proceed with NT medium because lower concentrations of the emulsifying salt were necessary to inhibit *Listeria innocua* ATCC 33090 and *Listeria monocytogenes* Scott A in the streak assay on agar. Furthermore, in all the protocols tested, the solid culture medium (NT agar) was always more effective for the inhibition of the target bacteria. This behavior is probably associated with the solubility of the analyzed emulsifying salt. When the emulsifying salt was added to the broth, even after stirring, the sedimentation of a portion of the ingredient was visible. Results of the streak assay on NT agar showed that 11 out the 21 tested strains were highly inhibited at 0.5% of JOHA® HBS (w/v); these results include *Bacillus* spp. targets, where in the results prior to this study concentrations above 2% (w/v) were necessary for inhibition. At adjusted pH levels, concentrations of 1.0% (w/v) were able to inhibit the same targets, confirming the antimicrobial effect of JOHA® HBS. Overall, it was possible to rapidly and effectively assess an *in vitro* protocol for screening the inhibitory activity of emulsifying salts with low solubility. The comparison of different culture media and protocols can provide a standard for future studies on this topic, thus avoiding obtaining untrue data and an incorrect association of inhibitory activity.

In out next step, we selected *B. thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124 to access the *in situ* inhibitory activity of two emulsifying salts (ESSP = short polyP and BSLP = long polyP), as reported in *Chapter III*. Two treatments were performed: T1 = 1.5% ESSP; and T2 = 1.0% ESSP + 0.5% BSLP. Processed cheese samples were produced through laboratory-scale (using a Thermomix homogenizer) and pilot-scale (using Stephan Geiger homogenizer), inoculated and analyzed until 45 days of storage at 6 ± 2 ºC. In brief, none of the conditions tested inhibited *C. perfringens* ATCC 13124. On the other hand, *B. thuringiensis* CFBP 3476 showed bacterial decrease in both of the treatments. The most interesting aspect of this study was that the sample production method influenced the inhibitory activity of *B. thuringiensis* CFBP 3476, we obtained a higher and faster reduction in samples produced by the laboratory-scale method (1.6 log cfu/g) when compared to the pilot-scale method (1.8 log cfu/g) ($p < 0.05$); and this motivated the construction of *Chapter V*. Hence, at this point it was clear to us the inhibitory potential of emulsifying salts, as well as a possible interaction between them.

Therefore, continuing to work with these emulsifying salts was important to explore a possible inhibitory combinations and to compare with antimicrobial additives commonly used in the dairy industry; and this we demonstrate in *Chapter IV*. Compared to the chapter mentioned above, we have added more bacterial targets (n=8; *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *C. perfringens* ATCC 13124, *Enterococcus faecalis* FAIR-E 179, *L. monocytogenes* Scott A, *Pseudomonas fluorescens* 07A, and *Staphylococcus aureus* ATCC 6538) and treatments (n=14; T1 = 1.5% ESSP + 0.00125% nisin; T2 = 1.5% ESSP + 0.1% potassium sorbate; T3 = 1.5% ESSP + 0.00125% nisin + 0.1% potassium sorbate; T4 = 1.5% trisodium citrate; T5 = 1.5% ESSP; T6 = 1.25% ESSP + 0.25% BSLP; T7 = 1% ESSP + 0.5% BSLP; T8 = 0.75% ESSP+ 0.75% BSLP; T9 = 0.5% ESSP+ 1% BSLP; T10 = 0.25% ESSP + 1.25% BSLP; T11 = 0.5% BSLP; T12 = 1% BSLP; T13 = 1.5% BSLP; and T14 = 2% BSLP). However, all processed cheese samples were produced through laboratory-scale (using a Thermomix homogenizer), inoculated and analyzed until 90 days of storage at 6 ± 2 ºC. As we would have 2,520 samples to be analyzed, we created a strategy on the day of processing and inoculation of the samples; all samples were divided into 25 g portions in appropriate bags for microbiological analysis. Thus, on the day of the analysis during storage, it was not necessary to weigh the samples, just add the dilution solution and proceed with the appropriate protocol for each bacterial target.

