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TESE

Bioconservantes contendo potenciais pós-bióticos como alternativa para o controle de *Listeria Monocytogenes* e deteriorantes em embutidos cárneos cozidos embalados a vácuo

Aloizio Lemos de Lima

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BIOCONSERVANTES CONTENDO POTENCIAIS PÓS-BIÓTICOS COMO ALTERNATIVA PARA O CONTROLE DE *LISTERIA MONOCYTOGENES* E DETERIORANTES EM EMBUTIDOS CÁRNEOS COZIDOS EMBALADOS A VÁCUO

ALOIZIO LEMOS DE LIMA

Sob a orientação da Professora
Prof. Dra. Rosa Helena Luchese

e Coorientação do Professor
Prof. Dr. André Fioravante Guerra

Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia de Alimentos**, no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração em Ciência de Alimentos.

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RESUMO GERAL

LIMA, Aloizio Lemos. **Bioconservantes contendo potenciais pós-bióticos como alternativa para o controle de *Listeria monocytogenes* e deteriorantes em embutidos cárneos cozidos embalados a vácuo**. 2024. 96p. Tese (Doutorado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia, Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2024.

Os produtos à base de carne são altamente suscetíveis à ação microbiana devido às suas características intrínsecas e riqueza de nutrientes. Além disso, são frequentemente expostos a variáveis de risco após saírem da indústria, como o fracionamento no varejo e o abuso de temperatura durante o transporte e estocagem. *Listeria monocytogenes* (*Lm*) é o agente patogênico causador de listeriose, uma doença grave com altas taxas de hospitalização e mortalidade. Esta patologia está estreitamente associada ao consumo de alimentos processados prontos para consumo, e os produtos cárneos têm se destacado pelo número de ocorrências. Neste trabalho, dois bioconservantes contendo potenciais pós-bióticos (BCPP_SP e BCPP_YE), produzidos por fermentação com *Lacticaseibacillus paracasei* DTA 83, foram investigados *in vitro* quanto à sua ação antilisterial. Nisina, lactato de sódio e outros quatro conservantes comerciais foram incluídos no estudo para comparação. Os bioconservantes também foram testados *in situ* em amostras de linguiça cozida embalada a vácuo (LCEV). O BCPP_YE foi aplicado por imersão de curta duração (1 minuto) em LCEV intencionalmente contaminadas com *Lm*. O BCPP_SP foi testado *in vitro* e *in situ* contra a microbiota natural de LCEV, tendo lactato de sódio como comparação. Neste teste, a aplicação do bioconservante foi realizada na massa (como ingrediente) ou adicionada dentro da embalagem antes do selamento a vácuo (superfície). Nos dois testes *in situ*, o software de modelagem preditiva, MicroLab_Shelf-Life, foi utilizado para estimar a vida de prateleira das LCEV em diferentes perfis de temperatura. Os resultados *in vitro* revelaram que os bioconservantes foram igualmente eficientes ($p > 0,05$) em sua ação antilisterial, apresentando uma concentração inibitória mínima e uma concentração listericida mínima de 1,00%. Entretanto, perderam a ação antilisterial em concentrações de até 10% quando foram submetidos a neutralização dos ácidos orgânicos; mas não foram afetados por tratamento com tripsina e apresentaram forte estabilidade ao calor. Os tratamentos por imersão em BCPP_YE apresentaram efeito bactericida, sendo capazes de reduzir carga microbiana inicial das LCEV. Todavia, não foram capazes de impedir o crescimento de *Lm*, bactérias ácido láticas e contagem total de bactérias em temperaturas mais elevadas. Os resultados preditivos revelaram que a manutenção da temperatura de refrigeração a 7ºC foi um fator de barreira eficiente para controlar a população de *Lm* por mais de 180 dias e estender a vida de prateleira das LCEV por até 135 dias. A adição de 1,00% de BCPP_SP na massa das LCEV foi tão eficaz quanto a adição de 2,00% de lactato de sódio para controlar a microbiota natural de LCEV. As mesmas concentrações aplicadas dentro das embalagens não apresentaram resultados eficazes em comparação com branco e controle. Estes resultados apontam para a importância de se avaliar a forma de aplicação de conservantes em produtos cárneos. O perfil de temperatura inserido no modelo preditivo influenciou no resultado de crescimento da microbiota natural das LCEV. A durabilidade foi inversamente proporcional ao aumento da temperatura. Este estudo demostrou que os bioconservantes BCPP_SP e BCPP_YE podem ser uma alternativa natural promissora para uso em produtos cárneos quando associados a outras medidas de controle. Todavia, mais pesquisas são necessárias, sobretudo para avaliar o melhor método de aplicação e realizar testes em outros produtos cárneos.

Palavras-chave: listeriose, bactérias láticas, doenças transmitidas por alimentos, microbiologia preditiva.

ABSTRACT

LIMA, Aloizio Lemos. **Biopreservatives containing potential postbiotics as an alternative for controlling *Listeria monocytogenes* and spoilage organisms in vacuum-packaged cooked meat products.** 2024. 96p. Thesis (Doctorate in Food Science and Technology). Technology Institute, Food Technology Department, Federal Rural University of Rio de Janeiro, Seropédica, RJ, 2022.

Meat-based products are highly susceptible to microbial action due to their intrinsic characteristics and nutrient richness. Additionally, they are often exposed to risk variables after leaving the industry, such as retail fractionation and temperature abuse during transport and storage. *Listeria monocytogenes* (*Lm*) is the pathogenic agent causing listeriosis, a severe disease with high hospitalization and mortality rates. This pathology is closely associated with the consumption of ready-to-eat processed foods, and meat products have stood out due to the number of occurrences. In this study, two biopreservatives containing potential postbiotics (BCPP_SP and BCPP_YE), produced by fermentation with *Lacticaseibacillus paracasei* DTA 83, were investigated *in vitro* for their antilisterial capacity. Nisin, sodium lactate, and four other commercial preservatives were included in the study for comparison. The biopreservatives were also tested *in situ* in samples of vacuum-packed cooked sausage (VPCS). BCPP_YE was applied by short-term immersion (1 minute) in VPCS intentionally contaminated with *Lm*. BCPP_SP was tested *in vitro* and *in situ* against the natural microbiota of VPCS, with sodium lactate as a comparison. In this test, the biopreservative was applied in the mass (as an ingredient) or added inside the packaging before vacuum sealing (surface). In both *in situ* tests, the predictive modeling software, MicroLab_Shelf-Life, was used to estimate the shelf life of VPCS under different temperature profiles. The *in vitro* results revealed that the biopreservatives were equally efficient ($p > 0.05$) in their antilisterial action, presenting a minimum inhibitory concentration and a minimum listericidal concentration of 1.00%. However, they lost antilisterial action at concentrations of up to 10% when subjected to organic acid neutralization; but were not affected by trypsin treatment and showed strong heat stability. The immersion treatments with BCPP_YE showed a bactericidal effect, capable of reducing the initial microbial load of VPCS. However, they were unable to prevent the growth of *Lm*, lactic acid bacteria, and total bacterial count at higher temperatures. Predictive results revealed that maintaining refrigeration temperature at 7°C was an effective barrier factor to control the population of *Lm* for more than 180 days and to extend the shelf life of VPCS up to 135 days. The addition of 1.00% BCPP_SP in the mass of VPCS was as effective as the addition of 2.00% sodium lactate to control the natural microbiota of VPCS. The same concentrations applied inside the packages did not yield effective results compared to the blank and control. These results point to the importance of evaluating the application form of preservatives in meat products. The temperature profile inserted into the predictive model influenced the growth result of the natural microbiota of VPCS. Durability was inversely proportional to the temperature increase. This study demonstrated that the biopreservatives BCPP_SP and BCPP_YE can be a promising natural alternative for use in meat products when combined with other control measures. However, further research is necessary, especially to evaluate the best application method and conduct tests on other meat products.

Keywords: listeriosis, lactic acid bacteria, foodborne illness, predictive microbiology.

LISTA DE ABREVIAÇÕES, SIGLAS E SÍMBOLOS

APC: alimentos prontos para o consumo
BAL: bactérias do ácido láctico
BCPP_SP: bioconservante contendo potenciais pós-bióticos com proteína isolada de soja.
BCPP_YE: bioconservante contendo potenciais pós-bióticos com extrato de levedura.
CIM: concentração inibitória mínima
CLM: concentração listericida mínima
CTB: contagem total de bactérias
GRAS: geralmente reconhecidas como seguras
LCEV: linguiça cozida embalado a vácuo
Lm: *Listeria monocytogenes*
pH_{MI}: pH inibitório mínimo
pH_{ML}: pH listericida mínimo

AR: preservative containing sodium lactate, vinegar powder, sodium citrate, and citric acid.
FCSDV: preservative containing dry vinegar and fermented cane sugar.
BCPP_SP: biopreservative containing potential postbiotics with isolated soy protein.
BCPP_YE: biopreservative containing potential postbiotics with yeast extract.
LAB: lactic acid bacteria
VPCS: vacuum-packed cooked sausage
Lm: *Listeria monocytogenes*
MIC: minimum inhibitory concentration
MLC: minimum listericidal concentration
NS: preservative containing nisin.
NSDR: preservative containing nisin, sodium diacetate and rosemary extract.
pH_{MI}: minimum inhibitory pH
pH_{ML}: minimum listericidal pH
RTE: ready-to-eat
SL: preservative containing sodium lactate (60%).
SLS: preservative containing sodium lactate (60%) and liquid smoke.
TBC: total bacterial count

SUMÁRIO

1 INTRODUÇÃO GERAL

As doenças transmitidas por alimentos afetam milhões de pessoas em todo o mundo, todos os anos. Ainda em 2024, são consideradas um problema saúde pública global, afetando inclusive os países desenvolvidos. Neste contexto, a listeriose tem se destacado pelo aumento no número de casos e gravidade da doença. Essa patologia, ocasionada pela bactéria *Listeria monocytogenes* (*Lm*), afeta principalmente idosos, mulheres grávidas e indivíduos imunocomprometidos. As manifestações clínicas podem resultar em meningite, septicemia, aborto espontâneo, infecções neonatais, gastroenterite, febre, dor muscular e morte (CDC, 2021; FDA, 2012). Em 2021, a listeriose ocupou o quinto lugar entre as zoonoses mais notificadas na União Europeia, com 2.183 casos registados. Ela também apresentou as maiores taxas de hospitalização e mortalidade, especialmente entre indivíduos com mais de 64 anos (EFSA e ECDC, 2022).

A listeriose é frequentemente associada ao consumo de alimentos processados prontos para consumo com pH ≥ 4,4, atividade de água (aw) ≥ 0,92 ou uma combinação de pH ≥ 5,0 e aw ≥ 0,94. Nesta categoria de alimentos, os produtos à base peixe e carnes (bovinos, suínos e aves) têm se destacado pelo número de casos e surtos (EFSA e ECDC, 2022). Esses produtos também foram prevalentes em relação a detecção de *Lm* em amostras de alimentos analisadas, seguidos por saladas, frutas e vegetais prontos para o consumo, queijos elaborados com leite cru, especiarias e ervas aromáticas (ECDC, 2021).

Dada a gravidade da listeriose, a indústria alimentícia, as agências de saúde pública e os pesquisadores têm trabalhado para implementar medidas rigorosas de prevenção e controle.

Neste estudo, foram avaliados dois bioconservantes contendo potenciais pós-bióticos (BCPP_SP e BCPP_YE) quanto à sua eficácia antilisterial e sua capacidade de controlar organismos de deterioração em salsichas cozidas embaladas a vácuo (LCEV). Esses conservantes foram produzidos por fermentação com *Lacticaseibacillus paracasei* DTA 83, uma cepa potencialmente probiótica com características tecnológicas bem definidas em outros estudos.

Na revisão geral da literatura, foram abordados alguns conceitos relacionados ao tema central do estudo.

No capítulo I os bioconservantes foram investigados *in vitro* quanto à sua ação antilisterial, utilizando o método de macrodiluição e um método espectrofotométrico. Nisina, lactato de sódio e outros 4 conservantes comerciais foram incluídos no estudo para comparação. Neste capítulo, os bioconservantes também foram pesquisados quanto à sua sensibilidade à tripsina e tratamento térmicos, bem como, sua eficácia após a neutralização dos ácidos orgânicos. As cepas de Listeria utilizadas no estudo foram investigadas quanto ao pH inibitório mínimo e pH listericida mínimo na presença de ácido láctico e ácido clorídrico.

Os Capítulos II e III apresentaram estratégias para o uso dos bioconservantes em linguiças cozidas embaladas a vácuo (LCEV).

No Capítulo II, o BCPP_YE foi aplicado por imersão de curta duração (1 minuto) em amostras de LCEV intencionalmente contaminadas com *Lm*, para simular uma contaminação extrema pós-tratamento térmico. Neste capítulo, além da ação antilisterial, também foi

verificado o efeito dos tratamentos sobre as bactérias ácido láticas e a contagem total de bactérias.

No Capítulo III, o BCPP_SP (denominado PPCP no artigo) foi testado *in vitro* e *in situ* contra a microbiota natural de LCEV, com lactato de sódio como comparação. O teste *in vitro* foi conduzido usando o método de turbidez com um espectrofotômetro (similar ao realizado no Capítulo I). No teste *in situ*, o bioconservante foi aplicado na massa de salsicha (como ingrediente) ou adicionado dentro da embalagem antes do selamento a vácuo (superfície).

Nos testes *in situ* conduzidos nos Capítulos II e III, o software de modelagem preditiva MicroLab_Shelf-Life foi usado para estimar a vida útil de LCEV sob diferentes perfis de temperatura.

Este trabalho teve como objetivo investigar se os bioconservantes BCPP_SP e BCPP_YE podem ser alternativas eficazes para controlar *Lm* e organismos de deterioração em produtos cárneos, bem como explorar estratégias de aplicação eficientes em LCEV.

A Figura 1 apresenta um resumo gráfico da tese.

2 OBJETIVO GERAL

Verificar se os bioconservantes BCPP_SP e BCPP_YE podem ser alternativas eficazes para controlar *Lm* e organismos de deterioração em embutidos cárneos embalados a vácuo.

3 OBJETIVOS ESPECÍFICOS

- Determinar a concentração inibitória mínima (CIM) e concentração listericida mínima (CLM) dos bioconservantes BCPP_SP e BCPP_YE, bem como, dos conservantes comerciais SL (lactato de sódio - 60%), SLS (lactato de sódio - 60% e fumaça liquida), AR (lactato de sódio, vinagre em pó, citrato de sódio e ácido cítrico), FCSDV (fermentado de cana de açúcar e vinagre em pó), NSDR (nisina, diacetato de sódio e extrato de alecrim) e NS (nisina 2.5% - 25000 mg/kg) em diferentes valores de pH.
- Traçar curvas de crescimento microbiano (*Lm*) em diferentes concentrações de BCPP_SP e BCPP_YE;
- Investigar a resistência térmica dos BCPP_SP e BCPP_YE;
- Investigar a sensibilidade dos bioconservantes a tripsina;
- Investigar a eficácia dos bioconservantes após a neutralização dos ácidos orgânicos.
- Determinar o pH inibitório mínimo (pH_{MI}) e pH listericida mínimo (pH_{LM}) do pool de *Lm* na presença de ácido láctico e ácido clorídrico;
- Verificar se a aplicação do BCPP_YE por imersão de curta duração (1 minuto) em LCEV intencionalmente contaminadas por *Lm* é eficaz para controlar *Lm* e aumentar a vida de prateleira;
- Estimar a vida de prateleira de LCEV submetidas a imersão de curta duração em BCPP_YE após contaminação intencional com *Lm*, utilizando o método preditivo MicroLab_Shelf-Life;
- Determinar *in vitro* a concentração de BCPP_SP capaz de impedir o crescimento da microbiota autóctone de LCEV;
- Estimar, por meio do método preditivo MicroLab_Shelf-Life, a vida de prateleira de LCEV tratadas com diferentes concentrações de BCPP_SP, aplicadas na massa da linguiça (como ingrediente) ou adicionadas dentro da embalagem antes do selamento a vácuo (tratamento superficial).

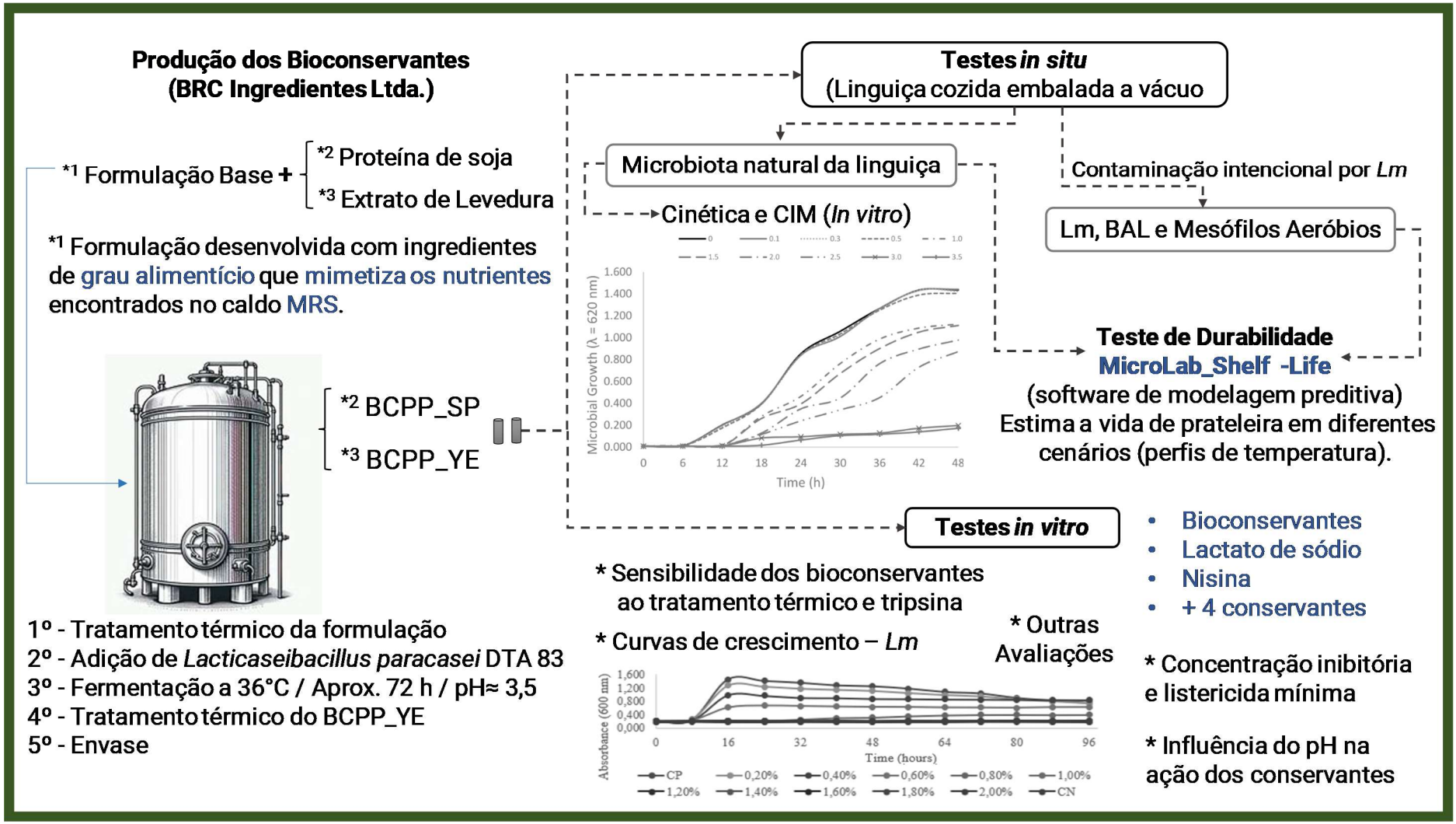

Figura 1. Resumo gráfico da tese.

4 REVISÃO DE LITERATURA GERAL

4.1 Probióticos, prebióticos e simbióticos

O avanço das pesquisas sobre a correlação entre microbiota e saúde tem revelado o importante papel dos microrganismos vivos e/ou inativados, bem como de seus componentes celulares e metabólitos, na promoção de benefícios para humanos e animais. Nesse contexto, conceitos como probióticos, prebióticos, pós-biótico, parabióticos e termos afins tem emergido como uma nova fronteira de interesse e discussão.

Os probióticos são definidos como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (Hill *et al.*, 2014), enquanto um prebiótico é "um substrato que é utilizado seletivamente pelos microrganismos hospedeiros conferindo um benefício à saúde", ou seja, não deve ser amplamente metabolizado, mas provocar um metabolismo tendenciosamente benéfico para microrganismos promotores da saúde dentro do ecossistema autóctone do hospedeiro (Gibson *et al.*, 2017). O conceito de simbióticos, inicialmente concebido como uma combinação de probióticos e prebióticos, foi redefinido como "uma mistura compreendendo microrganismos vivos e substrato(s) utilizados seletivamente pelos microrganismos hospedeiros que conferem um benefício à saúde do hospedeiro"(Swanson *et al.*, 2020).