In order to compare the obtained data, we separated the treatments into 4 groups (I = negative control for bacterial growth, T1, T2 and T3; II = positive control for bacterial growth, T4 and T5; III = partial replacement of ESSP by BSLP, T6, T7, T8, T9 and T10; and IV = BSLP alone, T11, T12, T13 and T14). Then, we selected one treatment from each of the 4 groups to compare the groups, which was named as Group V. Among the treatments in Group I, higher levels of inhibition were recorded with T3 against the bacterial targets. For Group II, lower counts were obtained in T5. In relation to Group III, in the treatments where the replacement of ESSP by BSLP was greater (T9 and T10), a higher level of bacterial inhibition was observed. Finally, in Group IV all treatments had a similar inhibitory profile, but the performance of T13 and T14. When the selected treatments were compared in Group V, similar results were observed for T10 and T13; however, T13 presented a better bacteriostatic effect against *E. faecalis* FAIR-E 179 and *P. fluorescens* 07A, and was able to reduce the *B. thuringiensis* CFBP 3476 populations in less time. Also, the performance of T13 was better than T3 against *B. cereus* INV 10(3), *B. thuringiensis* CFBP 3476, *C. perfringens* ATCC 13124 and *L. monocytogenes* Scott A; this fact may support the possibility that BSLP emulsifying salt has the potential to replace nisin and potassium sorbate in processed cheese. Furthermore, one of the most important aspects of *Chapter IV* was that our hypothesis from *Chapter III* regarding a possible interaction between the emulsifying salts was confirmed. The treatments in which BSLP was applied alone showed greater inhibitory activity; that is, equal concentrations of BSLP result in less bacterial inhibition when in the presence of ESSP. At this point in the PhD project, we had understood the other problem of the previous project, where 1.5% ESSP + 0.5% BSLP were not able to inhibit *Bacillus* spp. On the other hand, here we have provided relevant insights into the antimicrobial effect of emulsifying salts in processed cheese and that the effect is related to the type of emulsifying salt.

Based on the knowledge obtained from previous chapters (*Chapter III* and *IV*), *Chapter V* focused on the inhibitory effect of six emulsifying salts based on the P_2O_5 content using *B. cereus* as a target model. Since we had already explored two emulsifying salts, it was in our interest to evaluate other salts, as well as the influence of processing methods (PST = pasteurization, and CRE = creaming), storage temperature (6 ± 2 and 30

± 2ºC) and storage time (until 120 days). A mix of spores of *B. cereus* INV 10(3) and *B. cereus* DSM 626 was used as inoculum and six treatments were evaluated according to the final content of P_2O_5 of the combinations of emulsifying salts (F1 = 17.25 ± 1.0%; F2 = 59.7 ± 1.0%; F3 = 62.03 ± 1.0%; F4 = 63.0 ± 1.0%; F5 = 65.7 ± 1.0%; F6 = 66.0 ± 1.0%). *B. cereus* enumeration and mesophilic spore count were performed. Our results indicated that most samples stored at 6 ºC did not show bacterial growth, but it was possible to observe a bacteriostatic effect against vegetative cells and a bacterial decrease against spores during 120 of storage. For the samples stored at 30 ºC, a different behavior was observed, where F6 was the only treatment able to inhibit the multiplication of *B. cereus*. As no spore growth was observed, this indicated that the spores germinate from their resting form is able to return to the vegetative state and resume multiplication and normal metabolism. Regarding processing method, our results show that the growth of *B. cereus* vegetative cells occurred faster for the PST samples. There are no articles available in the literature that compare processing methods with the inhibitory effect of emulsifying salts in processed cheese; but the results obtained in that chapter agree with *Chapter III*. A possible explanation for this behavior is in the process of processed cheese, where the Ca^{2+} of the para-casein micelles is exchanged by Na^{+} ions (introduced by sodium phosphates) and it is enhanced through the extension of creaming process. Available Ca^{2+} will be less to maintain bacterial metabolism and this can be related to the inhibitory mechanism of melting salts, therefore the CRE samples were more stable. Hence, emulsifying salts formulation also affected the antibacterial potential; although F6 with the highest P_2O_5 content showed the best inhibitory effect, F5 has practically the same P_2O_5 content and did not show the same inhibitory potential. On the other hand, F4 had the same formulation as F6 and was not able to inhibit *B. cereus* growth; this behavior may be related to phosphorus units of the polyphosphates (main constituent in the formulation of F4 and F6). In this way, more detailed studies to determine the specific emulsifying salt composition and the relation of P_2O_5 content responsible for bacterial inhibition are needed.

Overall, this thesis provides further knowledge on the inhibitory activity of emulsifying salts against selected target bacteria in processed cheese. Furthermore, we have a broad area of knowledge that can be explored.