Os prebióticos têm o objetivo servir de nutrientes unicamente para microbiota benéfica de humanos e animais, incluindo cepas probióticas administradas e microrganismos autóctones, com o objetivo de melhorar a saúde. Como exemplo, temos os oligossacarídeos dietéticos, como os frutanos (frutooligossacarídeos (FOS) e inulina) e os galactanos (galactooligossacarídeos ou GOS) que estimulam seletivamente lactobacilos e bifidobacterias. As fibras alimentares, como pectinas, celulose e xilanas, que estimulam o crescimento de uma grande variedade de microrganismos intestinais não são prebióticos (Gibson *et al.*, 2017; Pineiro *et al.*, 2008).

Os simbióticos podem ser formulados usando duas abordagens. 1ª Probiótico(s) mais prebiótico(s) trabalhando de forma independente para alcançar um ou mais benefícios à saúde (simbiótico complementar). Neste caso, tanto o(s) probiótico(s) quanto o(s) prebiótico(s) devem atender aos critérios mínimos que os classifica como tais. 2ª Microrganismo(s) vivo(s) (não precisa se enquadrar nos critérios de probiótico) e um substrato utilizado seletivamente (não precisa se enquadrar nos critérios mínimos de prebiótico) (simbiótico sinérgico). Estes devem funcionar em conjunto (o substrato deve ser utilizado seletivamente pelo microrganismo coadministrado) (Swanson *et al.*, 2020).

Os efeitos benéficos à saúde dos probióticos, prebióticos e simbióticos devem ser comprovados e documentados para que possam ser considerados como tais (Gibson *et al.*, 2017; Hill *et al.*, 2014; Swanson *et al.*, 2020).

4.2 Conceito de pós-biótico: uma análise de consensos e divergências

Dois artigos recentes, o primeiro da International Scientific Association of Probiotics and Prebiotics (ISAPP) (Salminen *et al.*, 2021) e o segundo uma réplica crítica do primeiro artigo (Aguilar-Toalá *et al.*, 2021), discutem a definição e o escopo dos pós-bióticos. Ressalta-

se que ambos os artigos basearam suas argumentações em robustas pesquisas bibliográficas e contaram com uma extensa lista de autores/pesquisadores reconhecidos na área por sua experiência e expertise.

O artigo da ISAPP propõe que o termo pós-bióticos seja definido como “uma preparações de microrganismos inanimados e/ou seus componentes que conferem um benefício à saúde do hospedeiro”. Esta definição abrange células microbianas que foram deliberadamente inativadas e/ou seus componentes celulares (pili, componentes da parede celular e/ou outras estruturas), podendo ou não incluir metabólitos. É essencial que os efeitos benéficos dos pós-bióticos sejam confirmados no hospedeiro alvo. É importante ressaltar que metabólitos microbianos purificados e vacinas não são considerados pós-bióticos. Além disso, um pós-biótico não precisa ser derivado de um probiótico, ou seja, a versão inativada não precisa ter sido um probiótico para ser aceita como pós-biótico. A definição exclui produtos derivados de microrganismos indefinidos.

O artigo de Aguilar-Toalá et al. propõe a manutenção de uma definição enunciada em 2013 como "qualquer fator resultante da atividade metabólica de um probiótico ou de qualquer molécula liberada capaz de conferir efeitos benéficos ao hospedeiro de forma direta ou indireta" (Tsilingiri e Rescigno, 2013). Esta definição abrange moléculas bem definidas resultantes da atividade metabólica dos probióticos, excluindo células microbianas inativadas (definidas como paraprobióticos). Além disso, exclui os compostos liberados por microrganismos não probióticos e abrange benefícios indiretos ao hospedeiro, possibilitando um debate mais aprofundado sobre os tipos de benefícios que poderiam ser abrangidos.

Segundo o ISAPP, para que um pós-biótico seja considerado benéfico, seus efeitos devem ser confirmados no hospedeiro alvo, que pode incluir humanos, animais de companhia, gado e outras espécies. Os locais de ação dos pós-bióticos não se limitam ao intestino, podendo ser administrados em superfícies do hospedeiro como a cavidade oral, a pele, o trato urogenital ou a nasofaringe. Injeções não estão incluídas no escopo dos pós-bióticos. A segurança para o uso pretendido é um requisito implícito na definição de um pós-biótico.

De acordo com o ISAPP, para uma preparação (biomassa microbiana) ser qualificada como pós-biótica, é necessário cumprir vários critérios rigorosos. A caracterização molecular dos microrganismos progenitores, a descrição detalhada do procedimento de inativação e da matriz, a confirmação da inativação, evidências de benefícios à saúde a partir de ensaios clínicos controlados, e segurança da preparação no hospedeiro alvo avaliada para o uso pretendido.

A definição de pós-bióticos continua a evoluir, refletindo diferentes perspectivas sobre o que deve ser incluído no conceito. A definição original focava em moléculas bem definidas resultantes da atividade metabólica dos probióticos, enquanto a definição atualizada da ISAPP abrange preparações de microrganismos inativados. Ambas as abordagens visam promover benefícios ao hospedeiro, mas diferem em seus escopos e critérios.

4.3 O gênero *Listeria*

O gênero *Listeria* é composto por bactérias ubíquas, gram-positivas, não formadoras de esporos, capazes de crescer na presença ou ausência de oxigénio (anaeróbicas facultativas).

Possuem a forma de pequenos bastonetes com extremidades arredondadas, podendo aparecer isolados, agrupados em pares ou em cadeias curtas. Na observação direta ao microscópio podem parecer cocos e confundidas com estreptococos. Possuem motilidade em temperaturas de até 25ºC por meio de flagelos. Em temperaturas mais elevadas, como 37°C (temperatura corporal), os flagelos geralmente não são expressos, e a motilidade bacteriana é reduzida ou ausente. Essa característica é importante para a adaptação ambiental e para os processos infecciosos dessas bactérias. São catalase positivos, não produtoras de indol e H_2S, fermentam glicose com produção de ácido láctico e sem produção de gás, não produzem oxidase; apresentam resultados positivos nos testes de Voges-Proskauer e vermelho de metila; possuem capacidade de hidrolisar a esculina e incapacidade de utilizar a ureia. A atividade hemolítica em ágar sangue, juntamente com outras características bioquímicas, permite distinguir *Listeria monocytogenes* (*Lm*) das outras espécies pertencentes ao gênero *Listeria*. (ASAE, [s.d.]; Farber e Peterkin, 1991).

Embora a maioria das espécies do gênero *Listeria* seja saprofítica, vivendo de matéria orgânica em decomposição, algumas são reconhecidas como patogênicas para humanos e animais. Frequentemente são isoladas de fontes naturais, como rios, lagos, córregos, água do mar, solo, esgoto, fezes humanas e de animais, lama, silagem e pássaros, sendo consideradas contaminantes ambientais passíveis de serem transmitidos ao homem e animais (Farber e Peterkin, 1991).

O gênero é composto por 20 espécies, se destacando *L. monocytogenes* por ser capaz de causar enfermidades em humanos e animais(EURL Lm, 2021).

4.4 *Listeria monocytogenes* (*Lm*)

Lm é reconhecida como um microrganismo desafiador para indústria de alimentos por ser capaz de crescer em temperaturas de refrigeração, ausência ou presença de oxigênio e tolerar condições adversas de processamento e estocagem. *Lm* também é capaz de formar biofilmes nos equipamentos e demais superfícies das unidades beneficiadoras, o que torna difícil a sua eliminação durante os processos de limpeza e de desinfecção. Além disso, pode se espalhar facilmente pelo ambiente de fabricação e ocasionar contaminação cruzada de alimentos processados (Cruz *et al.*, 2008a; FDA, 2012; Nowak *et al.*, 2017).

Este microrganismo também apresenta boa resistência aos efeitos deletérios do congelamento, dessecamento e aquecimento, além de outras características que favorecem seu crescimento em alimentos processados e estocados por longos períodos. Pode crescer em uma ampla faixa de temperatura, que vai de -2,0 a 45°C, sendo 30 a 37°C a zona ótima para seu desenvolvimento. Ambientes com valores de pH entre 4,3 e 9,6 permitem seu crescimento. A taxa de crescimento máxima ocorre em valores de pH próximos a 7. Concentrações de CO_2 superiores a 80% são consideradas inibitórias e valores de atividade de água (Aa) superiores a 0,92 permitem seu crescimento. Pode tolerar concentrações de cloreto de sódio (NaCl) elevadas (até 12 % - dependendo das outras condições do meio). Consegue sobreviver (mas não se multiplicar) por longos períodos em níveis altos de sal (≥ 20 % de NaCl) (EURL Lm, 2021).

Nos alimentos, os fatores intrínsecos e extrínsecos podem influenciar no crescimento de *Lm*. Os fatores intrínsecos se relacionam ao próprio alimento (pH, Aa, teor de NaCl, umidade, microbiota nativa e contaminante, nutrientes, conservantes etc.), já os fatores extrínsecos se relacionam ao ambiente de armazenamento do alimento (embalagem, atmosfera, umidade relativa, temperatura de armazenamento, etc.). Esses fatores, ou combinações de fatores, podem propiciar, ou não, condições favoráveis para multiplicação do patógeno durante o armazenamento (EURL Lm, 2021).

O consumo de alimentos contaminados com *Lm* ocasiona a listeriose, com manifestações clínicas que podem resultar em meningite, septicemia, aborto espontâneo, infecções neonatais e gastroenterite. Esta doença de origem alimentar é considerada grave por apresentar altas taxas de hospitalização e mortalidade, especialmente em idosos, crianças, indivíduos imunocomprometidos e mulheres grávidas. O tratamento com antibióticos é indicado em casos de listeriose, sendo a ampicilina em associação com a gentamicina ou o sulfametoxazol-trimetoprim os mais utilizados (CDC, 2021; FDA, 2012; ILSI, 2005).

Segundo o relatório do Centro Europeu de Prevenção e Controle de Doenças (ECDC), no período de 2015 a 2019, foram reportados por 28 países membros da união europeia (UE) 12.324 casos invasivos de listeriose em humanos, com taxas variando de 0,43 a 0,48 casos por 100.000 habitantes. Informações sobre hospitalizações foram fornecidas em 51,1% dos casos confirmados, por 19 países membros. Entre os casos em que a informação sobre a hospitalização foi relatada, 92,1% resultaram em internação. Estes dados posicionam a Listeriose como a zoonose de maior proporção de casos de hospitalização da EU (ECDC, 2021).

O relatório ressalta ainda que houve um aumento constante no número de mortes entre 2010 e 2019, com média de 217 mortes por ano. A taxa de mortalidade entre os casos com desfecho conhecido aumentou em 2019 (17,6%), quando comparados a 2018 (13,6%) e 2017 (15,6%), números considerados altos, e que posicionam a Listeriose como uma das mais graves doenças de origem alimentar sob vigilância da UE. Em relação a faixa etária, a doença apresentou maior incidência na população acima dos 64 anos, passando de 56,1% em 2008 para 64,5% em 2019. Em indivíduos acima de 84 anos esse aumento foi mais acentuado, passando de 7,3% para 14,3% no mesmo período. A taxa de letalidade também foi maior na faixa etária dos 64-84 anos (19,5%) e acima dos 84 anos (23,0%) em 2019 (ECDC, 2021).

Em 2021, a listeriose ocupou o quinto lugar entre as zoonoses mais notificadas na União Europeia, com 2.183 casos registados e apresentou as taxas de hospitalização e mortalidade mais elevadas, especialmente entre indivíduos com mais de 64 anos (EFSA e ECDC, 2022).

A listeriose está frequentemente associada ao consumo de alimentos processados prontos para consumo (APC) com pH ≥ 4,4, aw (atividade de água) ≥ 0,92 ou uma combinação de pH ≥ 5,0 e aw ≥ 0,94. De acordo com relatórios do ECDC e da Autoridade Europeia para a Segurança dos Alimentos (EFSA), os APC à base de carne (carne bovina, suína e aves), à base de peixe, saladas prontas, frutas e vegetais prontos para o consumo, os queijos elaborados com leite cru e as especiarias e ervas aromáticas têm-se destacado nos últimos anos devido ao número de ocorrências em amostras de alimentos analisadas (ECDC, 2021a; EFSA e ECDC, 2022).

Embora tratamentos térmicos, como a pasteurização, sejam eficientes para eliminação de *Lm*, os produtos termicamente tratados ainda podem ser fontes do microrganismo. De forma abrangente, a presença de *Lm* nestes alimentos decorre de falhas no processamento térmico, matéria-prima com contagem elevada da bactéria e contaminação pós-tratamento térmico, sobretudo em unidades processadoras que não adotam medidas sanitárias de produção. A implementação de sistemas de controle em toda a cadeia de produção, como os 5S (5 sensos), as BPF (boas práticas de fabricação), os PPHO (procedimentos padrão de higiene operacional) e o APPCC (análise de perigos e pontos críticos de controle) são fundamentais para prevenção de infecções pelo patógeno (FDA, 2012; ILSI, 2005; Peiris *et al.*, 2009).

Um painel de especialistas em *Lm* em alimentos, reunidos pelo Risk Science Institute - ILSI Research Foundation (ILSI, 2005), apresentou três estratégias principais para redução do risco de listeriose: (1) prevenção da contaminação do alimento, (2) prevenção do crescimento (para números elevados) e (3) programas educativos com base científica direcionados a populações suscetíveis e seus cuidadores. Dessas estratégias, impedir o crescimento de *Lm* é apontado pelo grupo como a ação mais relevante para redução dos casos de infecção. Esta conclusão foi balizada por modelos de dose-resposta, que preveem um aumento do risco da doença diretamente proporcional ao aumento da concentração do microrganismo no alimento.

Devido à severidade da patologia e potencial de crescimento elevado durante a estocagem, o limite de tolerância zero (0 UFC/25g) ou (< 100 UFC/g) é definido para APC conforme seu público-alvo e capacidade do produto em suportar o crescimento de *Lm*. O Regulamento Europeu (CE) nº 2073/2005 (Comissão Europeia (CE), 2005) estabelece, entre outros, os critérios para *Lm* em APC, determinando: I) APC destinados a bebês e fins médicos especiais, ausência em 10 x 25g; II) APC que não sejam destinados a bebês e fins médicos especiais, adotar critérios baseados na capacidade do produto em suportar o crescimento de *Lm*, da seguinte forma: a) APC incapazes de suportar o crescimento de *Lm*, os níveis devem ser menores que 100 UFC/g ao longo de todo o prazo de validade (n = 5; c = 0); b) APC capazes de suportar o crescimento de *Lm*, ausência em 5 x 25g no momento que o produto sai da planta de produção; no entanto, se o produtor puder demonstrar, a contento da autoridade competente, que o produto não excederá o limite de 100 UFC/g ao longo de seu prazo de validade, o nível deve ser menor que 100 UFC/g ao longo do prazo de validade do produto (n = 5, c = 0). No Brasil, a instrução normativa n° 161/2022 estabelece para APC os seguintes critérios microbiológicos: I) destinados a lactentes ou para fins especiais, ausência em 25g ou mL (n = 10); II) Demais APC, < 100 UFC/g ou mL (n = 5) (Brasil, 2022).

O rigor exigido no controle de *Lm* em APC, reside no fato de não haver nestes produtos indicação da necessidade de tratamento térmico efetivo ou outro processo de eliminação ou de redução do patógeno a níveis seguros momentos antes de sua ingestão. Ademais, o processamento e/ou armazenamento de muitos destes produtos pode gerar condições favoráveis para o patógeno crescer e ser competitivo (Gillesberg Lassen *et al.*, 2016; Namiq e Milne, 2017; Peiris *et al.*, 2009).

4.5 Linguiças

As linguiças, segundo a legislação brasileira, são produtos cárneos industrializados elaboradas a partir de carnes de animais de açougue, embutidas em envoltórios naturais ou artificiais e submetidas ao processo tecnológico adequado. O sal é um ingrediente obrigatório. São ingredientes opcionais a gordura, água, açúcares, plasma, aditivos intencionais, aromas, especiarias e condimentos. É permitido a adição de proteínas não cárneas no teor máximo de 2,5% como proteína agregada (exceto nas linguiças toscana, calabresa, portuguesa, blumenau e colonial (Brasil, 2000).

No quadro 1 são apresentados os parâmetros físico-químicos obrigatórios para as linguiças no Brasil, segundo a instrução normativa nº4 de 2000. O quadro 2 espelha os padrões microbiológicos para linguiças conforme a IN nº161 de 2022. Nos casos de linguiças declaradas como alimentos prontos para o consumo (APC), em adição aos padrões apresentados no quadro 2, a normativa estabelece a obrigatoriedade de atendimento aos padrões para *Lm*, conforme exposto no quadro 3.

Quadro 1. Parâmetros físico-químicos obrigatórios para linguiças (Brasil, 2000).

PARÂMETRO FÍSICO-QUÍMICO	FRESCAIS	COZIDAS	DESSECADAS
Umidade (máximo)	70%	60%	55%
Gordura (máximo)	30%	35%	30%
Proteína (mínimo)	12%	14%	15%
Cálcio (base seca) (máximo)	0,1%	0,3%	0,1%

Quadro 2. Padrão microbiológico para linguiças (Brasil, 2022).

PRODUTO	MICRORGANISMO	n	c	m	M
Linguiça frescal (carne bovina e suína)	*Salmonella* / 25g (carne bovina e outras carnes)	5	0	Ausente	---
	Salmonella / 25g (carne suína)	5	1	Ausente	---
	Escherichia coli / g (carne bovina e outras carnes)	5	2	10	10^2
	Escherichia coli / g (carne suína)	5	3	10^2	10^3
	Aeróbios mesófilos / g	5	3	10^5	10^6
Linguiça frescal (carne de aves)	*Salmonella* Enteritidis / 25 g	5	0	Ausente	---
	Salmonella Typhimurium / 25 g	5	0	Ausente	---
	Escherichia coli / g	5	3	$5x10^2$	$5x10^3$
	Aeróbios mesófilos / g	5	3	10^5	10^6
Linguiça cozida	*Salmonella* sp / 25g	10	0	Ausente	---
	Clostridium perfringens / g	5	1	10^2	10^3
	Estafilococos coagulase positiva / g	5	1	10^2	10^3
	Escherichia coli / g	5	2	< 10	10^2

Quadro 3. Padrão microbiológico de *Listeria monocytogenes* em alimentos prontos para o consumo com vida útil maior que 5 dias, pH > 4,4, Aa > 0,92 ou combinação de pH > 5,0 e Aa > 0,94 (Brasil, 2022).

PRODUTO	MICRORGANISMO	n	c	m	M
APC	*Lm* / 25 g ou mL	5	0	10^2	---
APC destinados a lactentes ou para fins especiais	*Lm* / 25 g ou mL	10	0	Ausente	---

APC: alimentos prontos para o consumo. *Lm*: *Listeria monocytogenes*.

4.6 Bactérias ácido láticas

As bactérias ácido láticas (BAL) fazem parte do ecossistema natural dominante de muitos alimentos. São geralmente reconhecidas como seguras para consumo (GRAS) e potencialmente probióticas. Sua utilização na indústria de alimentos é extensa, uma vez que é capaz de conferir aos produtos alimentícios características peculiares de aroma, sabor e textura. Frequentemente são associadas a produção de compostos antimicrobianos (Stupar *et al.*, 2021; Wiernasz *et al.*, 2017).

As BAL são caracterizadas como bactérias Gram-positivas, catalase negativas, geralmente não moveis, não esporuladas e detentoras da capacidade de fermentar açúcares em ácido láctico. Entre os gêneros que compõem o grupo das láticas estão: *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Lastosphaera, Leuconostoc, Oenococcus, Pedicoccus, Streptococcus, Tetragenococcus, Vagococcus e Weissella* (Poffo e Silva, da, 2011).

Entretanto, mais recentemente, as famílias *Lactobacillaceae* e *Leuconostocaceae* foram taxonomicamente reclassificadas considerando que uma série de espécies destas famílias apresentam características fenotípicas, ecológicas e genotípicas diversas. Desta forma, o gênero *Lactobacillus* foi reclassificado em 25 gêneros, incluindo 23 novos. As espécies do grupo *Lactobacillus casei* foram reclassificadas no novo gênero *Lacticaseibacillus* enquanto aquelas anteriormente pertencentes ao grupo *Lactobacillus reuteri* e *Lactobacillus fermentum* foram alocadas em um novo gênero, *Limosilactobacillus* (Zheng *et al.*, 2020).

Entre os compostos com ação antimicrobiana passíveis de serem produzidos por BAL, citam-se bacteriocinas, como nisina, pediocina e reuterina além de ácidos orgânicos, ácidos graxos, diacetil, acetaldeído e peroxido de hidrogênio. Ademais, quando presentes nos alimentos exercem o efeito de competição, inibindo em muitos casos o crescimento de patógenos como *Lm* (Stupar *et al.*, 2021; Wiernasz *et al.*, 2017).

4.7 Curva de crescimento microbiano

O crescimento microbiano é caracterizado por uma curva sigmoidal composta por fases distintas: fase de latência (lag), fase de aceleração de crescimento (A), fase exponencial com taxa máxima de crescimento (log - μmáx), fase de desaceleração de crescimento (S), fase estacionária (E) e fase de declínio populacional (D).

A construção de uma curva de crescimento pode ser feita pela associação de métodos de quantificação da concentração celular diretos e indiretos, como a contagem em placas e medidas de absorbância em espectrofotômetro, respectivamente. Essa abordagem de

combinação de métodos pode otimizar o trabalho em laboratório, reduzindo custos e facilitando o monitoramento de muitas amostras simultaneamente.

Uma vez construída a curva de correlação entre a absorbância lida no espectrofotômetro e a contagem de unidades formadoras de colônias (UFC) nas placas, é possível monitorar o crescimento microbiano de forma mais rápida e prática e determinar a velocidade específica máxima de crescimento (μmáx). No entanto, esse método não é eficiente para concentrações iniciais de inóculo baixas devido ao seu limite de detecção, falhando na determinação da fase lag da curva de crescimento microbiano.

A leitura de turbidez de uma cultura microbiana pode ser utilizada para estimar a concentração celular de um ensaio microbiológico. Nesse método indireto de quantificação, um feixe de luz e focado em uma suspensão microbiana sofrendo dispersão (luz é parcialmente desviada pelas células presentes) e a luz não desviada (transmitância – T) é medida pelo espectrofotômetro. A quantidade de luz que atravessa a suspensão celular depende da concentração de células presentes na amostra, do tamanho das células, do comprimento de onda (λ), da intensidade da luz incidente (I_0) e do diâmetro do tubo onde é colocada a amostra. A densidade óptica (DO) da cultura corresponde à absorbância (que é uma medida logarítmica de uma razão de intensidades luminosas) sendo calculada pela equação $DO_{(\lambda)} = \log (I_0/I)$, onde I é a intensidade da luz transmitida. Frequentemente λ entre 540 e 640 nm são utilizados na medição da DO de culturas de leveduras e bactérias.

Esta metodologia não permite distinguir entre células viáveis e células mortas. Sua utilização é particularmente interessante quando se pretende confirmar se uma dada cultura se encontra em crescimento ou para acompanhar o crescimento microbiano ao longo do tempo. Dentro de certos limites ocorre uma relação linear entre a DO e o número total de células por mililitro de suspensão, ou seja, a concentração celular. Normalmente para suspensões celulares muito densas há necessidade de se realizar diluições, de modo que todos os valores de DO estejam incluídos na parte linear da curva DO *versus* concentração celular.

4.8 Preditor de crescimento microbiano - MicroLab_ShelfLife

O método preditivo, MicroLab_Shelf-Life, permite estimar a vida de prateleira de produtos cárneos. O método é desenvolvido por triagem *in vitro* e *in silico*. No software é possível inserir o limite máximo de microrganismos permitido em uma amostra e a curva preditiva do crescimento microbiano é plotada sob um perfil dinâmico de temperatura. O limite de utilização pode, por exemplo, ser baseado na carga microbiana a qual impacta as características originais do produto ou ser baseado em limites prescritos por padrão regulatório. A predição da vida de prateleira pode, por exemplo, ser realizada inserindo o perfil de temperatura que mimetiza o armazenamento dos produtos no mercado, com variação horária durante um dia, ou outro perfil de temperatura que reflita a forma de armazenamento do produto alvo.

O método é desenvolvido a partir de amostras (n = 5), provenientes de um mesmo lote e fabricadas nas mesmas condições. O método ABNT NBR ISO 4833-2 (2015) pode ser utilizado para obter contagem total de bactérias e os resultados expressos de acordo com o

método ABNT NBR ISO 7218:2019, seja pelo método em profundidade (pour-plate), espalhamento em superfície (spread-plate) ou microgota (drop plate).

4.8.1 Procedimento para utilização do MicroLab_ShelfLife

De acordo com o procedimento, uma unidade amostral (1 embalagem) (n = 1) deve ser analisada assim que o produto chegar ao laboratório (tempo zero). A contagem microbiana no tempo zero deve estar abaixo de 8,2 log UFC/g para validar o teste. A população microbiana nas embalagens restantes (n = 4) deve ser estimulada a crescer por incubação por pares a uma temperatura mais baixa (n = 2) e mais alta (n = 2). Os laboratórios podem determinar as temperaturas de incubação, sendo que, a temperatura mais baixa deve estar no intervalo de 4 a 20°C e a mais alta entre 25 e 36°C.

Exceto para o tempo zero, não há tempo pré-definido para realização das contagens microbianas, uma vez que, o algoritmo é capaz de processar qualquer período; no entanto, a fase de crescimento microbiano (log) deve ser incluída em pelo menos uma das contagens.

Os resultados relacionados à contagem de colônias são inseridos no software MicroLab_ShelfLife para obter informações sobre os parâmetros de crescimento da microbiota e a construção da curva preditiva do crescimento microbiano, sob um perfil dinâmico de temperatura.

4.8.2 Resultado das contagens de colônias

A curva de crescimento microbiano para cada temperatura é construída a partir das contagens de colônias em pelo menos dois níveis de diluição sucessivos, conforme equação 1.

$$N = \frac{\Sigma c}{V(n1+0{,}1n2)d} \qquad \text{(equação 1)}$$

Onde: ∑c - soma das colônias contadas nas duas placas retidas de duas diluições sucessivas (ao menos uma delas contém um mínimo de 10 colônias), V - volume de inóculo colocado em cada placa (mL), n1 e n2 - número de placas selecionadas na primeira diluição e número de placas selecionados na segunda diluição, respectivamente, e d - nível da primeira diluição retido. O número de microrganismos (log UFC/g) (N) X tempo (hora) é plotado em um gráfico de dispersão xy, para cada temperatura. Desta forma, é obtido o perfil do crescimento da população microbiana (curva de crescimento microbiano) a uma dada temperatura. O coeficiente angular da curva é utilizado para determinar as taxas de crescimento específicas, após a normalização das fases.

4.8.3 Modelagem da fase de crescimento (log)

As taxas de crescimento específicas por hora (log UFC/g/h) na temperatura mais baixa e mais alta são obtidas pela determinação do coeficiente angular da fase de crescimento (log) (L) em cada curva de crescimento. As taxas são calculadas para uma unidade de grau Celsius (log UFC/g/h/°C), dividindo-se o valor médio do coeficiente angular pela diferença entre a temperatura mais alta e a mais baixa (Equação 2). Este parâmetro é utilizado para calcular o crescimento microbiano por hora em cada perfil de temperatura. O crescimento microbiano horário é obtido multiplicando a taxa de crescimento específico (log UFC/g/h/°C) pelo valor

da temperatura durante 1 hora. O crescimento diário é obtido pela soma de todo o crescimento horário (Equação 3).

$$N(Tcrescimento)\ (log\ UFC/g/h/°C) = \frac{\left(\frac{\alpha(HT)-\alpha(LT)}{2}\right)*\left(\frac{1}{HT-LT}\right)}{24} \qquad \text{(equação 2)}$$

$$Ncrescimento\ (log\ UFC/g) = \sum_{k=1}^{24} n * (N(Tcrescimento)) \qquad \text{(equação 3)}$$

Onde: N(Tcrescimento) - taxa de crescimento microbiano por grau Celsius (log UFC/g/h/°C) na fase de crescimento (log), α(HT) e α(LT) - coeficientes angulares na maior (HT) e menor (LT) temperaturas (°C), respectivamente, n - temperatura horária variando de 4 a 36°C, Ncrescimento - crescimento microbiano diário (log UFC/g) na fase L, e k - tempo (hora).

4.8.4 Modelagem da fase de desaceleração

O fator variável de correlação FT(n) (equação 4) foi criado e inserido nas equações 2 e 3 para modelar o crescimento microbiano na fase de desaceleração (D) com base no valor da fase L (equações 5 e 6). Regressão linear é utilizado para modelagem matemática de valores. A equação de primeiro grau foi considerada para determinar o valor do fator variável FT(n) para qualquer perfil de temperatura. Pela estimativa do crescimento populacional diário nas fases L e D , é possível prever quando a população atingirá a fase estacionária (S).

$$FT(n) = L/D \qquad \text{(equação 4)}$$

$$N(Tdesaceleração\ (log\ UFC/g/h/°C) = \frac{N(Tcrescimento)}{FT(n)} \qquad \text{(equação 5)}$$

$$Ndesaceleração\ (log\ UFC/g/dia) = \sum_{k=1}^{24} n * \frac{N(Tdesaceleração)}{FT(n)} \qquad \text{(equação 6)}$$

Onde: N(Tdesaceleração) - taxa de crescimento microbiano por grau Celsius (log UFC/g/h/°C) na fase D, N(Tcrescimento) - taxa de crescimento microbiano por grau Celsius (log UFC/g/h/°C) na fase L, FT(n) - fator variável de correlação para descrever a taxa de crescimento específica entre as fases L e D por grau Celsius, n - temperatura horária variando de 4 a 36 °C, *N*desaceleração - crescimento microbiano diário (log UFC/g) na fase D, e k - tempo (hora).

4.8.5 Curva preditiva de crescimento microbiano

A modelagem preditiva computacional foi projetada para criar curvas preditivas de crescimento microbiano (figura 1) com base nos resultados do desenho experimental descrito acima. A correlação entre as fases exponencial (log) (L) e de desaceleração (D) foi realizada com base no valor do FT(n), que considera o perfil realístico de temperatura praticado para a matriz . A curva de crescimento microbiano inicia com o resultado da contagem (log UFC/g) obtido no tempo zero. As fases (L) e (D) foram modeladas com base nas Equações 4 e 5, respectivamente.

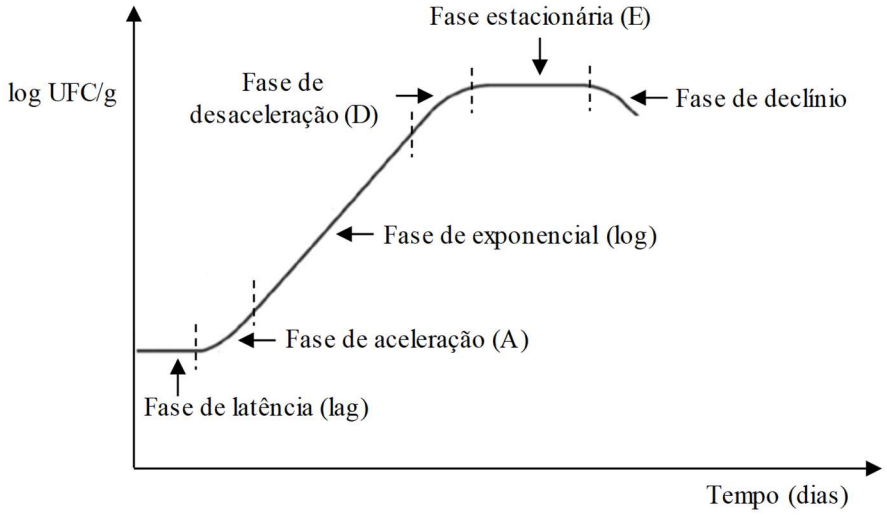

Figura 2. Ilustração esquemática da curva de crescimento microbiano.

CAPÍTULO I

ANTILISTERIAL ACTIVITY OF TWO NOVELS BIOPRESERVATIVES CONTAINING POTENTIAL POSTBIOTICS

ATIVIDADE ANTILISTERIAL DE DOIS NOVOS BIOCONSERVANTES CONTENDO POTENCIAIS PÓS-BIÓTICOS

Antilisterial activity of two novels biopreservatives containing potential postbiotics

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Highlights

- The pH of the growth medium directly influenced CIM and CLM.
- BCPP_SP and BCPP_YE are promising alternatives for preserving meat products.
- Use of preservatives without specific pathogen validation can pose a health risk.

[1]

[1] BCPP_SP: biopreservatives containing potential postbiotics with isolated soy protein.
BCPP_YE: biopreservatives containing potential postbiotics with yeast extract.
SL: preservative containing sodium lactate (60%).
SLS: preservative containing sodium lactate (60%) and liquid smoke.
AR: preservative containing sodium lactate, vinegar powder, sodium citrate, and citric acid.
FCSDV: preservative containing dry vinegar and fermented cane sugar.
NS: preservative containing nisin.
NSDR: preservative containing nisin, sodium diacetate and rosemary extract.
MIC: minimum inhibitory concentration
MLC: minimum listericidal concentration
pH_{MI}: minimum inhibitory pH
pH_{ML}: minimum listericidal pH
Lm: *Listeria monocytogenes*
LAB: lactic acid bacteria

5.1 Resumo

Neste estudo, dois bioconservantes contendo potenciais pós-bióticos (BCPP_SP e BCPP_YE), produzidos por fermentação axênica com *Lacticaseibacillus paracasei* DTA 83, foram investigados quanto à sua capacidade antilisterial. Lactato de sódio, nisina e outros quatro conservantes comerciais de produtos cárneos foram testados nas mesmas condições. O método de macrodiluição foi utilizado para determinar a concentração inibitória mínima (CIM) e a concentração listericida mínima (CLM) dos conservantes contra seis cepas de *Listeria monocytogenes* (*Lm*) e um pool dessas cepas. A dinâmica populacional do pool de *Lm* na presença dos conservantes foi monitorada por turbidimetria usando um espectrofotômetro. A ação antilisterial dos bioconservantes após a neutralização total e parcial dos ácidos orgânicos, bem como a sensibilidade à tripsina e ao aquecimento, foram investigadas. Ambos os BCPPs foram igualmente eficientes em inibir o crescimento de *Lm in vitro* ($p > 0,05$). Além disso, mostraram forte estabilidade ao calor e não foram afetados pelo tratamento com tripsina. A neutralização total ou parcial dos ácidos orgânicos resultou na ausência de ação antilisterial em concentrações de até 10%. O pool de *Lm* foi testado em diferentes valores de pH com ácido lático e ácido clorídrico. Verificou-se que o pH inibitório mínimo foi 4,5 em ambos os casos, enquanto o pH listericida mínimo foi 4,0 para o ácido lático e 3,5 para o ácido clorídrico. Todos os conservantes comerciais foram capazes de inibir *Lm in vitro*; no entanto, em alguns casos, isso ocorreu em concentrações bem acima da dosagem limite sugerida pelos fabricantes. A CIM e CLM foram diretamente influenciadas pelo pH do meio. BCPP_SP e BCPP_YE podem ser uma alternativa natural promissora para uso em produtos cárneos; e sua utilização, dentro de uma estratégia de múltiplas barreiras, pode contribuir para aumentar a robustez dos programas de segurança e qualidade na indústria da carne.

Palavras chaves: antimicrobianos, listeriose, bactérias ácido láctico, lactato de sódio, nisina, conservantes comerciais.

5.2 Abstract

In this study, two biopreservatives containing potential postbiotics (BCPP_SP and BCPP_YE), produced by axenic fermentation with *Lacticaseibacillus paracasei* DTA 83, were investigated for their antilisterial capacity. Sodium lactate, nisin, and four other commercial meat product preservatives were tested under the same conditions. The macrodilution method was used to determine the minimum inhibitory concentration (MIC) and minimum listericidal concentration (MLC) of the preservatives against six strains of *Listeria monocytogenes* (*Lm*) and a pool of these strains. The population dynamics of the *Lm* pool in the presence of the preservatives were monitored by turbidimetry using a spectrophotometer. The biopreservatives antilisterial action after total and partial neutralization of organic acids, as well as sensitivity to trypsin and to heating, were investigated. Both BCPPs were equally efficient in inhibiting the growth of *Lm in vitro* ($p > 0.05$). Besides, showed strong heat stability and were not affected by trypsin treatment. Total or partial neutralization of organic acids resulted in the absence of antilisterial action at concentrations up to 10%. The *Lm* pool was tested at different pH values with lactic acid and hydrochloric acid. It was found that the minimum inhibitory pH was 4.5 in both cases, while the minimum listericidal pH was 4.0 for lactic acid and 3.5 for hydrochloric acid. All commercial preservatives were able to inhibit *Lm in vitro*; however, in some cases, this occurred at concentrations well above the limit dosage suggested by the manufacturers. MIC and MLC were directly influenced by the medium pH. BCPP_SP and BCPP_YE could be a promising natural alternative for use in meat products; and their utilization, within a multi-hurdle strategy, may contribute to increasing the robustness of safety and quality programs in the meat industry.

Keywords: antimicrobials, listeriosis, lactic acid bacteria, sodium lactate, nisin, commercial preservatives.

5.3 Introduction

Foodborne diseases are a global public health problem, affecting millions of people every year and generating high costs associated with medical treatment and productivity loss. Among these diseases, listeriosis stands out as a bacterial infection caused by *Listeria monocytogenes* (*Lm*). In 2021, listeriosis ranked fifth among the most reported zoonoses in the European Union, with 2.183 registered cases and presenting the highest hospitalization and mortality rates, especially among individuals over 64 years old (EFSA e ECDC, 2022).

Listeriosis is often associated with the consumption of ready-to-eat (RTE) processed foods with pH ≥ 4.4, an water activity (aw) ≥ 0.92, or a combination of pH ≥ 5.0 and aw ≥ 0.94. According to reports from the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA), RTE meat-based (beef, pork, and poultry), RTE fish-based, RTE salads, RTE fruits and vegetables, cheeses made from raw milk, and spices and herbs have been standing out in recent years due to the number of occurrences in analyzed food samples (ECDC, 2021; EFSA e ECDC, 2022).

RTE meat-based are chemically complex, rich in nutrients to support microbial growth, and can easily undergo chemical, biochemical, microbiological, and sensory changes during their shelf life. When well established, the thermal treatment applied in the production of these foods can eliminate pathogenic microorganisms and reduce spoilage to acceptable levels. However, even in production units that adopt good manufacturing practices, there is a potential risk of recontamination from the production environment and/or excessive handling. Furthermore, RTE meat-based are often exposed as temperature abuse in retail (Freiberger *et al.*, 2016; Göransson, Nilsson e Jevinger, 2018; Yu, Chin e Paik, 2021).

Cold temperatures and oxygen suppression through vacuum packaging are commonly employed preservation technologies as hurdles in RTE meat-based products. Besides contributing to the reduction of oxidation, these methods enable the control of a great part of spoilage organisms, but they are not effective barriers against anaerobes and anaerobes/facultatives, such as *Lm* (Martinis, De, Alves e Franco, 2002). In this case, preservatives can be an additional strategy to mitigate microbial action (Freiberger *et al.*, 2016; Yu, Chin e Paik, 2021).

Among preservatives, natural ones have been gaining prominence due to the negative associations between chemical additives and health risks. In fact, the population's greater awareness of the role of food in health and the increase in the number of consumers looking for high-quality products without chemical additives have driven researchers and industry to seek healthier natural alternatives for use in their products. One of these options is the use of either protective cultures or antimicrobial metabolites produced by lactic acid bacteria (LAB). These bacteria and their metabolites are generally recognized as safe (GRAS) (Martinis, De, Alves e Franco, 2002; Wiernasz *et al.*, 2017).

Antimicrobial substances isolated from LAB, such as bacteriocins, have shown promising results in bio-controlling the growth of pathogens and spoilage organisms in meat products. However, the high cost of isolation and purification has limited their use to high-value-added products (Castellano *et al.*, 2017; Hernández-Aquino *et al.*, 2019). Conversely,

preservatives made from media fermented by LAB, thermally treated, without isolation and purification of antimicrobial substances, containing potential postbiotics, can be a promising and relatively low-cost alternative to prevent undesirable microorganisms in meat products (Jaramillo *et al.*, 2018; Lima *et al.*, 2022).

The term "postbiotics" has been widely used in the scientific literature and the food and health industries; however, there is still no universally accepted consensus on its definition. Two recent articles, one from the International Scientific Association of Probiotics and Prebiotics (ISAPP) (Salminen *et al.*, 2021) and the other a critical response to the first article (Aguilar-Toalá *et al.*, 2021), discussed the definition and scope of the term postbiotics, basing their arguments on robust bibliographic research.

The ISAPP article proposes that the term postbiotics be defined as “preparations of inanimate microorganisms and/or their components that confer a health benefit to the host.” This definition encompasses microbial cells that have been deliberately inactivated and/or their cellular components (pili, cell wall components, and/or other structures), which may or may not include metabolites. It is essential that the beneficial effects of postbiotics are confirmed in the target host. It is important to note that purified microbial metabolites and vaccines are not considered postbiotics. Additionally, a postbiotic does not need to be derived from a probiotic; that is, the inactivated version does not need to have been a probiotic to be accepted as a postbiotic. The definition excludes products derived from undefined microorganisms.

The article by Aguilar-Toalá *et al*. proposes maintaining a definition stated in 2013 as "any factor resulting from the metabolic activity of a probiotic or any molecule released capable of conferring beneficial effects to the host either directly or indirectly" (Tsilingiri e Rescigno, 2013). This definition encompasses well-defined molecules resulting from the metabolic activity of probiotics, excluding inactivated microbial cells (defined as paraprobiotics). Furthermore, it excludes compounds released by non-probiotic microorganisms and includes indirect benefits to the host, allowing for a more in-depth discussion on the types of benefits that could be covered.