General recommendations for future study

Behind the application of emulsifying salts in processed cheese there are many advantages, which go beyond technological issues. In particular, I believe that the results of this thesis can be useful to inspire other groups and also for companies to expand research into the antimicrobial activity of emulsifying salts. Through the knowledge obtained so far, I conclude the PhD with more questions than at the beginning. Several experimental works can be considered and explored in the future. The following are recommendations for further potential study:

Influence of Ca^{2+} and Mg^{2+} content on the inhibitory activity of emulsifying salts

According to the literature, the mechanism of action of polyphosphates is related to the high affinity for divalent ions, such as Ca^{2+} and Mg^{2+}, essential for the integrity of Gram-positive bacteria cell walls. The free content of Ca^{2+} and Mg^{2+} can be explored by supplementation in processed cheese and, consequently, evaluate the behavior of emulsifying salts in the inhibitory activity. Field emission scanning electron microscopy could be used to observe structural damages in bacterial cells.

Effect of cremification conditions on the inhibitory activity of emulsifying salts

In relation to the above recommendation, in processed cheese processing the Ca^{2+} of the para-casein micelles is exchanged by Na^{+} ions and is potentiated through the extension of the creaming process. My hypothesis is that with increasing cremification time, the available Ca^{2+} will be lower to maintain bacterial metabolism and this may be related to the inhibitory mechanism of the emulsifying salts. Furthermore, for this approach it would be important to associate the texture parameters for processed cheese and the mechanisms of structure formation by light and transmission electron microscopy.

Challenge tests with different processed cheese formulations

Several factors have previously been reported to control bacterial growth in processed cheese; these include low moisture, fat content, monoacylglycerols, concentration and type of emulsifying salt, encapsulated and un-encapsulated emulsifying salts, NaCl, pH, water activity, lactate levels, nisin, potassium sorbate, sorbic acid, propionic acid, adjunct cultures. In this way, a series of tests with a varied formulation of processed cheese can determine the influence of each component on bacterial growth. These challenge tests can achieve the safety of processed cheese.

Evaluation of lower initial concentrations of bacterial inoculum and open shelf-life

In the studies performed, relatively high initial inoculum concentrations were used (around 10^5 to 10^6 cfu/g). Under more realistic conditions, initial contamination is lower in processed cheese. However, to work with lower inoculum concentrations, such as 10^2 to 10^3 cfu/g, it would be necessary to employ real-time PCR to measure the bacterial population in the matrix. With the recognized enumeration methods (pour plate and spread plate), the detection limit does not allow the correct visualization of a possible bacteriostatic or bactericidal effect. Hence, studies with lower initial inoculum concentrations may bring different results to those described in this thesis, and probably the concentration of emulsifying salts will be lower to control bacterial growth. This approach can be considered in parallel with the evaluation of open shelf-life, that is, after the package of processed cheese is opened. Under these conditions, it would be possible to visualize the bacterial behavior in samples that theoretically will already be in the consumer's refrigerator.

Inhibitory effect of the composition of emulsifying salts and formulation of a new blend

In the *in situ* studies of the thesis, we reported a possible interaction between the emulsifying salts. Polyphosphates tend to have a greater inhibitory effect than phosphates and, in some cases, this may be related to the P_2O_5 content. However, what can actually explain the synergistic and/or inhibitory effect is the number of phosphorus units and the molecular mass in an emulsifying salt. This characteristic is not directly linked to the P_2O_5 content. As we use commercial emulsifying salts, it was not possible to make this association; the exact composition of each emulsifying salt is confidential. Therefore, future studies are relevant to evaluate separately the different groups of emulsifying salts, such as citrates (monosodium citrate, trisodium citrate), orthophosphates (monosodium phosphate, disodium phosphate, trisodium phosphate), pyrophosphates (disodium pyrophosphate, trisodium pyrophosphate, tetrasodium pyrophosphate), polyphosphates (pentasodium tripolyphosphate, pentasodium hexahydrate, sodium tetrapolyphosphate, sodium hexametaphosphate). This approach could be used against bacteria, fungi and yeasts. By obtaining the inhibitory effect of each emulsifying salt, there is the possibility to formulate a new blend for the ingredients industry, which would meet the technological and microbiological needs. Additionally, it would collaborate with the clean label concept, as there is the possibility of replacing additives that are commonly added in processed cheese, such as nisin and potassium sorbate.

Development and validation of a growth model to optimize the use emulsifying salts

Growth and growth boundary model with terms for melting salts to predict growth responses were already reported for *Listeria monocytogenes* in processed cheese.

However, considering all the possibilities and variables mentioned above, a new model could be developed to achieve microbiological stability of processed cheese. Cardinal parameter terms for emulsifying salts, processed cheese composition, Ca^{2+} and Mg^{2+} content, creaming process, initial inoculum concentrations and open shelf-life can be developed and used to expand the available knowledge on growth and growth limit models. In addition, other microbial targets could be tested, mainly spore-forming bacteria. The mathematical model can be an important tool to support product development, reformulation or risk assessment for processed cheese.

Evaluation of inhibitory behavior in 3D-printed processed cheese

As we have described, the processing method of processed cheese can affect the antimicrobial activity of the emulsifying salts. Considering that we are under continuous innovations, three-dimensional (3D) food printing has increasingly become a topic of discussion in the food sector in recent years as a disruptive and novel food technology. As it is a relatively unexplored area in food science, it would be interesting to study further this topic by comparing the feasibility with traditional processing methods, as well as introducing emulsifying salts to evaluate shelf-life behavior.

CHAPTER VII.

PHD OUTPUT

Published papers

Diversity of filamentous fungi associated with dairy processing environments and spoiled products in Brazil

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Abstract

Few studies have investigated the diversity of spoilage fungi from the dairy production chain in Brazil, despite their importance as spoilage microorganisms. In the present study, 109 filamentous fungi were isolated from various spoiled dairy products and dairy production environments. The isolates were identified through sequencing of the internal transcribed spacer (ITS) region. In spoiled products, *Penicillium* and *Cladosporium* were

the most frequent genera of filamentous fungi and were also present in the dairy environment, indicating that they may represent a primary source of contamination. For dairy production environments, the most frequent genera were *Cladosporium*, *Penicillium*, *Aspergillus*, and *Nigrospora*. Four species (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae*, and *Paecilomyces maximus*) were identified for the first time in dairy products or in dairy production environment. Phytopathogenic genera were also detected, such as *Montagnula*, *Clonostachys*, and *Riopa*. One species isolated from the dairy production environment is classified as the pathogenic fungi, *R. similis*. Regarding the phylogeny, 14 different families were observed and most of the fungi belong to the Ascomycota phylum. The understanding of fungal biodiversity in dairy products and environment can support the development of conservation strategies to control food spoilage. This includes the suitable use of preservatives in dairy products, as well as the application of specific cleaning and sanitizing protocols designed for a specific group of target microorganisms.

Bacteriocin-like inhibitory substances (BLIS) synthesized by *Lactococcus lactis* LLH20: Antilisterial activity and application for biopreservation of minimally processed lettuce (*Lactuca sativa* L.)

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Abstract

This work aimed to evaluate the ability of the isolate *Lactococcus lactis* LLH20 to synthesize bacteriocin-like inhibitory substances (BLIS) with antilisterial activity and its potential application for the biopreservation of minimally processed lettuce (*Lactuca sativa* L.). The BLIS showed antilisterial activity, sensitivity to proteolytic enzymes and stability at 100 ºC, pH 2 to 10, chemical agents and temperatures of -20 ºC for 105 days. The isolate LLH20 did not present positive results for lantibiotics related genes (*lanB, lanC, lanM*) and nisin, lacticin 481, lacticin 3147, and lactococcin 972. In 14 h of fermentation (MRS broth), the highest BLIS produced by LLH20 was identified, and the best condition found was a medium containing 1.25% glucose, pH 7 at 32 ºC. The LLH20 has been shown to be safe for use in food by phenotypic tests. Lettuce was prepared, inoculated with *Listeria monocytogenes* (4.0 log CFU/g) and antimicrobial agents LLH20 (8.0 log CFU/g), nisin (2.5 ppm), BLIS of LLH20 (1600 AU/mL), or association of BLIS and nisin (1600 AU/mL and 2.5 ppm), and stored at 4 ºC for 7 days. The *L. monocytogenes* counts in lettuce decreased by 3.14 and 1.63 log CFU/g, when inoculated with LLH20 or with BLIS from LLH20, respectively, being significantly different from the positive control ($p < 0.05$). The association of BLIS and nisin eliminated *L. monocytogenes* in minimally processed lettuce. These results indicate that *L. lactis* LLH20 synthesized a BLIS capable of controlling the growth of *L. monocytogenes* in lettuce stored at 4 ºC, demonstrating its potential as a biopreservative.

The *Weissella* genus in the food industry: A review

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Abstract

The genus *Weissella* is composed of bacteria classified as Gram-positive, catalase negative, non-spore forming, coccoid morphology or short bacilli. They belong to the group of lactic acid bacteria (LAB), mainly by production of lactic acid from the fermentation of carbohydrates. *Weissella* species are distributed in different habitats, such as soils, milking machines, sugar cane and some strains with interesting technological features can be isolated from fermented foods, such as cheeses made from raw milk, fermented vegetables and fermented milk. From the point of view of food technology, some strains have potential in the production of exopolysaccharides, non-digestible oligosaccharides, which is beyond their probiotic potential. Therefore, the bacteria belonging to the genus *Weissella* might have great technological importance, being also involved in the control of foodborne diseases by production of bacteriocins and hydrogen peroxide. This genus has great potential for use in the food industry.