Several studies have evaluated the effect of different preservatives in meat products, but there are still gaps in knowledge regarding the most effective use of these compounds in inhibiting *Lm* (Bodie *et al.*, 2019). Therefore, this study aimed to verify, *in vitro*, if the biopreservatives BCPP_SP and BCPP_YE can be effective alternatives for the control of *Lm*, using commercial preservatives for meat products as a comparison parameter.

5.4 Materials and methods

5.4.1 Microbial culture for BCPP production

The BCPPs were produced by axenic fermentation from the strain of *L. paracasei* DTA 83. This bacterial strain, belonging to the culture collection of the food microbiology laboratory-DTA-UFRRJ, was isolated from the feces of newborns (7 to 21 days old) in Rio de Janeiro, Brazil, and identified by 16S rDNA sequencing using RAPD-PCR (Guerra *et al.*, 2018). The complete genome data were deposited in GenBank under accession number

QRBH00000000 (Lemos-Junior, Fioravante Guerra, *et al.*, 2019). The culture was classified as GRAS status, characterized as potentially probiotic (Lemos-Junior, Guerra, *et al.*, 2019), reported to have potential to release postbiotics compounds (Oliveira *et al.*, 2021; Silva *et al.*, 2021), capable of biocontrolling the growth of *L. innocua*, *Salmonella Typhimurium*, *Candida albicans*, and *Escherichia coli* even after partial reduction of cell viability due to gastrointestinal transit stress (Tarrah *et al.*, 2019), and demonstrated good results in controlling the natural microbiota of vacuum packed cooked sausages when confronted with sodium lactate (Lima *et al.*, 2022).

5.4.2 Production of BCPPs

The BCPPs were individually produced on a pilot industrial scale at the BRC Ingredients Ltda. industrial unit in Rio Claro, SP, Brazil, as described by (Lima *et al.*, 2022). In summary, two formulations that mimic MRS broth in relation to most ingredients, but without the addition of polysorbate 80 (Tween 80). The biopreservatives were prepared using food-grade ingredient. They differ from each other only in the nitrogen source used in the fermentation medium (BCPP_SP, isolated soy protein and BCPP_YE, yeast extract). These formulations were heat-treated in a 330 L stirred-tank bioreactor with automatic pH and temperature control. *L. paracasei* DTA 83 culture was then added for fermentation at 36°C. After 72 hours of fermentation the medium was heat-treated at 95°C for 5 minutes. The BCPPs were hot-bottled in 10 L polypropylene containers. The presence of viable *L. paracasei* cells or contaminants was assessed by plate counting on MRS medium, plate count agar, and potato dextrose agar acidified to pH 3.5 with tartaric acid.

5.4.3 Commercial meat product preservatives

In addition to biopreservatives, six commercial meat product preservatives were included in this study. A code was assigned to each preservative, as shown in Table 1.

Table 1. Coding (acronym) used to denote the preservatives in this article, label-composition, and manufacture-recommended dosage.

Preservative	Preservative Label-Composition	Recommended Dosage
SL	Sodium lactate (60%)	1.0 – 2.0%
SLS	Sodium lactate (60%), and liquid smoke	1.0 – 2.0%
AR	Sodium lactate, vinegar powder, sodium citrate, and citric acid	0.5 – 1.0%
FCSDV	Fermented cane sugar, and Dry vinegar	0.5 – 1.0%
NSDR	Nisin, sodium diacetate, and rosemary extract	Up to 0.13%
NS	Nisin (2.5% - 25000 mg/kg)	Up to 0.1% - 25 mg/kg

* According to the manufacturer, the dosage of 0.13% NSDR applied to meat products mass provides a final concentration of 0.1% sodium diacetate and 0.00035% (3.5 mg/kg) nisin. The Brazilian legislation establishes the limit of 0.1% sodium diacetate and 0.0025% (25 mg/kg) nisin for cooked industrialized meat products (Brasil, 2019).

5.4.4 Strains of *Listeria monocytogenes* and preparation of the inocula

In this study, six strains of *Lm* were used, with five isolated from processed meat products (CLIST 4165 - serotype 1/2a, CLIST 4396 - serotype 1/2b, CLIST 4405 - serotype 1/2a, CLIST 4642 - serotype 1/2b, and CLIST 4645 - serotype 1/2c) and one reference strain (CLIST 3436 (Scott A) - serotype 4b), all sourced from the Listeria collection (CLIST) at the Laboratory of Bacterial Zoonoses (LABZOO) of the Oswaldo Cruz Foundation (FIOCRUZ).

The *Lm* strains were cultured three times in BHI broth (Kasvi - Spain), and the third subculture (14-16 hours) was diluted 1:2, 1:5, 1:10, 1:25, and 1:50 in BHI broth. The absorbance of each dilution was read at 600 nm in a spectrophotometer (model UV-M51 UV-VIS from BEL Engineering - Italy) and, at the same time, subjected to colony counting on BHI agar plates (Kasvi - Spain). Standard growth curves were obtained for each of the six *Lm* strains and for a pool of these strains, correlating absorbance with colony counts (CFU/mL) using simple linear regression.

The individual inocula of *Lm* were prepared in BHI broth, adjusting the concentration of the cell suspension, obtained after 14-16 hours of the third subculture, to *ca* 10^8 CFU/mL using the spectrophotometer at 600 nm. The *Lm* pool was obtained by mixing equal aliquots of the individual *Lm* inocula. The purity of the inocula and confirmation of the cell concentration were verified by plating on agar Listeria according to Ottaviani and Agosti (ALOA) from Himedia (India) without the addition of inhibitors. Plates were incubated at 36°C for 24/48 h, followed by counting and morphological evaluation of the colonies and counting .

5.4.5 Macrodilution

The macrodilution method was employed to evaluate the antilisterial activity and determine the MIC of the preservatives. Briefly, the preservatives were diluted in BHI broth (Table 2) and inoculated with individual *Lm* inoculums or a pool of *Lm*. The final concentration in each tube was approximately 10^5 CFU/mL. Tubes containing BHI broth and inoculum were used as positive controls (PC), and tubes containing only BHI broth were used as negative controls (NC). The tubes were incubated at 36°C and visually examined for turbidity at 24, 48, 72, and 96 h, comparing the test tubes with the PC and NC. The lowest concentration at which *Lm* growth was not observed was considered the MIC.

Table 2. Preservative concentrations used to evaluate the Minimum Inhibitory Concentration (MIC) of individual *Lm* inocula or *Lm* pool, using the macrodilution method in test tubes.

Preservative	Concentrations of preservatives in percentage (%).											
BCPP_SP	3.00	2.25	1.69	1.27	0.95	0.71	0.53	0.40	0.30	0.23	0.17	0.13
BCPP_YE	3.00	2.25	1.69	1.27	0.95	0.71	0.53	0.40	0.30	0.23	0.17	0.13
SL	50.00	37.50	28.13	21.09	15.82	11.87	8.90	6.67	5.01	3.75	2.82	2.11
SLS	10.00	7.50	5.63	4.22	3.16	2.37	1.78	1.33	1.00	0.75	0.56	0.42
AR	10.00	7.50	5.63	4.22	3.16	2.37	1.78	1.33	1.00	0.75	0.56	0.42
FCSDV	10.00	7.50	5.63	4.22	3.16	2.37	1.78	1.33	1.00	0.75	0.56	0.42
NSDR	1.00	0.75	0.56	0.42	0.32	0.24	0.18	0.13	0.10	0.08	0.06	0.04
NS	0.20	0.15	0.13	0.10	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01

* %w/v: NSDR and NS. %v/v: BCPP_SP, BCPP_YE, SL, SLS, AR, and FCSDV. The preservative NS contains 2.5% nisin in its formulation. Therefore, the percentage dilutions of NS presented in the table reflect calculated concentrations of nisin in mg/L of 50.00, 37.50, 31.25, 25.00, 20.00, 17.50, 15.00, 12.50, 10.00, 7.50, 5.00, and 2.50.

5.4.6 Growth kinetics of a pool of *Lm* strains

The impact of the preservatives BCPP_SP, BCPP_YE, NSDR, and NS on the population dynamics of the *Lm* pool was monitored for up to 96 hours through reading the absorbance at 600 nm in a spectrophotometer. In summary, a test tube battery was used to dilute the preservatives and add the *Lm* pool. All dilutions were made with BHI broth, as shown in Table 3. No preservatives were added to the CP and CN. The final microbial concentration in each tube was approximately 10^5 CFU/mL, except in the CN tubes, which did not receive inoculum.

The tubes were incubated at 36ºC, with absorbance readings at time zero (time of inoculation) and every 8 hours, up to 96 hours. The tubes were vortexed before each reading. A tube containing BHI broth was used as a blank. To avoid possible errors due to differences between tubes, the absorbance of the empty tubes was read. The corrected absorbance was determined by subtracting the absorbance reading of the "filled" tube (with diluted preservatives and inoculum) from the absorbance reading of the empty tube, for all sampling times. Absorbance was measured directly in the tubes, and they were carefully placed in the same position in the spectrophotometer. Microbial growth curves were plotted on an XY scatterplot, correlating absorbance values with time (hours) for each tested concentration.

Table 3. Preservative concentrations used to evaluate the growth kinetics of a pool of *Lm* strains.

Preservative	Concentrations of preservatives in percentage (%).									
BCPP_SP	2.00	1.80	1.60	1.40	1.20	1.00	0.80	0.60	0.40	0.20
BCPP_YE	2.00	1.80	1.60	1.40	1.20	1.00	0.80	0.60	0.40	0.20
NSDR	1.00	0.75	0.56	0.42	0.32	0.24	0.18	0.13	0.10	0.08
NS	0.20	0.15	0.13	0.10	0.08	0.05	0.04	0.03	0.02	0.01

* %w/v: NSDR and NS. %v/v: BCPP_SP and BCPP_YE. The preservative NS contains 2.5% nisin in its formulation. Therefore, the percentage dilutions of NS presented in the table reflect calculated concentrations of nisin in mg/L of 50.00, 37.50, 31.25, 25.00, 18.75, 12.50, 10.00, 7.50, 5.00, and 2.50.

5.4.7 Minimum listericidal concentration (MLC)

The assay used to stablish MIC (spectrophotometric and macrodilution), were subsequently investigated for MLC. In both cases, aliquots of 0.1 *mL* from all tubes where *Lm* growth was not observed were transferred to tubes containing 4.9 mL of sterile BHI broth. The tubes were then incubated at 36°C, with readings taken at 24/48 hours. The first concentration where *Lm* growth was not observed was considered as the MLC.

5.4.8 Effect of lactic acid, hydrochloric acid, and pH on the pool of *Lm* strains

To investigate the influence of lactic acid, hydrochloric acid, and pH on the *Lm* pool, tubes containing BHI broth (pH 7.20±0.05) were acidified with 1M hydrochloric acid or 85%_AG lactic acid to final pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. The tubes were inoculated with the *Lm* pool to achieve a final concentration of approximately 10^5 CFU/mL, then incubated at 36ºC and read at 24, 48, 72, and 96 hours. The lowest pH at which no *Lm* growth was observed was considered the minimum inhibitory pH (pH_{MI}). Tubes that did not show visible turbidity were investigated for minimum listericidal pH (pH_{ML}), similarly to the procedure described in section 2.7.

5.4.9 Neutralization of organic acids in BCPPs

The impact of total and partial neutralization of organic acids on the antilisterial action of BCPPs was evaluated by macrodilution against the *Lm* pool (*ca* 10^5 CFU/mL) at concentrations of 0.60, 0.80, 1.00, 1.20, 1.40, 1.60, 1.80, 2.00, 2.50, 5.00, and 10.00%. BCPP_SP and BCPP_YE were adjusted to pH 7 and pH 6 by addition of 4 M NaOH using a pH meter (model PHS-3E, Even). Subsequently, the treated BCPPs were sterilized by filtration through a sterile 0.20 µm cellulose acetate membrane filter (Dismic-25cs, Advantec, USA). The innocuity was verified by duplicate plating of 0.1 mL aliquots on PCA agar. The volume of NaOH was considered in the calculation of the dilution of the biopreservatives.

5.4.10 Assessment of the temperature sensitivity of BCPPs

The effect of thermal treatments on the antilisterial activity of BCPPs was evaluated. Briefly, sterile BHI broth was used to prepare individual stock solutions of BCPP_SP and BCPP_YE, both at a concentration of 10%. These solutions were transferred to well-sealed tubes and subjected to three treatments: boiling in a water bath for 20 and 30 minutes and autoclaving at 121°C for 15 minutes. After cooling, the BCPPs were tested by macrodilution against the *Lm* pool (*ca* 10^5 CFU/mL), at concentrations of 0.60, 0.80, 1.00, 1.20, 1.40, 1.60, 1.80, 2.00, 2.50, e 5.00%. Positive controls with unheated BCPPs and a negative control with BHI broth subjected to the same conditions were used.

5.4.11 Trypsin Sensitivity Assessment

The sensitivity of biopreservatives to the enzyme trypsin was evaluated in three independent assays. In brief, solutions containing 50% sterile BHI broth and BCPPs (BCPP_SP - pH 2.13±0.02 and BCPP_YE - pH 2.24±0.02) were treated with 1 mg/mL trypsin for 2 hours

at 36±1°C. Trypsin inactivation was performed by boiling for 2 minutes. The macrodilution method was used to investigate the MIC of treated BCPPs against the *Lm* pool (*ca* 10^5 CFU/mL).

5.4.12 Statistical analysis

The assays were performed in triplicate. The results of pH were presented as Mean ± Standard deviation (SD) of the replicates, with a 95% confidence interval (CI) ($p < 0.05$).

Three-way ANOVA was used to compare the growth curves of the biopreservatives BCPP_SP and BCPP_YE. The analysis involved a factorial design with three study factors: biopreservatives (BCPP_SP and BCPP_YE), concentrations (0.20%, 0.40%, 0.60%, 0.80%, 1.00% (CIM), CP, and CN), and times (T8, T16, T24, T32, T40, T48, T56, T64, T72, T80, T88, and T96). Each concentration was treated as an independent sample, and the absorbance values used in the ANOVA were obtained by subtracting the absorbance observed at each sampling time from the absorbance at time zero for the respective concentration (Δ_absorbance = absorbance_t(x) – absorbance_t(0)). In this way, the interference of the inherent absorbance of each biopreservative and each dilution was eliminated. The data were processed using the software Jamovi version 2.3.

5.5 Results and discussion

5.5.1 pH of preservatives and their dilutions in BHI broth

The complete data from pH values of pure and diluted preservatives in BHI broth (used for MIC, MLC, and microbial growth curve construction research - sections 3.2 and 3.3) are presented in Table S1 (Supplementary Material).

The pH of biopreservatives BCPP_SP and BCPP_YE, in dilutions ranging from 0.40% to the maximum tested for MIC (3.00%), ranged between 6.17–3.59 and 6.17–3.62. In 1.00% (the concentration at which the biopreservatives exhibited MIC), the pH was 4.55.

The preservative SL did not cause pH changes in the dilutions tested *in vitro* for MIC, with the pH of the medium remaining close to neutrality. Likewise, the preservatives NS (1.00% w/v) and NSDR (2.00% w/v) did not change the pH of the growth medium.

In dilutions ranging from 1.00% to the maximum tested for MIC (10.00%) of the preservatives SLS, AR and FCSDV, the pH ranged, respectively, between 7.00–5.41, 6.21–5.00 and 7.17–6.17.

5.5.2 Minimum Inhibitory Concentration (MIC) and Minimum Listericidal Concentration (MLC).

The MIC and MLC results, obtained by macrodilution, are presented in Table 4, and the tested concentrations are shown in Table 2.

The biopreservatives BCPP_SP and BCPP_YE showed an MLC of 1.27% and MIC of 0.71% or 0.95%, depending on the strain tested. In previous studies, BCPP_SP (named PPCP) was tested *in vitro* under similar conditions against the natural microbiota of vacuum-packed

cooked sausages, providing partial inhibition at concentrations between 1.0 and 3.0% and total inhibition at a concentration of 3.5% (Lima *et al.*, 2022). In this case, the higher concentration required can be explained by the presence of less sensitive microorganisms in the sausage microbiota, compared to the *Lm* strains.

In another study (Almeida Godoy, de *et al.*, 2022), the biopreservative was produced in a semi-culture fermentation system, adding *Saccharomyces cerevisiae* var. boulardii 17 to the medium pre-fermented by the *L. paracasei* DTA 83 strain. The action on the microbiota isolated from chicken sausages and semi-finished chicken parts was verified *in vitro*. Partial inhibition was achieved by adding 1.0 to 2.5% of PPCP, and total inhibition was reached at concentrations above 3.0%. According to the authors, the improved efficacy can be attributed to the synergistic action of the main metabolites, lactic acid (*L. paracasei* DTA 83) and acetic acid (*S. cerevisiae* var. boulardii 17), in addition to other biocides produced by the cultures (not measured).

The SL preservative exhibited MIC values of 11.87% or 15.82%, depending on the tested strain, and no MLC was observed in dilutions up to 50.00%. The SL preservative's label indicates that it contains 60% sodium lactate (unverified concentration), which corresponds to 7.12% or 9.49% sodium lactate in the found MICs. At the evaluated concentrations, SL did not cause significant changes in the medium's pH, which remained between 7.18 and 7.24 (Table S1 - Supplementary Material). Temperature, pH, sodium lactate concentration, the type of microorganism, and the presence of other ingredients in a product's formulation are factors that influence the antimicrobial activity of sodium lactate (Williams e Phillips, 1998).

In different pH conditions, Apostolidis; Kwon; Shetty (2008) verified that 2.00% sodium lactate had an inhibitory effect on *Lm* Scott A 4b, cultured in Tryptic Soy Broth supplemented with 1.5% yeast extract and acidified with lactic acid to pH 5.5 or pH 6.0, at 4°C and 37°C. The antimicrobial effect of lactate was more effective at pH 5.5 and at the temperature of 4°C. The authors pointed out that the antimicrobial effect of lactate is more effective at lower pH values, as under these conditions, there is a higher concentration of the non-dissociated form of the antimicrobial.

The SLS preservative did not exhibit MLC at concentrations up to 10.0% and its MIC was 7.50% for all strains. Its label indicates a percentage of 60% sodium lactate (unverified concentration) in its composition, in addition to an undisclosed concentration of liquid smoke.

Pathogens such as *L. monocytogenes, Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* have shown variable sensitivity to liquid smoke *in vitro* and in food systems, including variable susceptibility among different strains of the same species. The antimicrobial action of liquid smoke occurs mainly through the action of phenolic compounds on the cytoplasmic membrane, causing leakage of intracellular fluids; and carbonyls, which inhibit microbial growth by inactivating cytoplasmic and membrane-bound enzymes (Lingbeck *et al.*, 2014).

The FCSDV preservative exhibited an MIC of 7.50% and no MLC at concentrations up to 10.00% for all strains. The MIC of the AR preservative was 2.37% or 3.16%, depending on the strain, and the MLC was 10.00% for all strains.

Under the pH conditions of this test, the NSDR preservative showed only listeriostatic action, and its MIC ranged from 0.18 to 0.42%, depending on the resistance of the target strain.

NSDR also exhibited a considerable decay of its antilisterial action during the 96 hours of the test. Similarly, the NS preservative, under the pH conditions of this assay, showed a decay of its action against *Lm* during the incubation period at 36ºC. All strains treated with NS showed visible growth within 96 hours, except for strain 4165, which had its growth inhibited at the concentration of 0.02% (50.00 mg/L of nisin). The gradual decrease in the ability of the NSDR and NS preservatives to inhibit the growth of *Lm* can be graphically visualized in Figure 2.

Other authors have also observed a decrease in the antimicrobial action of nisin over time. (Brasileiro *et al.*, 2016) observed that the preservatives NovaGARD®LM100 (similar to NSDR - encapsulated nisin, free nisin, rosemary extract, and sodium diacetate), NovaGARD®NR100 (free nisin and rosemary extract), Nisaplin® (NS preservative), or Purasal S® (similar to LS) gradually lost their antilisterial action in mortadellas intentionally contaminated with *Lm* during storage at 8°C for 30 days.

Mortadellas formulated with NovaGARD®NR100 and Purasal S® did not differ significantly from the control ($p > 0.05$) without the addition of preservatives. Until the 5th day, the mortadella added with Nisaplin® (12.5 ppm) showed the best inhibitory effect, however, it did not differ significantly from the control in the 10th day count. On the 10th and 20th days, mortadellas formulated with NovaGARD®LM100 showed a *Lm* count approximately 3 log and 2 log lower than the other formulations. This difference dropped to 0.5 log on the 30th day. According to the authors, the best inhibitory effect of NovaGARD®LM100 was due to the combined action of encapsulated nisin with free nisin. Free nisin would act immediately after contact with target cells and gradually decrease its activity due to degradation or interaction with components of the food matrix. Encapsulated nisin, protected from the unfavorable environment by lipid encapsulation, would present a controlled release, improving the availability of the antimicrobial.

Table 4. Minimum Inhibitory Concentration (MIC) observed at 24, 48, 72, and 96 hours by the broth macrodilution method, and Minimum Listericidal Concentration (MLC).