Weissella: An emerging bacterium with promising health benefits

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Abstract

Weissella strains have been the subject of much research over the last 5 years because of the genus' technological and probiotic potential. Certain strains have attracted the attention of the pharmaceutical, medical, and food industries because of their ability to produce antimicrobial exopolysaccharides (EPSs). Moreover, *Weissella* strains are able to keep foodborne pathogens in check because of the bacteriocins, hydrogen peroxide, and organic acids they can produce; all listed have recognized pathogen inhibitory activities. The *Weissella* genus has also shown potential for treating atopic dermatitis and certain cancers. *W. cibaria*, *W. confusa*, and *W. paramesenteroides* are particularly of note because of their probiotic potential (fermentation of prebiotic fibers) and their ability to survive in the gastrointestinal tract. It is important to note that most of the *Weissella*

strains with these health-promoting properties have been shown to be save safe, due to the absence or the low occurrence of virulence or antibiotic-resistant genes. A large number of scientific studies continue to report on and to support the use of *Weissella* strains in the food and pharmaceutical industries. This review provides an overview of these studies and draws conclusions for future uses of this rich and previously unexplored genus.

Biodiversity and technological features of *Weissella* isolates obtained from Brazilian artisanal cheese-producing regions

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Abstract

Weissella spp. strains (n = 57) were isolated from different Brazilian food-related environments and characterized based on their genetic profiles and technological potential. PFGE and Rep-PCR revealed the high level of biodiversity among isolates: PFGE grouped the isolates in three major profiles (similarity from 60 to 80%), while rep-PCR characterized the isolates as belonging to a single profile. Based on PFGE, isolates with identical pulsotypes were found within a same geographical region (Campo das Vertentes, Minas Gerais state). Based on these profiles, 26 isolates were selected and characterized based on their inhibitory and bacteriocinogenic activity against *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* subsp. *aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *Escherichia coli* ATCC 11229. Most isolates (n = 20) were able to inhibit the targets through organic

acids. Most of the isolates (n = 12) were able to produce diacetyl, proteases, and/or coagulating milk, but none were able to produce exopolysaccharides. Isolate 16, in particular, was characterized as possessing high acidification ability, diacetyl and protease production, with promising technological potential in the dairy industry. The obtained results allowed the understanding of the *Weissella* spp. strains role as starter cultures in the dairy industry.

Microbiological quality and safety of Brazilian artisanal cheeses

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Abstract

The establishment of norms that regulates the production and trade of Brazilian Artisanal Cheeses (BAC) has been stimulating many small farmers for this activity. The predominance of lactic acid bacteria (LAB) is a typical characteristic of BAC, which confers desirable attributes to artisanal cheeses. However, these products can be contaminated by other microbial groups, including those that indicate hygienic failures during production and may cause spoilage, or even microorganisms that pose risks to consumers' health. A systematic review of the literature published from January 1996 to November 2020 was carried out to identify scientific data about production characteristics and microbiological aspects of BAC, with a major focus on quality and safety status of these traditional products. Studies that fulfilled the inclusion criteria indicated that artisanal chesses produced in Brazil still do not satisfactorily meet the microbiological criteria established by the national laws, mainly due to the high counts of coagulase-positive *Staphylococcus* and coliforms. Despite low prevalence, pathogens such as

Salmonella and *Listeria monocytogenes* were isolated in some BAC. This review contributed to better understanding microbiological aspects of BAC, the data compiled by the authors highlight the need to improve hygiene practices along the production chain of these traditional cheeses.

Microbial shifts through the ripening of the "Entre Serras" Minas artisanal cheese monitored by high-throughput sequencing

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Abstract

Minas Gerais is a Brazilian state known as the largest cheese producer in Brazil. Minas Artisanal Cheese (MAC) is produced in different regions of this Brazilian state using raw cow milk to which a natural starter culture ("pingo") is added. "Entre Serras" is one of these regions, in which the MAC production had decreased (even stopped) for decades until recently, when artisanal cheeses production has been resurrected. Here, we aimed to gain insights on the bacterial diversity of "Entre Serras" MAC. 16S rRNA gene amplicon sequencing was used to assess the bacterial community in cheeses produced by four farms (A, B, C, and D) over 60 days of ripening. Overall, *Lactococcus lactis* was the predominant species found, regardless of the producer/farm. *Enterococcus*, *Streptococcus*, *Lactobacillus* and *Leuconostoc* genera were also prevalent in the samples microbiota and their levels varied according to the producer/farm. Cheeses produced by

Farms A and B presented high contaminant levels (mainly Enterobacteriaceae and *S. aureus*), which may be attributed to poor hygiene during cheese production and/or herd health management. Chao1 indices varied significantly when the estimated species richness values of the producers/farms were compared ($p < 0.05$). A principal coordinate analysis also revealed distinct microbial communities for some farms ($p < 0.001$). However, no statistical significance was identified when samples were grouped by ripening time. Core microbiota analysis indicated that "Entre Serras" MAC microbiota includes not only LAB, but also spoilage and potentially pathogenic bacteria. We provide the first insights on the bacterial diversity of "Entre Serras" MAC, helping the understanding of the inter-regional microbiological diversity of the samples.

Short communication: Potential use of passion fruit (*Passiflora cincinnata*) as a biopreservative in the production of coalho cheese, a traditional Brazilian cheese

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Abstract

Passion fruit (*Passiflora cincinnata* Mast.) is a native fruit from the Caatinga, a typical ecoregion in northeastern Brazil, and it has potential for use by the food and pharmaceutical industries. In this study, we characterized the antimicrobial activity of *P. cincinnata* and its application in the production of coalho cheese, a traditional Brazilian product. Aqueous extract of *P. cincinnata* exhibited high inhibitory activity against *Listeria* spp. (n = 4, reference strains), *Staphylococcus aureus* (n = 3, reference strains), and multidrug-resistant *Staph. aureus* (n = 8), and low inhibitory activity against lactic acid bacteria (LAB, n = 3, reference strains). Based on these results, we produced coalho cheese using goat milk with and without (control) passion fruit. Cheeses were stored at 10°C for 14 d and populations of mesophilic aerobes, *Staph. aureus*, and presumptive LAB were monitored at d 1, 7 and 14. The passion fruit cheese had lower counts of

mesophilic aerobes, *Staph. aureus* (after 7 and 14 d), and presumptive LAB (after 14 d) than the control cheese. Adding ground passion fruit contributed to a reduction of *Staph. aureus* counts in goat cheese, although these differences were not significant. These results indicated the inhibitory potential of passion fruit and its potential use for controlling microbial populations in a cheese model; further studies are needed to characterize the active molecules that are responsible for such activity.

Novel sequence types of *Lactococcus lactis* subsp. *lactis* obtained from Brazilian dairy production environments

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[1] Contributed equally

Abstract

Lactococcus lactis subsp. *lactis* strains are widely used by the dairy industry in fermentation processes. This study aimed to characterize the genetic diversity of *L. lactis* subsp. *lactis* isolated from Brazilian dairy environments. A collection of 23 isolates of *L. lactis* subsp. *lactis* was subjected to rep-PCR (GTG$_5$) and PFGE (SmaI) to determine their genetic profiles. rep-PCR allowed a maximum similarity of 97.2% among strains, while PFGE grouped isolates in four clusters, and it was possible to identify isolates with 100% of similarity. The selected strains were also subjected to MLST (*pepXP, pgk, glyA, recN, bcaT and pdp*), resulting in the characterization of 11 STs; nine of them were firstly described in the present study. ST grouping allowed for the characterization of 2 CC: CC1

with 3 isolates and CC2 with 2 isolates. The remaining STs were distributed as *singletons*. These results show that *L. lactis* subsp. *lactis* obtained from Brazilian dairy environments present a high genetic diversity, highlighting the relevance of further studies to characterize their beneficial potential to be exploited by the food industry.

The ability of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis strains in producing nisin

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Abstract

Lactococcus lactis subsp. *lactis* bv. diacetylactis is a relevant microorganism for the dairy industry because of its role in the production of aromatic compounds. Despite this technological property, the identification of bacteriocinogenic potential of obtained strains can offer the additional positive aspect of biosafety. A panel of 15 *L. lactis* subsp. *lactis* bv. diacetylactis strains was characterized for the presence and expression of bacteriocin related genes, and further investigated regarding the nisin operon. Eight strains were positive only for *nisA*, and one strain (SBR4) presented a full nisin operon, with sequencing that was shown to be similar to nisin Z. Only SBR4 presented inhibitory activity against 16 microbial target strains. The growth curves of selected targets strains confirmed the inhibitory activity of SBR4 and consequently the nisin production. This research has demonstrated the inhibitory potential of *L. lactis* subsp. *lactis* bv.

diacetylactis strain, SBR4, due to its ability to produce nisin Z. This biopreservative potential, associated to previously characterized technological properties, allow the indication of this strain as a promising candidate to be used by the dairy industry as a starter or adjunct culture.