Preservative	Time (hours)	*Listeria monocytogenes* strain						
		4645	4396	4642	4405	4165	3436	Pool
BCPP_SP (biopreservative prepared with soy protein)	24	0.71	0.71	0.71	0.53	0.71	0.71	0.71
	48	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	72	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	96	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	MLC	1.27	1.27	1.27	1.27	1.27	1.27	1.27
BCPP_YE (biopreservative prepared with yeast stratum)	24	0.71	0.71	0.71	0.53	0.71	0.71	0.71
	48	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	72	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	96	0.71	0.95	0.95	0.71	0.71	0.71	0.95
	MLC	1.27	1.27	1.27	1.27	1.27	1.27	1.27
SL (sodium lactate 60%)	24	15.82	15.82	11.87	11.87	15.82	11.87	15.82
	48	15.82	15.82	11.87	11.87	15.82	11.87	15.82
	72	15.82	15.82	11.87	11.87	15.82	11.87	15.82
	96	15.82	15.82	11.87	11.87	15.82	11.87	15.82
	MLC	---	---	---	---	---	---	---
SLS (sodium lactate (60%) and liquid smoke)	24	7.50	7.50	5.63	5.63	7.50	7.50	7.50
	48	7.50	7.50	7.50	5.63	7.50	7.50	7.50
	72	7.50	7.50	7.50	7.50	7.50	7.50	7.50
	96	7.50	7.50	7.50	7.50	7.50	7.50	7.50
	MLC	---	---	---	---	---	---	---
FCSDV (fermented cane sugar and dry vinegar)	24	7.50	5.63	5.63	5.63	5.63	7.50	7.50
	48	7.50	7.50	5.63	5.63	7.50	7.50	7.50
	72	7.50	7.50	7.50	7.50	7.50	7.50	7.50
	96	7.50	7.50	7.50	7.50	7.50	7.50	7.50
	MLC	---	---	---	---	---	---	---
AR (sodium lactate, vinegar powder, sodium citrate, and citric acid)	24	2.37	1.78	1.78	1.78	2.37	2.37	2.37
	48	2.37	2.37	2.37	2.37	3.16	3.16	3.16
	72	2.37	2.37	2.37	2.37	3.16	3.16	3.16
	96	2.37	2.37	2.37	2.37	3.16	3.16	3.16
	MLC	10.00	10.00	10.00	10.00	10.00	10.00	10.00
NSDR (nisin, sodium diacetate and rosemary extract)	24	0.10	0.10	0.10	0.10	0.10	0.10	0.10
	48	0.18	0.32	0.32	0.18	0.13	0.13	0.32
	72	0.24	0.42	0.42	0.24	0.18	0.18	0.42
	96	0.24	0.42	0.42	0.24	0.18	0.18	0.42
	MLC	---	---	---	---	---	---	---
NS (nisin)	24	0.04	0.05	0.05	0.04	0.03	0.03	0.05
	48	0.10	0.10	0.10	0.10	0.10	0.10	0.10
	72	0.15	0.20	0.20	0.15	0.15	0.15	0.20
	96	GACT	GACT	GACT	GACT	50.00	50.00	GACT
	MLC	***	***	***	***	---	***	***

* MIC and MLC expressed in percentage concentrations of preservatives (%). GACT - growth at all tested concentrations. (---) Did not exhibit listericidal effect at the tested concentrations. (***) Did not exhibit listericidal or listeriostatic effect at the tested concentrations. NS concentrations (% NS - mg nisin/L): 0.03-7.50, 0.04-10.00, 0.05-12.50, 0.10-25.00, 0.15-37.50, and 0.20-50.00.

5.5.3 Microbial Growth Curves

The growth curves of the *Lm* pool in BHI broth, with added preservatives BCPP_SP, BCPP_YE, NS, and NSDR, are presented in Figure 2.

The spectrophotometric evaluation revealed a MIC as well as MLC of 1.00% for the biopreservatives BCPP_SP and BCPP_YE against the *Lm* pool. The results obtained confirm the values found by the macrodilution method.

The CP and concentrations of 0.20, 0.40, and 0.60% of BCPP_SP and BCPP_YE took 8 to 9 hours to enter the exponential growth phase, presenting maximum absorbance around 18 hours of incubation. The 0.80% concentrations prevented the growth of the *Lm* pool for 24 hours. In both biopreservatives, the increase in concentration resulted in a gradual decrease in maximum population density (maximum absorbance) at subinhibitory concentrations of 0.20, 0.40, 0.60, and 0.80%.

The statistical analysis of absorbance variation in the growth curves, using three-way ANOVA, revealed that the biopreservatives BCPP_SP and BCPP_YE did not differ statistically from each other at equal concentrations over the 96 hours of study ($p > 0.05$). These results suggest that the efficacy of the biopreservatives is comparable when used at the same concentrations. However, significant differences were observed between the different concentrations ($p \leq 0.05$), demonstrating that variations in the concentration of the biopreservatives significantly influenced the growth of the *Lm* pool over time.

The adjusted means of the different concentrations of the biopreservatives are presented in Figure 1. The remaining statistical analysis data are presented in the supplementary material.

The similarity in the antilisterial action of BCPP_SP and BCPP_YE may allow manufacturers to choose the raw material with the most advantageous price, reducing the final cost of the product. However, the significant difference between concentrations highlights the importance of adjusting the dosage to achieve the desired efficacy in controlling *Lm*.

The NS preservative, at concentrations up to 0.02% (50.00 mg/L of nisin), did not retain the growth of the *Lm* pool during the 96 hours of incubation at 36ºC. The inhibition time was proportional to the concentration, reaching 70 hours at the 0.02% dilution. In this assay, the pH of the growth solutions remained close to neutrality (pH 7.19±0.03). In this pH range, the antibacterial action of nisin is reduced due to decreased solubility and storage instability (Adhikari, Das e Ramesh, 2012; Tan *et al.*, 2015), which may explain the absence of antilisterial activity retention during the evaluated period.

Indeed, nisin is more soluble in acidic environments, becoming less soluble at pH close to neutrality. Moreover, the retention of the antimicrobial activity is favored at low temperatures. Therefore, the medium's pH and the temperature are factors that influence the activity of nisin during storage (Tan *et al.*, 2015; Wu *et al.*, 2023).

The NSDR preservative showed a MIC of 0.42% and no bactericidal effect at concentrations of up to 1.00%. In the subinhibitory dilutions of 0.08, 0.10, 0.13, 0.18, 0.24, and 0.32%, the gradual increase in concentration resulted in a decrease in the maximum population density (maximum absorbance) and an extension of the lag phase to 16, 24, 32, 36, 48, and 66 hours, respectively. The MIC value found was three times higher than the dosage recommended

by the manufacturer. However, it is crucial to emphasize that the pH of the growth solutions remained close to neutrality in this assay (pH 7.17±0.03). As nisin shows instability and low solubility in this pH range, the residual antilisterial action may have been predominantly a function of the other components of the preservative.

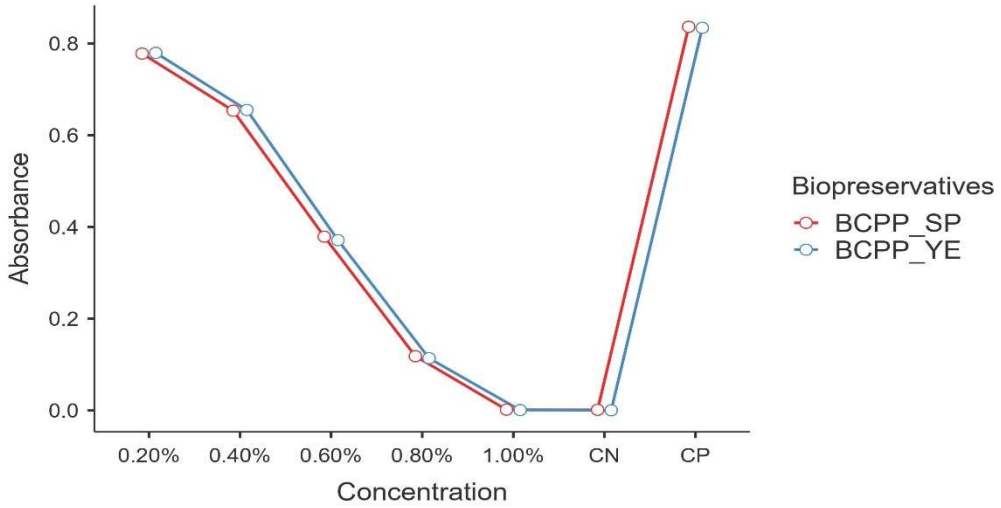

Figure 1. Estimated Marginal Means Graph. Adjusted averages of different concentrations of biopreservatives BCPP_SP and BCPP_YE. Software: Jamovi version 2.3.

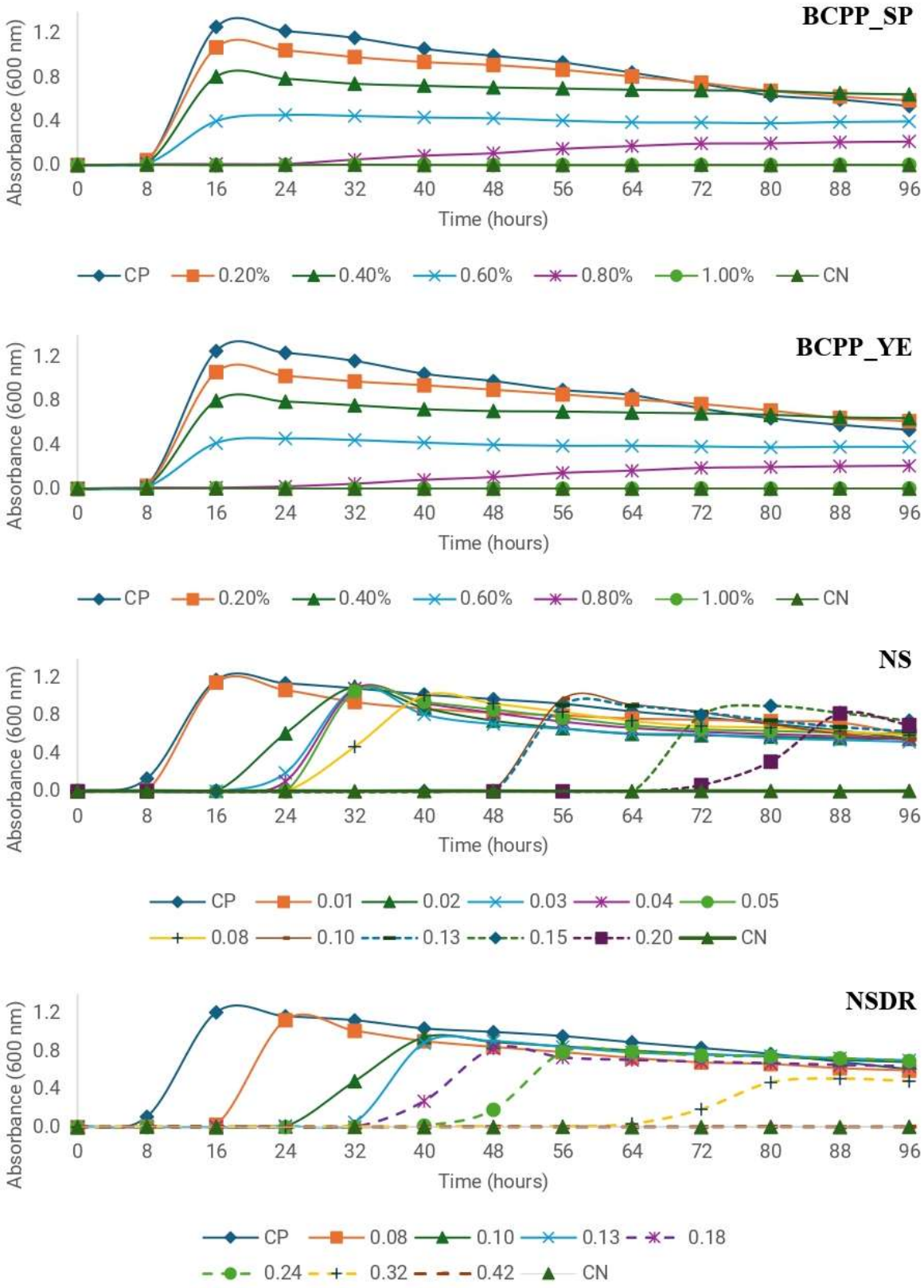

Figure 2. Growth curves of the *Lm* pool at different concentrations of the preservative. BCPP_SP (biopreservatives containing potential postbiotics with isolated soy protein). BCPP_YE (biopreservatives containing potential postbiotics with yeast extract). NS (preservative containing nisin). NSDR (preservative containing nisin, sodium

diacetate and rosemary extract). CP (positive control). CN (negative control). The NS percentage dilutions sequentially reflect the calculated concentrations of nisin in mg/L of 2.50, 5.00, 7.50, 10.00, 12.50, 18.75, 25.00, 31.25, 37.50, and 50.00. Curves with solid lines mean concentrations within the limit recommended by the manufacturer. Curves with dashed lines mean concentrations above that recommended by the manufacturer or above that permitted by the health authority.

5.5.4 Antilisterial activity at pH 6

The antilisterial activity and MIC were further investigated by macrodilution (section 2.5) against the *Lm* pool (*ca* 10^5 CFU/mL), after adjusting the pH of the growth medium (BHI broth with antimicrobials - Table 2) to pH 6, with the addition of 1M HCl. These results of this evaluation can be seen in Table S2 (Supplementary Material).

The MICs of the SLS and AR preservatives remained unchanged at 7.50% and 3.16%, respectively.

The LS, FCSDV, NSDR, and NS preservatives exhibited a decrease in MIC at pH 6. In LS, the MIC was reduced from 15.82% (pH ≈ 7.19) to 6.67%. In FCSDV, the MIC was reduced from 7.50% (pH ≈ 6.40) to 5.63%. In NSDR, the MIC was reduced from 0.42% (pH ≈ 7.17) to 0.10%. NS showed an absence of MIC after 96 hours of storage at pH ≈ 7.19. At pH 6, it presented a MIC of 0.04% (10.00 mg/L of nisin).

These results demonstrated that the antimicrobial activity of a preservative can be directly influenced by the medium's pH. Therefore, it is recommended to challenge the potential antimicrobial agent under extreme conditions that cover the range of fluctuations of intrinsic factors related to microbial growth, in order to prevent the growth of pathogens or deterioration that would influence its intended use and shelf life (Anonymous, 2003; EURL Lm, 2021).

5.5.5 Effect of lactic acid, hydrochloric acid, and pH on the pool of Lm strains

Lm exhibits considerable variability among its strains in terms of resistance to environmental stresses, such as pH, water activity, salt concentration, refrigeration temperatures, and sensitivity to different preservatives (HEREDIA et al., 2024; TIGANITAS et al., 2009). Thus, the minimum inhibitory pH (pH_{MI}) and minimum listericidal pH (pH_{ML}) were investigated for the *Lm* pool used in this study. These results of this evaluation can be seen in Table S3 and Figure S1 (Supplementary Material).

The *Lm* pool minimum inhibitory pH was 4.5 either in the presence of lactic acid or hydrochloric acid, whereas the minimum listericidal pH was 4.0 and 3.5 for exposure to lactic acid and HCl, respectively. Heredia *et al*. (2024) investigated the pH_{MI} of *Lm* strains isolated from different sources in BHI broth acidified with HCl, finding values between 4.29 and 5.04, with an average of 4.66±0.24. The authors hypothesized that adaptation to the acid stress presented by some strains could have been due to exposure to disinfectants and acidic solutions applied during the cleaning and disinfection of food processing plants. Tiganitas *et al*. (2009) reported total inactivation of *Lm* at pH 4.0 acidified with lactic acid and a significant reduction in the number of cells at pH 4.5.

The biopreservatives BCPP_SP and BCPP_YE exhibited MIC and MLC at a concentration of 1.00%. At this dilution, the growth solutions had an average pH of 4.54 (Table S1 - Supplementary Material). The pH results of the MICs were equivalent to the pH_{MI} values found in the tubes of BHI broth acidified with lactic acid, indicating a strong relationship between growth inhibition and pH.

5.5.6 Sensitivity of BCPPs to thermal treatment and neutralization of organic acids

There was no change in the MIC of the biopreservatives after treatments by boiling in a water bath for 20 and 30 minutes and autoclaving at 121°C for 15 minutes. This thermoresistance that BCPP_SP and BCPP_YE are promising options for application in thermally treated products.

BCPP_SP and BCPP_YE subjected to total neutralization of organic acids (pH 7) and partial neutralization (pH 6) did not exhibit antilisterial action when tested at concentrations of up to 10%. These results reinforce the role of organic acids as the main antagonistic substances of the biopreservatives.

Different results were obtained by other authors. Jawan *et al*. (2020) found that cell-free supernatant (CFS) produced by *Lactococcus lactis* remained stable after neutralization (pH 7), indicating that the antimicrobial activity of the CFS was not due to the acidity of lactic acid. The CFS showed optimal stability at pH values ranging from 4 to 8 and a reduction in activity of approximately 50% at pH 2, 3, 10, and 11. Wannun; Piwat; Teanpaisan (2014) also observed that the antimicrobial protein produced by *L. paracasei* SD1 was stable over a wide pH range (3.0 – 8.0), being most effective at pH between 5.0 and 6.0 and completely losing antimicrobial activity at pH 9.0.

5.5.7 Sensitivity of BCPPs to trypsin

There was no change in the MIC of BCPP_SP and BCPP_YE against the *Lm* pool (*ca* 10^5 CFU/mL) after treatment with 1 mg/mL trypsin. Thus, four hypotheses were proposed:

First, the biopreservatives may not contain peptide/protein compounds with antilisterial activity. Apparently, there was no formation of protein-like substances similar to bacteriocins, or they were inactivated by the severe heat treatment that BCPP_SP and BCPP_YE underwent before bottling.

The low thermal resistance of bacteriocin-like substances has been observed in other studies, such as that of paracasin SD1, produced by *L. paracasei* SD1 (Wannun, Piwat e Teanpaisan, 2014); and the cell-free supernatant produced by *Lactococcus lactis*, which had its antagonistic activity reduced to 57-78% at 60-90°C for 5-20 minutes and was completely inactivated at 100°C and 121°C in the shortest time tested. However, bacteriocin F1, produced by *L. paracasei* subsp. tolerans, was stable when heated to 60, 80, and 100°C for 60 minutes and 121°C for 20 minutes (Miao *et al.*, 2014).

Second, the trypsin treatment may not have been effective in inactivating potential peptide/protein compounds with antilisterial activity. Proteinaceous antagonistic substances are not always degraded by proteases. Jawan *et al*. (2020) found a bacteriocin-like inhibitory

substance, produced by *Lactococcus lactis* Gh1, was sensitive to proteinase K and resistant to trypsin. In their discussion, the authors argued that the stability of bacteriocins in the presence of proteolytic enzymes may be due to unusual amino acids in the active site structure of the peptide.

However, in other studies, BLIS was completely inactivated by treatment with proteinase K or 1 mg/mL trypsin, as in the case of the paracasin produced by *L. paracasei* SD1, (Wannun, Piwat e Teanpaisan, 2014), and purified bacteriocin plantaricin 163 produced by *L. plantarum* 163, which was completely inactivated by treatment with trypsin, proteinase K, α-chymotrypsin, and pepsin (Hu *et al.*, 2013); or partially reduced after treatment with pepsin or trypsin, such as bacteriocin F1 from *L. paracasei* subsp. tolerans (Miao *et al.*, 2014)

Third, the strong antilisterial action of the biopreservatives may have been predominantly due to the action of organic acids. The results presented and discussed in sections 3.5 and 3.6 shed light on the influence of pH and organic acids on the inhibition of the *Lm* pool. Indeed, the pH_{MI} of the tubes with lactic acid was equivalent to the MIC pH of BCPP_SP and BCPP_YE; and there was total loss of antilisterial action at concentrations of up to 10%, after total and partial inactivation of the acids with NaOH.

Fourth, possible bacteriocins present in the biopreservatives would be active at pH values different from those tested in the growth solutions, that is, below 3.59 or above 6.17. Bacteriocins produced by LABs usually exhibit antimicrobial activity over a wide range of pH values. However, the antagonistic action of some bacteriocins has only been observed at restricted pH values, such as E20 produced by *L. paracasei* CNCM I-5369, which showed effectiveness only at pH 4.5-5.0 (Belguesmia *et al.*, 2020).

The results presented throughout this work, along with the exclusion of hydrogen peroxide in the biopreservatives (as they undergo severe heat treatment before bottling), suggest that the strong antilisterial action of the biopreservatives occurs mainly due to the action of organic acids formed during the fermentative production process.

5.6 Conclusions

The BCPP_SP and BCPP_YE can be a promising natural alternative for use in meat products. The inclusion of these biopreservatives within a multi-hurdle strategy can significantly contribute to enhancing the robustness of safety and quality programs in the meat industry.