Technological properties of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtained from dairy and non-dairy niches

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DOI: https://doi.org/10.1007/s42770-019-00182-3

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Abstract

Lactococcus lactis subsp. *lactis* bv. diacetylactis strains are often used as starter cultures by the dairy industry due to their production of acetoin and diacetyl, important substances that add buttery flavor notes in dairy products. Twenty-three *L. lactis* subsp. *lactis* isolates were obtained from dairy products (milk and cheese) and dairy farms (silage), identified at a biovar level, fingerprinted by rep-PCR and characterized for some technological features. Fifteen isolates presented molecular and phenotypical (diacetyl and citrate) characteristics coherent with *L. lactis* subsp. *lactis* bv. diacetylactis and rep-PCR allowed the identification of 12 distinct profiles (minimum similarity of 90%). Based on technological features, only two isolates were not able to coagulate skim milk and 10 were able to produce proteases. All isolates were able to acidify skim milk: two isolates, in special, presented high acidifying ability due to their ability in reducing more than two pH units after 24 h. All isolates were also able to grow at different NaCl concentrations

(0 to 10%, w/v), and isolates obtained from peanut and grass silages presented the highest NaCl tolerance (10%, w/v). These results indicate that the *L. lactis* subsp. *lactis* bv. diacetylactis isolates presented interesting technological features for potential application in fermented foods production. Despite presenting promising technological features, the isolates must be assessed according to their safety before being considered as starter cultures.

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Fusieger, A.; Silva, R. R.; Silva, S. R. J.; Honorato, J.A.; Natali, I.S.; Costa, N.A.S.; Pena, M.L; Caggia, C.; Nero, L.A.; Carvalho, A.F. (2021). Inhibitory activity of an emulsifying salt polyphosphate used in processed cheese: an *in vitro* analysis of its antibacterial potential. In: *6th Conference on Microbial Diversity – MD21*. On-line.

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APPENDICES

Appendix 1. Main keywords of the thesis.

Appendix 2. Author affiliations during the execution of the thesis.

Conceptualization, resources, research, methodology & investigation.
Principal research site of the PhD activity where *in vitro* and *in situ* studies were performed.

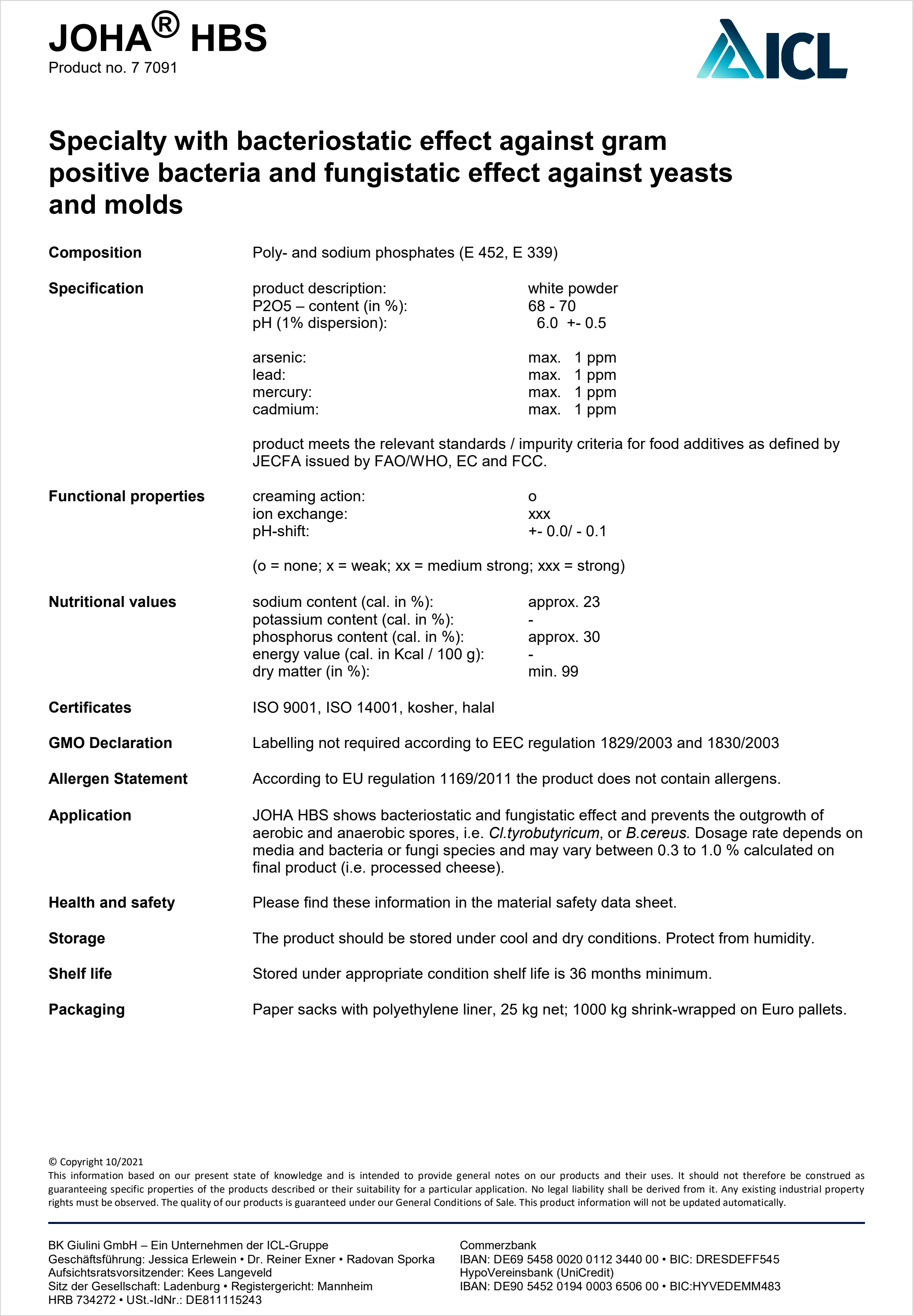
Investigation.
Partner for the use of equipment in the production of processed cheese in Brazil and Germany.

Validation, investigation & research.
Effect of melting salts against spores and evaluation of creaming process and storage temperature.
Period done: 12 months.

Formal analysis and visualization.
Creation and preparation of published articles, specifically data visualization and presentation.

ANNEXES

Annex 1. Datasheet of the emulsifying salt used to evaluate the *in vitro* inhibitory activity (ref. chapter: II).



JOHA® HBS

Product no. 7 7091

Specialty with bacteriostatic effect against gram positive bacteria and fungistatic effect against yeasts and molds

Composition Poly- and sodium phosphates (E 452, E 339)

Specification

product description:	white powder
P2O5 – content (in %):	68 - 70
pH (1% dispersion):	6.0 +- 0.5
arsenic:	max. 1 ppm
lead:	max. 1 ppm
mercury:	max. 1 ppm
cadmium:	max. 1 ppm

product meets the relevant standards / impurity criteria for food additives as defined by JECFA issued by FAO/WHO, EC and FCC.

Functional properties

creaming action:	o
ion exchange:	xxx
pH-shift:	+- 0.0/ - 0.1

(o = none; x = weak; xx = medium strong; xxx = strong)

Nutritional values

sodium content (cal. in %):	approx. 23
potassium content (cal. in %):	-
phosphorus content (cal. in %):	approx. 30
energy value (cal. in Kcal / 100 g):	-
dry matter (in %):	min. 99

Certificates ISO 9001, ISO 14001, kosher, halal

GMO Declaration Labelling not required according to EEC regulation 1829/2003 and 1830/2003

Allergen Statement According to EU regulation 1169/2011 the product does not contain allergens.

Application JOHA HBS shows bacteriostatic and fungistatic effect and prevents the outgrowth of aerobic and anaerobic spores, i.e. *Cl.tyrobutyricum*, or *B.cereus*. Dosage rate depends on media and bacteria or fungi species and may vary between 0.3 to 1.0 % calculated on final product (i.e. processed cheese).

Health and safety Please find these information in the material safety data sheet.

Storage The product should be stored under cool and dry conditions. Protect from humidity.

Shelf life Stored under appropriate condition shelf life is 36 months minimum.

Packaging Paper sacks with polyethylene liner, 25 kg net; 1000 kg shrink-wrapped on Euro pallets.

BK Giulini GmbH – Ein Unternehmen der ICL-Gruppe
Geschäftsführung: Jessica Erlewein • Dr. Reiner Exner • Radovan Sporka
Aufsichtsratsvorsitzender: Kees Langeveld
Sitz der Gesellschaft: Ladenburg • Registergericht: Mannheim
HRB 734272 • USt.-IdNr.: DE811115243

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Influence of Emulsifying Salts on the Growth of *Bacillus thuringiensis* CFBP 3476 and *Clostridium perfringens* ATCC 13124 in Processed Cheese

Andressa Fusieger; Raiane Rodrigues da Silva; Valéria Quintana Cavicchioli; Rafaela da Silva Rodrigues; Jaqueline Aparecida Honorato; Sidney Rodrigues de Jesus Silva; Mariana Lage Pena; Cinzia Caggia; Luís Augusto Nero; Antonio Fernandes de Carvalho

Foods 2022, Volume 11, Issue 20, 3217

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