Additionally, the results of this study showed that all preservatives were capable of inhibiting *Lm in vitro*; however, in some cases, this occurred at concentrations much higher than the manufacturer's suggested limit. Although the product labels do not indicate specific use for *Lm* (except for NSDR), these results are concerning and suggest that the concentrations employed by the industry in meat-based RTE formulations may not be an effective barrier to *Lm*. However, further studies are needed, especially *in situ* evaluations, as food matrices are complex and subject to numerous physicochemical, microbiological, and sensory changes during processing and storage.

5.7 Supplementary material

Table S1. pH values of BHI broth, preservatives (NS, NSDR, SL, SLS, AR e FCSDV), and biopreservatives (BCPP_SP e BCPP_YE), undiluted and diluted in BHI broth.

Concentration	Product	Mean	SD	CI 95%
100%	BHI broth	7.20	0.05	7.09 - 7.32
1.00% w/v	NS	7.19	0.03	7.12 - 7.26
2.00% w/v	NSDR	7.17	0.03	7.10 - 7.23

% v/v	BCPP_SP			BCPP_YE		
	Mean	SD	CI 95%	Mean	SD	CI 95%
100.00	1.37	0.02	1.32 - 1.43	1.58	0.02	1.53 - 1.64
50.00	2.13	0.02	2.09 - 2.16	2.24	0.02	2.19 - 2.30
10.00	2.91	0.02	2.88 - 2.95	3.02	0.02	2.96 - 3.07
7.50	3.07	0.02	3.03 - 3.10	3.16	0.02	3.10 - 3.21
5.00	3.31	0.02	3.28 - 3.35	3.37	0.02	3.32 - 3.42
4.00	3.46	0.02	3.41 - 3.52	3.50	0.02	3.45 - 3.55
3.00	3.59	0.04	3.50 - 3.69	3.62	0.02	3.58 - 3.66
2.00	3.91	0.01	3.89 - 3.93	3.91	0.02	3.88 - 3.95
1.00	4.55	0.03	4.47 - 4.63	4.54	0.04	4.44 - 4.63
0.80	4.87	0.03	4.80 - 4.95	4.88	0.03	4.80 - 4.96
0.60	5.55	0.03	5.49 - 5.62	5.53	0.03	5.46 - 5.61
0.40	6.17	0.05	6.06 - 6.29	6.17	0.03	6.08 - 6.26

% v/v	SL			SLS			AR			FCSDV		
	Mean	SD	CI 95%	Mean	SD	CI 95%	Mean	SD	CI 95%	Mean	SD	CI 95%
100.00	7.65	0.01	7.62 - 7.68	5.86	0.01	5.85 - 5.88	5.20	0.01	5.19 - 5.22	6.42	0.01	6.39 - 6.45
50.00	7.24	0.02	7.20 - 7.27	5.29	0.02	5.26 - 5.33	5.01	0.02	4.95 - 5.06	6.09	0.03	6.02 - 6.16
37.50	7.20	0.02	7.17 - 7.24	---	---	--- - ---	---	---	--- - ---	---	---	--- - ---
15.00	7.19	0.02	7.15 - 7.22	---	---	--- - ---	---	---	--- - ---	---	---	--- - ---
10.00	7.19	0.02	7.15 - 7.23	5.41	0.01	5.38 - 5.44	5.00	0.01	4.97 - 5.03	6.17	0.01	6.14 - 6.20
5.00	7.18	0.03	7.12 - 7.25	6.15	0.02	6.10 - 6.20	5.07	0.01	5.05 - 5.08	6.58	0.01	6.55 - 6.61
2.50	7,18	0,01	7,15 - 7,21	6.67	0.03	6.60 - 6.73	5.33	0.03	5.26 - 5.39	6.92	0.03	6.85 - 6.99
1.00	7.20	0.02	7.16 - 7.23	7.00	0.02	6.96 - 7.03	6.21	0.02	6.17 - 6.24	7.17	0.03	7.11 - 7.24

* % w/v - percent weight by volume; % v/v - percent volume by volume; SD – standard deviation; CI 95% - 95% confidence interval; NS – preservative containing nisin; NSDR - preservative containing nisin, sodium diacetate and rosemary extract; BCPP_SP - biopreservatives containing potential postbiotics with isolated soy protein; BCPP_YE - biopreservatives containing potential postbiotics with yeast extract; SL - preservative containing sodium lactate (60%); SLS - preservative containing sodium lactate (60%) and liquid smoke; AR - preservative containing sodium lactate, vinegar powder, sodium citrate, and citric acid; FCSDV - preservative containing dry vinegar and fermented cane sugar.

Table S2. Minimum Inhibitory Concentration (MIC) at pH 6.0, observed at 24, 48, 72 and 96 hours.

Preservative	Time (hours)	*Listeria monocytogenes* Pool
LS (sodium lactate 60%)	24	5.01
	48	6.67
	72	6.67
	96	6.67
SLS (sodium lactate (60%) and liquid smoke)	24	5.63
	48	7.50
	72	7.50
	96	7.50
FCSDV (fermented cane sugar and dry vinegar)	24	3.16
	48	5.63
	72	5.63
	96	5.63
AR (sodium lactate, vinegar powder, sodium citrate, and citric acid)	24	3.16
	48	3.16
	72	3.16
	96	3.16
NSDR (nisin, sodium diacetate and rosemary extract)	24	0.08
	48	0.10
	72	0.10
	96	0.10
NS (nisin)	24	0.01
	48	0.02
	72	0.04
	96	0.04

***** MIC expressed in percentage concentrations of preservatives (%). NS concentrations (% NS - mg nisin/L): 0.01-2.50, 0.02-5.00, and 0.04-10.00.

Table S3. Minimum Inhibitory pH (pH_{MI}) and Minimum Listericidal pH (pH_{ML}), observed at 24, 48, 72 and 96 hours.

Acid	Time (hours)	pH_{MI}	pH_{ML}
Hydrochloric acid (HCl)	24	4.5	4.0
	48	4.5	3.5
	72	4.5	3.5
	96	4.5	3.5
Lactic acid ($C_3H_6O_3$)	24	5.0	4.0
	48	4.5	4.0
	72	4.5	4.0
	96	4.5	4.0

***** pH_{MI} and pH_{ML} investigate against the *Listeria monocytogenes* Pool.

Figure S1. Effect of lactic acid on the pool of Lm strains. Tubes acidified with lactic acid (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0). pH_{MI} 4.5 (1st photo) and pH_{ML} 4.0 (2nd photo). Photos taken at 48 hours. The turbidity gradient observed in the first photo suggests the influence of lactic acid on the population dynamics of the *Lm* pool.

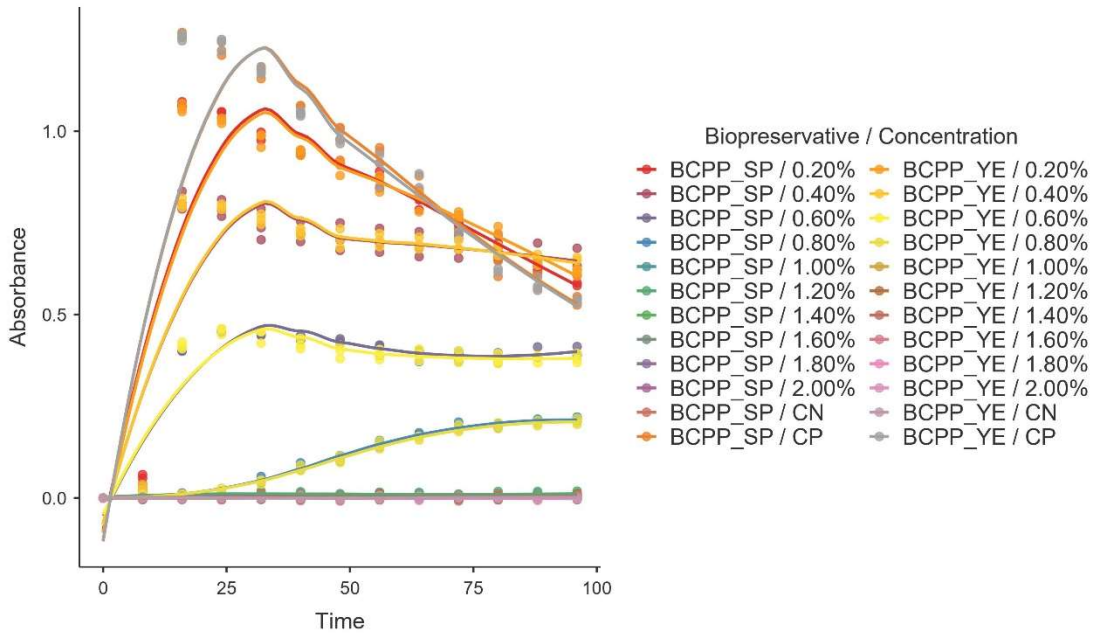

Figure S2. Scatterplot. Population dynamics of the *Lm* pool at different concentrations of BCPP_SP and BCPP_YE. Triplicate data on absorbance variation over time. Software: Jamovi version 2.3.

6 CHAPTER II - Biopreservatives containing potential postbiotics: *in silico* evaluation of the effectiveness of immersion application for controlling *Listeria monocytogenes* and extending the shelf life of vacuum-packed cooked sausage.

CHAPTER II

BIOPRESERVATIVES CONTAINING POTENTIAL POSTBIOTICS: *IN SILICO* EVALUATION OF THE EFFECTIVENESS OF IMMERSION APPLICATION FOR CONTROLLING *LISTERIA MONOCYTOGENES* AND EXTENDING THE SHELF LIFE OF VACUUM-PACKED COOKED SAUSAGE.

BIOCONSERVANTES CONTENDO POTENCIAIS PÓS-BIÓTICOS: AVALIAÇÃO *IN SILICO* DA EFICÁCIA DA APLICAÇÃO POR IMERSÃO NO CONTROLE DE *LISTERIA MONOCYTOGENES* E PROLONGAMENTO DA VIDA ÚTIL DE LINGUIÇAS COZIDAS EMBALADAS A VÁCUO

Bioconservantes contendo potenciais pós-bióticos: Avaliação *in silico* da eficácia da aplicação por imersão no controle de *Listeria monocytogenes* e prolongamento da vida útil de linguiças cozidas embaladas a vácuo.

6.1 Resumo

Os produtos à base de carne são altamente suscetíveis à ação microbiana e necessitam de medidas de controle para prevenir a deterioração prematura e riscos ao consumidor. *Listeria monocytogenes* (*Lm*) é o agente patogênico causador de listeriose, uma doença grave com altas taxas de hospitalização e mortalidade. Neste estudo, linguiças cozidas embaladas a vácuo (LCEV) foram intencionalmente contaminadas por um pool de cepas de *Lm* para simular uma contaminação pós processamento térmico extrema. As LCEV contaminadas foram submetidas a tratamentos de imersão de curta duração (1 minuto) com um bioconservante contendo pós-bióticos potenciais (BCPP_YE) e controles. A eficácia dos tratamentos foi avaliada em relação a *Lm*, bactérias ácido láticas (BAL) e contagem total de bactérias (CTB). A vida de prateleira das LCEV foi estimada em diferentes perfis de temperatura com auxílio do software de modelagem preditiva MicroLab_Shelf-Life. Os tratamentos por imersão em BCPP_YE apresentaram efeito bactericida, sendo capazes de reduzir carga microbiana inicial das LCEV. Todavia, não foram capazes de impedir o crescimento de *Lm*, BAL e CTB em temperaturas mais elevadas. Os resultados preditivos revelaram que a manutenção da temperatura de refrigeração a 7ºC foi um fator de barreira eficiente para controlar a população de *Lm* por mais de 180 dias e estender a vida de prateleira das LCEV por até 135 dias. Bioconservantes como o BCPP_YE podem ajudar a reduzir o crescimento microbiano em produtos cárneos prontos para o consumo quando associados a outras medidas de controle. Este trabalho corrobora os resultados apresentados em pesquisas anteriores e reforça o potencial do BCPP_YE como conservante para produtos cárneos.

Palavras chaves: listeriose, bactérias ácido láticas, teste de durabilidade, microbiologia preditiva, deterioração de alimentos.

Biopreservatives containing potential postbiotics: *in silico* evaluation of the effectiveness of immersion application for controlling *Listeria monocytogenes* and extending the shelf life of vacuum-packed cooked sausage.

6.2 Abstract

Meat-based products are highly susceptible to microbial action and require control measures to prevent premature spoilage and risks to consumers. *Listeria monocytogenes* (*Lm*) is the pathogenic agent causing listeriosis, a severe disease with high rates of hospitalization and mortality.

In this study, vacuum-packed cooked sausages (VPCS) were intentionally contaminated with a pool of *Lm* strains to simulate extreme post-thermal processing contamination. The contaminated VPCS were subjected to short-duration immersion treatments (1 minute) with a biopreservative containing potential postbiotics (BCPP_YE) and controls. The efficacy of the treatments was evaluated in relation to *Lm*, lactic acid bacteria (LAB), and total bacterial count (TBC). The shelf life of the VPCS was estimated under different temperature profiles using the predictive modeling software MicroLab_Shelf-Life. The immersion treatments with BCPP_YE exhibited a bactericidal effect, being able to reduce the initial microbial load of VPCS. However, they were not able to prevent the growth of Lm, LAB, and TBC at higher temperatures. Predictive results revealed that maintaining refrigeration temperature at 7°C was an effective barrier factor to control the population of *Lm* for more than 180 days and to extend the shelf life of VPCS up to 135 days. Biopreservatives like BCPP_YE can help reduce microbial growth in ready-to-eat meat products when combined with other control measures. This work corroborates the results presented in previous research and reinforces the potential of BCPP_YE as a preservative for meat products.

Keywords: listeriosis, lactic acid bacteria, durability test, predictive microbiology, food spoilage.

6.3 Introduction

Foodborne illnesses are a constant concern for processing industries and public health agencies worldwide. Among the most concerning pathogens is *Listeria monocytogenes* (*Lm*), the bacterium responsible for listeriosis. Listeriosis is a severe infection that can result in septicemia, meningitis, and other complications that can lead to death, posing a particular danger to pregnant women, newborns, the elderly, and individuals with compromised immune systems (CDC, 2021; FDA, 2012).

Lm is recognized as a challenging microorganism for the food industry due to its ability to grow at refrigeration temperatures, in the absence of oxygen, and tolerate adverse processing and storage conditions. *Lm* can also form biofilms on equipment and other surfaces in processing facilities, making it difficult to eliminate during cleaning and disinfection processes. Additionally, it can easily spread throughout the manufacturing environment, leading to cross-contamination of processed foods. Therefore, efforts should not be spared in its control, especially in ready-to-eat (RTE) foods. In this category of foods, there is no indication for the need of effective thermal treatment or other processes to eliminate or reduce pathogens to safe levels before consumption. If the bacteria find favorable conditions for multiplication, which is very likely in various types of RTE foods, contamination levels high enough to cause listeriosis can occur, particularly in individuals with weakened immune systems (Cruz *et al.*, 2008b; EURL Lm, 2021; ILSI, 2005).

Meat-based RTE foods have frequently been associated with cases of listeriosis. Additionally, a high percentage of samples from the industry or collected at retail by regulatory agencies have tested positive for *Lm* when analyzed (EFSA e ECDC, 2022). These data raise an alarm and demonstrate that, despite constant investments and technological advancements in the meat industry, compliance with microbiological quality and safety standards throughout the product's shelf life is often not achieved.

This situation can be exacerbated when meat-based RTE foods are subject to fractioning at retail and/or temperature abuse during transport and storage. Such occurrences are not uncommon. For example, in some regions of Brazil, products like vacuum-packed cooked sausages (VPCS) and bacon are traditionally sold at room temperature, displayed on counters or shelves without refrigeration. Figure 1 illustrates temperature abuse, inadequate fractioning, and exposure to contaminating factors of meat products in a large retail supermarket in Rio de Janeiro.

Therefore, additional measures should be researched to contribute to mitigating consumer health risks in a multi-hurdle strategy.

Preservatives produced from broth media fermented by lactic acid bacteria, thermally treated, without isolation and purification of antimicrobial substances, and containing potential postbiotics have been reported as a promising and relatively low-cost alternative to prevent undesirable microorganisms in meat products (Jaramillo *et al.*, 2018; Lima *et al.*, 2022). However, evaluations testing the most effective application method of these products are scarce.

Short-duration immersion antimicrobial treatments have been presented in other works (Bodie *et al.*, 2022; Geornaras *et al.*, 2006) as an alternative for applying preservatives to

sausages before packaging. This approach was designed to take advantage of the ice bath cooling step that occurs after sausage cooking. This would be a practical way to include preservatives in a multi-hurdle approach to the production of cooked sausages.

Fermentations by lactic acid bacteria can produce a variety of cellular structures and metabolites, such as cell surface components, lactic acid, short-chain fatty acids, and bioactive peptides, among other metabolites with potential antimicrobial action (Salminen *et al.*, 2021).

The biopreservative BCPP_YE is produced by fermentation of a broth medium from a propagated culture of *Lacticaseibacillus paracasei* DTA 83. This strain, isolated from newborn stool, was identified by 16S rDNA sequencing using RAPD-PCR (Guerra *et al.*, 2018) and integrated into the culture collection of the food microbiology laboratory-DTA-UFRRJ. The complete genome data were deposited in GenBank under the accession number QRBH00000000 (Lemos-Junior, Fioravante Guerra, *et al.*, 2019).

L. paracasei DTA 83 was classified with GRAS (generally recognized as safe) status, characterized as potentially probiotic (Lemos-Junior, Guerra, *et al.*, 2019), and reported to have the potential to release postbiotic compounds (Oliveira *et al.*, 2021; Silva *et al.*, 2021). This strain was able to biocontrol the growth of *L. innocua*, *Salmonella Typhimurium*, *Candida albicans*, and *Escherichia coli* even after partial reduction of cell viability due to gastrointestinal transit stress (Tarrah *et al.*, 2019), and demonstrated good results in controlling the natural microbiota of VPCS when compared to sodium lactate (Lima *et al.*, 2022).

This study aimed to verify, through *in situ* tests and predictive evaluations, whether BCPP_YE would be effective in extending shelf life and controlling *Lm* when applied by short-duration immersion in VPCS.

Figure 1. Photographs taken on 10/05/2024. Temperature abuse, inadequate fractioning, and exposure to contaminating factors of meat products in a large retail supermarket in metropolitan region of Rio de Janeiro.

6.4 Materials and methods

6.4.1 Biosafety

The experiments described in this study were conducted in strict adherence to laboratory biosafety regulations, ensuring the health protection of researchers and the proper handling of biological materials (Ministério da Saúde, 2006).

6.4.2 Vacuum-packed cooked sausage (VPCS)

Samples of Calabrese-type sausages, cooked and vacuum-packed (VPCS), were obtained from local commerce. The 2.5 kg, intact, and refrigerated packages were kept at 6ºC±2ºC until the *in situ* test. All samples were from the same production batch.

6.4.3 Microbial culture used in the production of BCPP_YE

The biopreservative BCPP_YE was produced by fermentation from the homogeneous culture of *L. paracasei* DTA 83.

6.4.4 Production of BCPP_YE

The production of BCPP_YE was carried out on a pilot industrial scale at the BRC Ingredients Ltda. facility in Rio Claro, SP, Brazil, as described by Lima *et al*. (2022). A formulation prepared with food-grade ingredients, mimicking the nutrients found in MRS broth but without the addition of polysorbate 80 (Tween 80), was used as the fermentation medium base. The nutrient broth was thermally treated in a 330 L stirred tank bioreactor with automatic temperature control and a pH meter. A propagated culture of *L. paracasei* DTA 83 was added for fermentation. The process, conducted at 36°C for approximately 72 hours, was accompanied by a pH drop to about 3.5. The fermentation was halted with thermal treatment at 95°C for 5 minutes. The BCPP_YE was hot-bottled in 10 L polypropylene containers. The presence of viable cells of *L. paracasei* DTA 83, and/or contaminants, was investigated by plate counts on MRS agar, plate count agar (PCA), and potato dextrose agar acidified to pH 3.5 with tartaric acid. Figure 2 illustrates the manufacturing process of BCPP_YE.

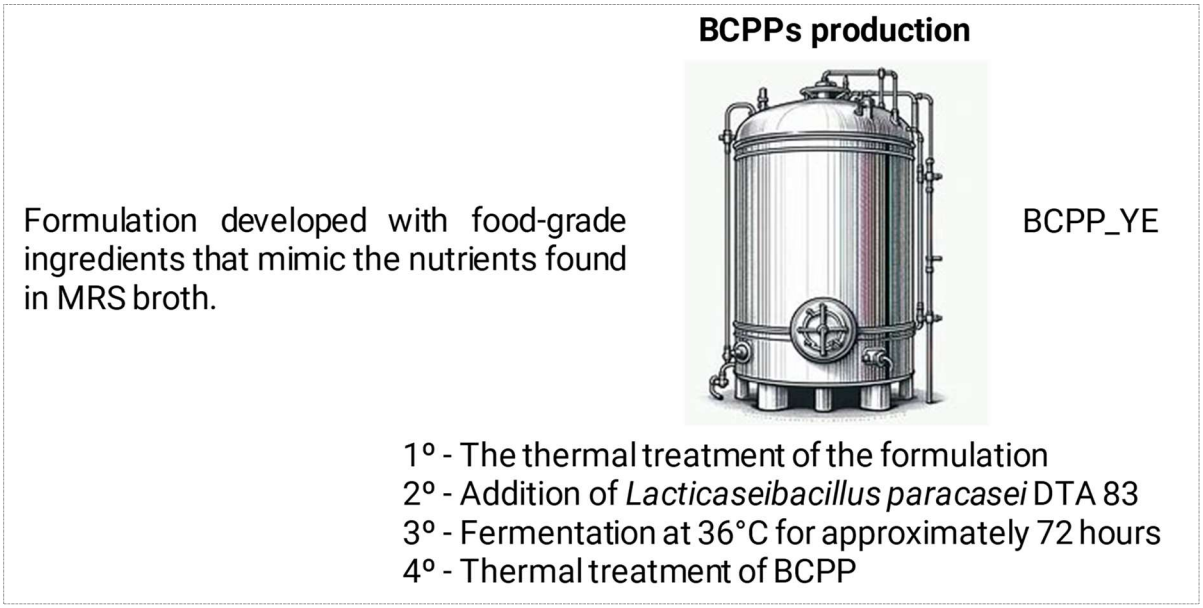

Figure 2. Production of BCPP_YE.

6.4.5 *Listeria monocytogenes* strains and inoculum preparation

The *Lm* inoculum was prepared by combining six strains of *Lm*, five of which were isolated from processed meat products (CLIST 4165 - serotype 1/2a, CLIST 4396 - serotype 1/2b, CLIST 4405 - serotype 1/2a, CLIST 4642 - serotype 1/2b, and CLIST 4645 - serotype 1/2c), and one reference strain (CLIST 3436 (Scott A) - serotype 4b), all sourced from the Listeria Collection (CLIST) at the Bacterial Zoonoses Laboratory (LABZOO) of the Oswaldo Cruz Foundation (FIOCRUZ).

The *Lm* strains were cultured three times in BHI broth (Kasvi - Spain), and the third subculture (14-16 hours) was diluted 1:2, 1:5, 1:10, 1:25, and 1:50 in BHI broth. The absorbance of each dilution was read at 600 nm in a spectrophotometer (model UV-M51 UV-VIS from

BEL Engineering - Italy) and simultaneously subjected to colony counting on BHI agar plates (Kasvi - Spain). Standard growth curves were obtained by correlating absorbance with colony count (CFU/mL) using simple linear regression.

Individual *Lm* inocula were prepared in BHI broth, adjusting the concentration of the cell suspension obtained after 14-16 hours of the third subculture to approximately 10^8 CFU/mL using the spectrophotometer at 600 nm. The *Lm* pool was obtained by mixing equal aliquots of individual *Lm* inocula. The purity of the inocula and confirmation of cell concentration were verified by plating on Listeria agar according to Ottaviani and Agosti (ALOA) from Himedia (India) without addition of inhibitors. Plates were incubated at 36°C for 24/48 h, followed by colony counting and morphological evaluation. The inoculum preparation scheme is presented in Figure 3.

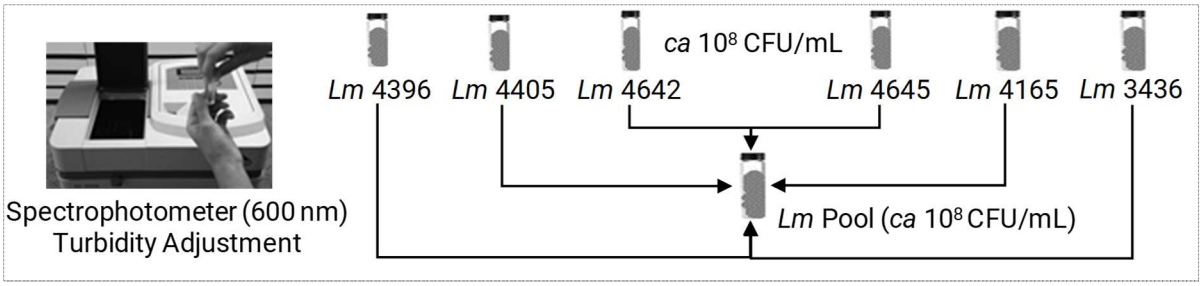

Figure 3. The preparation of the *Listeria monocytogenes* (*Lm*) pool was conducted using individually adjusted *Lm* suspensions (approximately 10^8 CFU/mL) in a spectrophotometer.

6.4.6 Preparation of sample and inoculation with the pool de *Lm*

Samples of VPCS weighing 100±0.5 g were obtained by transversely cutting the sausages with a sterile scalpel. This method exposed the cooked sausage mass without the cellulose casing on the cut side.

The *Lm* pool (*ca* 10^8 CFU/mL) was diluted in peptone water until a standardized inoculum containing *ca* 10^4 CFU/mL was obtained.

The VPCS samples were placed on a pre-sterilized stainless steel tray (Figure 4). 1 mL of the standardized inoculum was evenly distributed over each sample using a pipette and disposable spreader, to achieve a final concentration of *ca* 10^2 CFU/g of *Lm*. The tray was covered with two layers of PVC film and kept refrigerated at 6ºC±2ºC for 1 hour to allow the *Lm* inoculum to adhere.

Figure 4. Samples of vacuum-packed cooked sausages inoculated with the pool of *Listeria monocytogenes*.

6.4.7 Application of the biopreservative

The application of BCPP_YE to the VPCS samples was carried out using a short-duration immersion method, as suggested in other studies (Bodie *et al.*, 2022; Geornaras *et al.*, 2006), with some modifications.

In summary, the VPCS samples were individually submerged in a beaker containing 500 mL of diluted BCPP_YE (1% or 5%). Controls were prepared using 500 mL of sterile distilled water. The immersion time was 1 minute.

6.4.8 Microbiological Analyses

The counts of *Lm*, lactic acid bacteria (LAB), and total bacterial count (TBC) were performed according to the following standards: ABNT NBR ISO 11290-2:2020 (ABNT, 2020), e ISO 15214:1998 e ABNT NBR ISO 4833-2:2015 (ABNT, 2015).

6.4.9 Experimental design

The BCPP_YE was tested *in situ* at concentrations of 1% and 5%, with the assays conducted on different days. Each assay comprised 3 treatments: T1: VPCS samples individually immersed in BCPP_YE solution (x)% for 1 minute (x = 1% or 5%), T2: VPCS samples individually immersed in sterile distilled water for 1 minute, and T3: Control without immersion (VPCS samples inoculated with *Lm* only). The samples were randomly assigned to each treatment.

Each treatment consisted of 5 samples: one analyzed at time zero and the others incubated in pairs at temperatures of 7°C and 30°C. These samples were analyzed on different days for Lm, LAB, and TBC, as shown in Table 1.

A photo of the vacuum-packed samples, after incubation (9 days at 7ºC), can be seen in Figure 5.

Tabe 1. *In situ*. Treatments, temperatures, and incubation times of the VPCS samples.

Sample	Treatment	Incubation	
S_1	T_1 - **Treatment with BCPP_YE**. Immersion of VPCS samples, previously inoculated with the *Lm* pool, in a BCPP_YE solution at (x)% for 1 minute (x = 1% or 5%).	---	---
S_2		30ºC	3 days
S_3		30ºC	7 days
S_4		7ºC	5 days
S_5		7ºC	9 days
S_6	T_2 - **Water control**. Immersion of VPCS samples, previously inoculated with the *Lm* pool, in sterile distilled water for 1 minute.	---	---
S_7		30ºC	3 days
S_8		30ºC	7 days
S_9		7ºC	5 days
S_{10}		7ºC	9 days
S_{11}	T_3 - **No immersion control**. Only VPCS samples inoculated with the Lm pool and vacuum-packed.	---	---
S_{12}		30ºC	3 days
S_{13}		30ºC	7 days
S_{14}		7ºC	5 days
S_{15}		7ºC	9 days

* All samples were individually vacuum-packed after the treatments.

Figure 5: Vacuum-packed samples. Time = 9 days (7ºC). From left to right: control sample (without immersion), sample immersed in sterile distilled water, and sample immersed in BCPP_YE 5%.

6.4.10 Efficacy and Durability Study

The predictive modeling software, MicroLab_Shelf-Life, was used to estimate the shelf life of VPCS under different temperature profiles. The profiles were: 7ºC, 12ºC, 22ºC, and 30ºC.

The end of shelf life for *Lm* was established when the population reached 3.3 log CFU/g (WHO, 2022), for BAL when the population reached 6.0 log CFU/g, and for CTB when the total microbial count reached 9.33 log CFU/g (Guerra *et al.*, 2023).

The samples were incubated and analyzed as described in section 2.6.4 (experimental design). The results of the microbiological analyses were input into MicroLab_Shelf-Life, and the predictive simulations were organized into a table.

6.4.11 Statistical Analyses

The computational predictive modeling package, MicroLab_Shelf-Life, was used to analyze the results of the microbiological analyses and to predict the shelf life of LCEV. The software was also used to evaluate the effect of temperature associated with the treatments—immersion in BCPP_YE 1% or 5%, immersion in sterile distilled water, and no immersion treatment (control), on the shelf life of VPCS.

6.5 Results and Discussion

Counts of the *Lm*, BAL, and TBC for the treatments involving immersion in BCPP_YE 1% and BCPP_YE 5%, along with their respective controls (immersion in sterile distilled water and no immersion control) are shown in Figures 6 and 7. The times considered were t = 0 days, t = 3 and 7 days for samples incubated at 30ºC, and t = 5 and 9 days for samples incubated at 7ºC.

Tables 4 and 5 present the predictive evaluation results for the treatments involving immersion in BCPP_YE 1% and BCPP_YE 5%, with their respective controls.

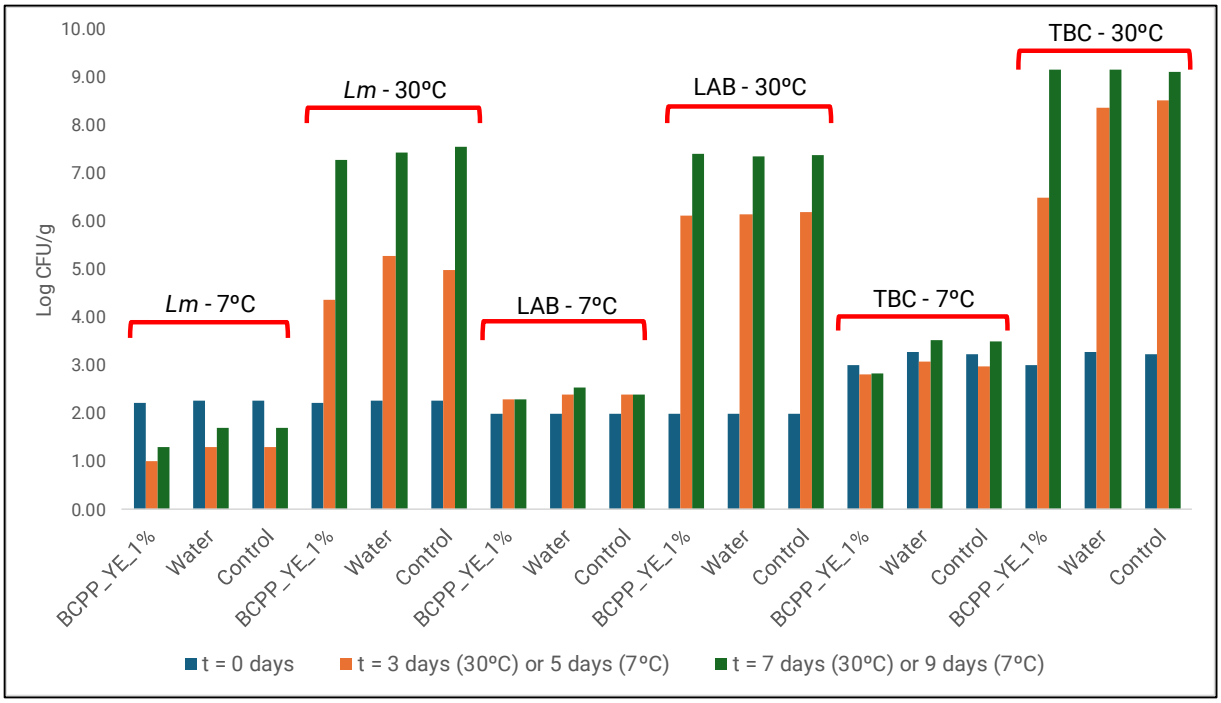

Figure 6: Counts of *Listeria monocytogenes* (*Lm*), lactic acid bacteria (LAB), and total bacterial counts (TBC) at times t = 0 days and at times t = 3 and 7 days for samples incubated at 30ºC, and at times t = 5 and 9 days for samples incubated at 7ºC. Treatments: immersion in BCPP_YE 1%, immersion in sterile distilled water, and control without immersion.

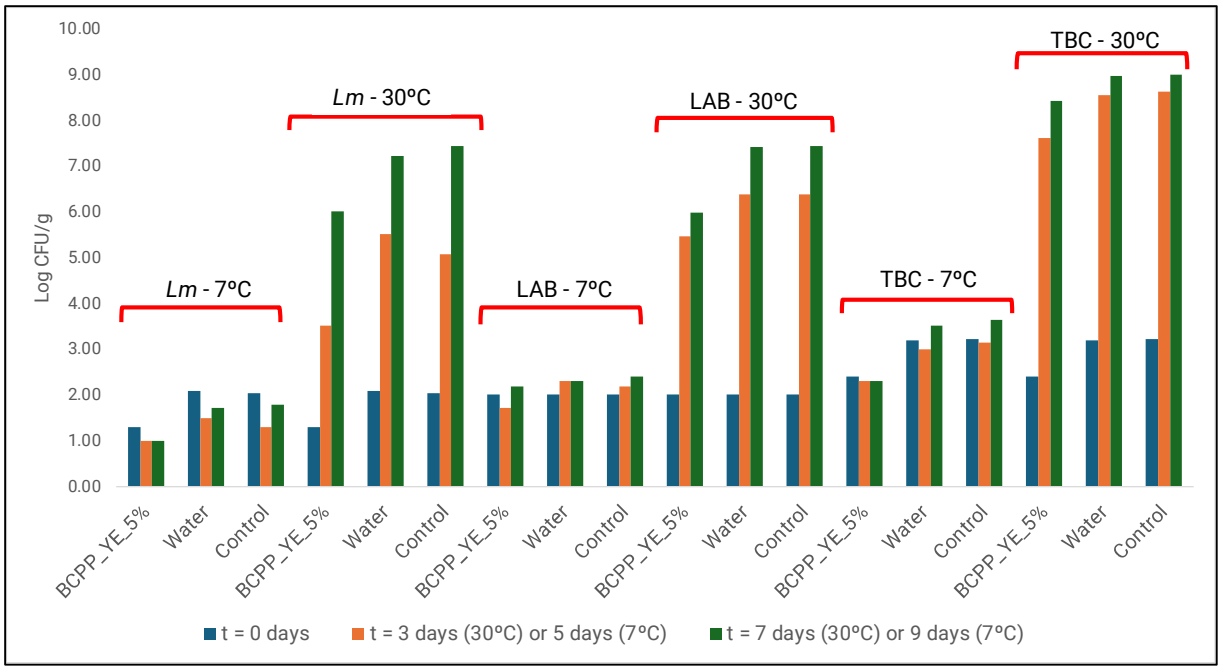

Figure 7: Counts of *Listeria monocytogenes* (*Lm*), lactic acid bacteria (LAB), and total bacterial counts (TBC) at times t = 0 days and at times t = 3 and 7 days for samples incubated at 30ºC, and at times t = 5 and 9 days for samples incubated at 7ºC. Treatments: immersion in BCPP_YE 5%, immersion in sterile distilled water, and control without immersion.

Despite the immersion treatments with the biopreservative not being effective in controlling microbial populations at higher temperatures (reaching the maximum stipulated limit well before the expiration date – as verified in the predictive analysis), there was a reduction in microbial counts compared to treatments with immersion in sterile distilled water and the control without immersion, as observed in Tables 2 and 3.

The immersion treatment in 5% BCPP_YE showed a strong bactericidal effect. At the first hour (time zero), there was a reduction of 81.82% in the *Lm* population and 84.85% in TBC, compared to the control without immersion. The reduction in the 1% treatment was 10.53% for *Lm* and 41.18% for TBC. At time zero, the immersion in water and the control without immersion showed similar counts. The lower microorganism count observed at time zero compared to the control was also verified in samples incubated at 7ºC and 30ºC.

Other studies have also reported a decrease in the initial microbial load of sausages subjected to immersion treatments in 2.5% acetic acid solution, 2.5% lactic acid, 5% potassium benzoate, 0.5% nisin, and combinations of antimicrobials. The immersion was performed for 2 minutes, and the initial reduction varied from 1 to 1.8 log CFU/cm2, reaching 3.8 log CFU/cm2 in treatments combined with nisin, depending on the origin of the inoculum. However, significant growth was observed in all treatments during storage at 10ºC (Geornaras *et al.*, 2006).

The assays conducted in this work, compared to tests performed on sausages by other authors, present an additional challenging factor: the exposed meat mass on the cut side and the less “smooth” cellulose casing. However, reductions of 0.74 log CFU/g were obtained in the

treatment with 5% BCPP_YE, indicating the potential of this biopreservative for application in meat products.

Satisfactory results regarding the extension of the shelf life of meat products can be achieved by reducing the initial microbial load. In this sense, the immersion of VPCS in BCPP_YE before vacuum packaging could contribute as an additional barrier factor.

Despite the reduction in microbial load compared to controls, the treatments with 1% and 5% BCPP_YE did not show sufficient bacteriostatic effect to prevent the increase of Lm, LAB, and TBC populations at higher temperatures. The high growth of these microorganisms, especially those favored by higher temperatures, can lead to health risks and/or the appearance of spoilage attributes before the expiration date stipulated by the manufacturer (Freiberger *et al.*, 2016; Kolbeck *et al.*, 2020).

When compared to their respective controls, the 5% BCPP_YE immersion treatment showed better results than 1% in terms of the percentage reduction of microbial load. This greater difference was maintained at subsequent sampling times, probably due to the higher initial reduction observed at time zero. However, this difference did not impact resilience in days, as verified in the predictive model. Resilience in days indicates the estimated number of days until the microbial population reaches the stipulated limit.

The predictive results revealed that maintaining the refrigeration temperature at 7°C was an effective barrier factor to control the *Lm* population for more than 180 days and to extend the shelf life of the VPCS for up to 135 days. Regarding the *Lm* counts in the samples incubated at 7°C, there was a reduction in the population between time zero (day of inoculation) and times 5 and 9. In the control without immersion, the average reduction of *Lm* compared to time zero was 86.67% (5 days) and 63.33% (9 days).

Lm are psychrotrophic bacteria, with an optimal growth temperature between 30 and 37°C. Thus, other barrier effects may have contributed to maintaining the limit of 3.3 log CFU/g for the 180-day refrigerated period. Stress factors, such as the abrupt change in environment and growth temperature (from BHI broth at 37°C to VPCS at 7°C), combined with competition with the indigenous microbiota of the VPCS, may explain the population decrease of *Lm*.

In fact, the LAB population grew under the same incubation conditions. LAB are recognized as potential producers of bacteriocins with antilisterial activity (Stupar *et al.*, 2021; Wiernasz *et al.*, 2020). Furthermore, the remaining indigenous microbiota may also have had a competitive effect with *Lm*. These data demonstrate that the growth results observed in the controlled laboratory environment can be affected by the complex ecological interactions present in the in situ food environments (Geornaras *et al.*, 2006).

Biopreservatives such as BCPP_YE can help reduce microbial growth in ready-to-eat meat products, such as VPCS, minimizing the risks associated with pathogens and extending the shelf life. However, the addition strategy must be carefully designed (Lima *et al.*, 2022).

In another study (Lima *et al.*, 2022), the addition of 1.00% biopreservative (PPCP) in the VPCS mass showed a result similar to the addition of 2.00% sodium lactate to control the natural microbiota of VPCS. The same concentrations applied inside the packages did not show effective results compared to blank and control. However, only treatments with 3.0% PPCP on

the surface or 2.0% or more PPCP in the sausage mass during winter reached the proposed shelf life. Likewise, cold temperature was a predominant factor in controlling the indigenous microbiota growth of the sausages.

Table 2. Percentage reduction of the population of *Listeria monocytogenes*, lactic acid bacteria, and total bacterial counts from treatments by immersion in BCPP_YE 1% compared to treatments by immersion in sterile distilled water and control without immersion.

Incubation	Count /	*Listeria monocytogenes*			Lactic acid bacteria			Total bacterial counts		
	% reduction	BCPP_YE 1%	Water	Control	BCPP_YE 1%	Water	Control	BCPP_YE 1%	Water	Control
t0	Log CFU/g	2.23	2.28	2.28	< 2.00	< 2.00	< 2.00	3.00	3.27	3.23
	% reduction*	---	10.53%	10.53%		0.00%	0.00%		45.95%	41.18%
t = 3 days (30ºC)	Log CFU/g	4.37	5.29	4.97	6.11	6.15	6.19	6.48	8.37	8.51
	% reduction*		88.09%	75.01%		9.25%	16.94%		98.73%	99.06%
t = 7 days (30ºC)	Log CFU/g	7.28	7.42	7.54	7.40	7.34	7.37	9.16	9.14	9.10
	% reduction*		28.49%	45.07%		-13.64%	-6.38%		-5.07%	-14.62%
t = 5 days (7ºC)	Log CFU/g	1.00	1.30	1.30	2.30	2.40	2.40	2.81	3.08	2.98
	% reduction*		50.00%	50.00%		20.00%	20.00%		45.83%	31.58%
t = 9 days (7ºC)	Log CFU/g	1.30	1.70	1.70	2.30	2.54	2.40	2.85	3.54	3.51
	% reduction*		60.00%	60.00%		42.86%	20.00%		79.71%	78.46%

* Percentage reduction of the treatment with BCPP_YE 1% compared to treatments by immersion in sterile distilled water and control without immersion.

Table 3. Percentage reduction of the population of *Listeria monocytogenes*, lactic acid bacteria, and total bacterial counts from treatments by immersion in BCPP_YE 5% compared to treatments by immersion in sterile distilled water and control without immersion.

Incubation	Count /	*Listeria monocytogenes*			Lactic acid bacteria			Total bacterial counts		
	% reduction	BCPP_YE 5%	Water	Control	BCPP_YE 5%	Water	Control	BCPP_YE 5%	Water	Control
t0	Log CFU/g	1.30	2.08	2.04	< 2.00	< 2.00	< 2.00	2.40	3.20	3.22
	% reduction*		83.33%	81.82%		0.00%	0.00%		84.38%	84.85%
t = 3 days (30ºC)	Log CFU/g	3.51	5.51	5.07	5.47	6.38	6.37	7.61	8.56	8.63
	% reduction*		99.02%	97.26%		87.81%	87.45%		88.61%	90.35%
t = 7 days (30ºC)	Log CFU/g	6.01	7.20	7.44	5.97	7.41	7.43	8.43	8.96	8.98
	% reduction*		93.64%	96.31%		96.38%	96.52%		70.17%	71.73%
t = 5 days (7ºC)	Log CFU/g	<1.00	1.48	1.30	1.70	2.30	2.18	2.30	3.00	3.13
	% reduction*		66.67%	50.00%		75.00%	66.67%		80.00%	85.19%
t = 9 days (7ºC)	Log CFU/g	1.00	1.70	1.78	2.18	2.30	2.40	2.30	3.52	3.63
	% reduction*		80.00%	83.33%		25.00%	40.00%		93.94%	95.29%

* Percentage reduction of the treatment with BCPP_YE 5% compared to treatments by immersion in sterile distilled water and control without immersion.

Table 4. MicroLab_Shelf-Life software. Durability testing under four temperature profiles: 7ºC (refrigeration), 12ºC, 22ºC, and 30ºC. Treatments: immersion in BCPP_YE 1%, immersion in sterile distilled water, and control without immersion.

	Listeria monocytogenes (log CFU/g)			Lactic acid bacteria (log CFU/g)			Total bacterial counts (log CFU/g)		
Incubation	Water	Control	BCPP_YE 1%	Water	Control	BCPP_YE 1%	Water	Control	BCPP_YE 1%
0 days	2.28	2.28	2.23	2.00	2.00	2.00	3.23	3.27	3.00
7°C / 5 days	1.30	1.30	1.00	2.40	2.40	2.30	2.98	3.08	2.81
7°C / 9 days	1.70	1.70	1.30	2.40	2.54	2.30	3.51	3.54	2.85
30°C / 3 days	4.97	5.29	4.37	6.19	6.15	6.11	8.51	8.37	6.48
30°C / 7 days	7.54	7.42	7.28	7.37	7.34	7.40	9.10	9.14	9.16
Simulation	Ngrowth (log CFU/g/days)*								
7ºC	-0.3554	-0.2427	-0.2427	0.1393	0.1758	0.1054	0.0451	0.0489	-0.0574
12ºC	0.0155	0.1507	0.1418	0.5272	0.5546	0.4968	0.5178	0.5137	0.3679
22ºC	0.7574	0.9376	0.9108	1.3031	1.3120	1.2798	1.4633	1.4432	1.2186
30ºC	1.3509	1.5672	1.5261	1.9238	1.9180	1.9061	2.2196	2.1869	1.8992
Simulation	Ndeceleration (log CFU/g/days)**								
7ºC	-0.2349	-0.1604	-0.1604	0.0920	0.1162	0.0696	0.0298	0.0323	-0.0380
12ºC	0.0080	0.0772	0.0726	0.2699	0.2839	0.2544	0.2651	0.2630	0.1884
22ºC	0.2673	0.3310	0.3215	0.4599	0.4631	0.4517	0.5165	0.5094	0.4301
30ºC	0.3819	0.4431	0.4314	0.5439	0.5423	0.5389	0.6275	0.6183	0.5369
Simulation	Resilience (days)***								
7ºC	> 180	> 180	> 180	29	23	38	147	135	> 180
12ºC	8	7	9	8	8	9	14	14	20
22ºC	2	2	2	4	4	4	5	5	6
30ºC	1	1	1	3	3	3	3	3	5

* Daily growth of microbial population (log CFU/g) in phase L

** Daily growth of microbial population (log CFU/g) in phase D.

*** Resilience for *Listeria monocytogenes* was established when the population reached 3.3 log CFU/g (WHO, 2022). Resilience for lactic acid bacteria was established when the population reached 6.0 log CFU/g. Shelf-life was established when the total microbial count reached 9.33 log CFU/g (Guerra *et al.*, 2023).

Table 5. MicroLab_Shelf-Life software. Durability testing under four temperature profiles: 7ºC (refrigeration), 12ºC, 22ºC, and 30ºC. Treatments: immersion in BCPP_YE 5%, immersion in sterile distilled water, and control without immersion.

	Listeria monocytogenes (log CFU/g)			Lactic acid bacteria (log CFU/g)			Total bacterial counts (log CFU/g)		
Incubation	Water	Control	BCPP_YE 5%	Water	Control	BCPP_YE 5%	Water	Control	BCPP_YE 5%
0 days	2.04	2.08	1.30	2.00	2.00	2.00	3.22	3.20	2.40
7°C / 5 days	1.30	1.48	1.00	2.18	2.30	1.70	3.13	3.00	2.30
7°C / 9 days	1.78	1.70	1.00	2.40	2.30	2.18	3.63	3.52	2.30
30°C / 3 days	5.07	5.51	3.51	6.37	6.38	5.47	8.63	8.56	7.61
30°C / 7 days	7.44	7.20	6.01	7.43	7.41	5.97	8.98	8.96	8.43
Simulation	Ngrowth (log CFU/g/days)*								
7ºC	-0.1398	-0.1553	-0.1054	0.1171	0.1054	0.0139	0.0940	0.0582	-0.0339
12ºC	0.2474	0.2403	0.2106	0.5194	0.5096	0.3323	0.5594	0.5268	0.4568
22ºC	1.0219	1.0315	0.8426	1.3241	1.3179	0.9692	1.4902	1.4640	1.4381
30ºC	1.6415	1.6644	1.3482	1.9678	1.9647	1.4786	2.2349	2.2138	2.2232
Simulation	Ndeceleration (log CFU/g/days)**								
7ºC	-0.0924	-0.1026	-0.0696	0.0774	0.0696	0.0092	0.0621	0.0385	-0.0224
12ºC	0.1267	0.1230	0.1078	0.2659	0.2609	0.1702	0.2864	0.2697	0.2339
22ºC	0.3607	0.3641	0.2974	0.4674	0.4652	0.3421	0.5260	0.5168	0.5076
30ºC	0.4641	0.4705	0.3812	0.5563	0.5554	0.4180	0.6318	0.6259	0.6285
Simulation	Resilience (days)***								
7ºC	> 180	> 180	> 180	35	38	> 180	71	115	> 180
12ºC	6	6	10	8	8	13	12	14	18
22ºC	2	2	3	4	4	5	5	5	5
30ºC	1	1	2	3	3	3	3	3	4

* Daily growth of microbial population (log CFU/g) in phase L

** Daily growth of microbial population (log CFU/g) in phase D.

*** Resilience for *Listeria monocytogenes* was established when the population reached 3.3 log CFU/g (WHO, 2022). Resilience for lactic acid bacteria was established when the population reached 6.0 log CFU/g. Shelf-life was established when the total microbial count reached 9.33 log CFU/g (Guerra *et al.*, 2023).

6.6 Conclusions

Storage temperatures had a direct impact on the control of *Lm* and the shelf life of VPCS. Although treatments involving immersion in BCPP_YE at 1% and 5% reduced the initial population of *Lm* and the total microbial count, they were not able to maintain low levels at higher temperatures.

The adoption of good manufacturing practices, the implementation of raw material and process controls, along with cold chain management throughout the entire shelf life of a food product, is essential to achieve the desired shelf life and avoid health risks to consumers.

Biopreservatives such as BCPP_YE are promising alternatives for use in ready-to-eat meat products, and their use in a multi-obstacle strategy can contribute to improving the robustness of safety and quality programs in the meat industry. However, the method of application of these products has a direct impact on their efficacy. Therefore, further studies are necessary to evaluate the most efficient way to use these products in VPCS.

CHAPTER III

A NATURAL TECHNOLOGY FOR VACUUM-PACKAGED COOKED SAUSAGE PRESERVATION WITH POTENTIALLY POSTBIOTIC-CONTAINING PRESERVATIVE

UMA TECNOLOGIA NATURAL PARA CONSERVAÇÃO DE LINGUIÇA COZIDA EMBALADA A VÁCUO COM CONSERVANTE POTENCIALMENTE PÓS-BIÓTICO

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A Natural Technology for Vacuum-Packaged Cooked Sausage Preservation with Potentially Postbiotic-Containing Preservative

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Abstract: In this study, a potentially postbiotic-containing preservative (PPCP) was produced in an axenic fermentation system with *Lacticaseibacillus paracasei* DTA 83 as a natural technology alternative for vacuum-packaged cooked sausage preservation. Cooked sausage-related microorganisms were obtained during the induced spoiling process in packages by pair incubation of sausages at different temperatures. The turbidity method was used to determine the microbiota susceptibility to PPCP. A controlled in situ design was performed by adding PPCP on the surface or to the mass of the sausages. Sodium lactate FCC85, which was used according to the manufacturer's recommendation, was included in the design for comparison. The results revealed that PPCP was as efficient as FCC85, which indicates PPCP as a promising alternative to the use of natural technologies to preserve and develop functional cooked sausages. Moreover, a strategy to use preservatives in vacuum-packaged cooked sausages was presented: the concentration needed to achieve the total inhibition of the microbiota determined by an in vitro trial should be respected when adding PPCP on the sausages' surface. When adding PPCP to the mass of the sausages, the concentration that showed a partial inhibition in vitro can also be applied in situ.

Keywords: biocontrol; biocin; heat-inactivated microorganism; food safety; sustainability

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1. Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group that has extensively reported on in the literature because of its potential benefits for consumer health [1,2]. *Lacticaseibacillus paracasei* DTA 83 has been described as a candidate strain to deliver probiotics in food matrices [3–5]. In contrast, since microorganisms may present invasive potential, studies have shown the administration of viable cells by healthy people as a subject of great concern. Thus, the use of postbiotics may be highlighted as a suitable alternative.

The presence of spoilage microorganisms in food represents a critical issue with repercussions on massive food waste and food loss worldwide [6]. The safety and stability of food may be affected by numerous factors, such as microbial presence and/or activity; biochemical, physical–chemical, and sensory alterations; nutritional losses; and others. When intrinsic and extrinsic aspects of food allow microbial growth, the microbial spoilage pathway becomes dominant [7,8].

Biopreservation is an alternative food preservation technology applied to replace artificial preservatives. The US Food and Drug Administration (FDA) recognizes both probiotics and their metabolites as Generally Recognized as Safe (G.R.A.S) (Section 2.1 CFR184). Thus, they are useful for controlling the development of pathogens and spoilage microorganisms in food and foodstuff. Moreover, FDA has determined that conditions for their use are prescribed in the referent regulations and are predicated on the use of nonpathogenic and nontoxicogenic strains of the respective organisms and on the use of current good manufacturing practice (184.1(b)). Despite all the advantages, the use of bacteriocins is still limited because of the high cost of their isolation and purification, mainly when considering their application in products of low cost. In this context, the use of precultured broth mediums by LAB, without bacteriocin isolation and purification, may be a promising strategy to prevent spoilage in meat products [9].

Heat-treated meat products, such as vacuum-packaged cooked sausages, are traditionally marketed at room temperature in Brazil, leading to food waste due to spoilage processes that may occur before the shelf life determined by the manufacturer [10]. The vacuum atmosphere selectively suppresses the growth of specific microbial groups, attributing the initial microbiota to anaerobic and facultative groups [11]. These microorganisms overgrow and produce metabolites that cause the rejection of the products by consumers [12]. As a solution, the food industry often increases the concentration of preservatives in meat products, which may result in abusive use.

Sodium lactate is a widespread commercial preservative commonly used in sausages to control microbial growth and increase shelf life [13]. However, the higher the concentration of sodium lactate added to a food product, the higher the content of sodium. Therefore, although sodium lactate is a safe preservative for food and foodstuff, its excessive intake may result in increased blood pressure for consumers [14]. Indeed, natural technologies to preserve food are of growing interest to food industries and consumers.

Metabolites produced by LAB have been extensively tested to biocontrol the growth of pathogenic and spoilage microorganisms in meat ecosystems [15–17]. *L. paracasei* DTA 83 is a strain of human origin of great functional and technological interest. It is a ɤ-hemolytic and nonantibiotic resistant strain. Previous studies have demonstrated its potential to control the growth of *Escherichia coli*, *Salmonella Typhimurium*, *Listeria innocua*, and *Candida albicans* even after partial reduction in cell viability due to stress in the gastrointestinal transit. Technological features associated with the ability of *L. paracasei* DTA 83 to assimilate sugar in hardship conditions, such as brewer wort and plant extract solutions, were presented by Silva et al. and Oliveira et al. [18,19]. These aspects were decisive for selecting the strain for bioproduct processing. Moreover, maternal supplementation with *L. paracasei* DTA 83 reduced the expression of GAD 65, GAD 67, and GABAA receptor α3 subunit in the hippocampus, modulating Swiss mice offspring [4].

Thus, this study aimed to compare the efficacy of potentially postbiotic-containing preservative (PPCP) produced by an axenic fermentation system with *L. paracasei* DTA 83 and sodium lactate in extending the use-by date of vacuum-packaged cooked sausages. Moreover, a strategy based on the co-use of preservative and cold chain management was presented to retain the original properties of the sausages during the proposed shelf-life period of 90 days.

2. Materials and Methods

2.1. Microbial Collection and Inoculum Preparation

L. paracasei DTA 83 was isolated from newborns' stools at Rio de Janeiro (Brazil) in selective Lawvab agar medium as reported by Lemos Junior et al. [20]. The strain was genotypically identified by sequencing of the 16S rDNA region and clustered by genetic similarity with other *Lacticaseibacillus* strains of the collection (Figure S1a) [21]. Furthermore, the complete genome data was deposited in GenBank under the accession number QRBH00000000 [22]. The strain has been classified as G.R.A.S. and characterized as a potential probiotic according to Tarrah et al. and Laureano-Melo et al. [3,4]. The technological

features of the strain were assessed in food matrices by Silva et al. (Figure S1b) [5,18]. Additionally, it was described as a potential strain for delivering postbiotic compounds by Oliveira et al. [18].

L. paracasei DTA 83 cultures were thawed at 7 °C for approximately 4 h and centrifuged at 6000× *g* for 5 min (2K15, Sigma Laborzentrifugen, Osterode am Harz, Germany) for pellet separation. The liquid fraction was discarded. Then, the remaining cell pellet was reconstituted with MRS broth and then incubated overnight at 36 °C for the microbial growth. To obtain sufficient biomass to produce PPCP on a pilot-industrial scale, the cultures were scaled up 1/10 (*vol*/*vol*) at 36 °C in an axenic cultivation in a sterile MRS broth medium prepared with food-grade ingredients to obtain 30 L of inoculum.

2.2. PPCP Production

A stirred tank bioreactor of 300 L, with automatic control of temperature and pH, was used to produce PPCP in an axenic fermentation system with *L. paracasei* DTA 83. This part of the experiment was carried out at BRC Ingredientes Ltda., located in the city of Rio Claro, state of São Paulo, Brazil. Modified MRS broth was prepared with food-grade ingredients without the addition of polysorbate 80 (Tween 80). The heat treatment was performed in a tank (heating up of 1 °C per minute) by the electrical activation of three resistors (3 kW). During heating, the medium was axially agitated at 84 rpm. The binomial 75 °C per 2 h was used to reduce the contaminants to an acceptable level (ca. 3 log cfu/g) and provide a competitive advantage to *L. paracasei* DTA 83 during the fermentation. After the heat treatment, the temperature of the medium was reduced to 36 °C (heating down of 0.5 °C per minute). *L. paracasei* DTA 83 biomass was produced in laboratory, scaling up 1/10 (*vol*/*vol*) of the culture into sterile modified MRS broth. A biological oxygen demand was used for incubation at 36 °C to obtain 30 L of inoculum. A culture with 18 h of growth, comprehended into the growth (log) phase, was added (1/10 of inoculum) into the bioreactor containing 270 L of modified MRS medium to obtain a final inoculum concentration of ca. 7 log cfu/mL. After 72 h of fermentation coupled with a pH decay to around 3.5, the medium was heat treated at 95 °C for 5 min (heating up of 1 °C per minute). PPCP was hot bottled in polypropylene containers of 10 L. The presence of remaining cells of *L. paracasei* DTA 83 or contaminants was assessed by plate counting on MRS and plate count agar and potato dextrose agar acidified to pH 3.5 with tartaric acid (all media from HiMedia, Mumbai, India).

2.3. In Vitro Efficacy of PPCP

Cooked sausage-related microorganisms were obtained from five packages of sausages, with collection at zero time ($n = 1$) and after pair incubation of samples at 7 °C (collection on days 3 and 6) and 36 °C (collection on days 2 and 4). A decimal suspension was prepared by weighing the sausages and adding 0.1% of peptone sterile water to the package. This step was conducted to count the microorganisms in the sausages, as well as those accumulated in the liquid inside the package after syneresis. After the samples were homogenized in a stomacher (SP-190, SPLabor, Brazil) for 90 s at 230 revolutions per minute (rpm), aliquots (100 µL) was transferred to tubes with 5 mL of brain–heart infusion, Casoy, deMan, Rogosa, and Sharp, and yeast–peptone–dextrose extract. The tubes were incubated at 36 °C for 24–48 h. The inoculum was obtained separately from each culture medium by transferring 1 mL of the tube content, with expressive growth (turbidity above 0.5 MacFarland standard), to an empty sterile screw-cap tube. Cells free of toxic compounds were obtained by twice washing the biomass cell pellets with a routine of centrifugation at 6000× *g* for 6 min for pellet sedimentation at the bottom of the tube, discarding the liquid fraction, adding 2 mL of phosphate buffer pH 7.2, and homogenizing in vortex. The turbidity of the microbial suspension was adjusted to 0.5 McFarland standard and 2-fold diluted. PPCP was randomly outlined to final concentrations of 0.0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5% (*vol*/*vol*) in the brain–heart infusion broth. The dilutions were prepared in the same media used in the test to avoid a shortage of nutrients for microbial growth. Finally,

100 µL of the microbial suspension was added into the tubes to achieve a final microbial concentration of ca. 5 log cfu/mL. A digital stirred water bath (SP-156/22, SPLabor, Brazil), with automatic temperature control, was used to incubate the tubes at 36 °C for 72 h. The absorbance was read in a photometry device at 600 nm (Spectrum SP-2000UV/2000UVPC, Shanghai, China) for a regular 6 h period. Before reading, the tubes were vortexed, and the absorbance was directly measured in the tubes. A tube without inoculum was used as blank and for equipment calibration at each reading.

2.4. In Situ Efficacy of PPCP

PPCP was tested in situ at concentrations of 1.0, 2.0, and 3.0% by adding the preservatives on the surface or to the mass of the sausages. The sausages were manufactured on an industrial-pilot scale for the meat industry located in Rio de Janeiro, Brazil. The production was performed according to the meat products' standard procedures, as follows: input of feed of raw materials, defrosting or breaking in frozen block crusher, grinding through industrial grinder knife (8–12 mm hole diameter (Ø) plate) (PC 106, Canoas, Brazil), mixing and addition of food ingredients (lean meat, pork fat, spices, and food additives) (250L, Cataguases, Brazil), stuffing in 15 and 250 mm inner (diameter × length) natural pork casing (NDX 22 Viscofan, Spain), cooking to achieve 72 °C (approximately 2 h) (MECA2G, Pará de Minas, Brazil) at the coldest point of the sausage, cooling by immersion in a cold water bath, and packing using a vacuum-package system with 5 to 7 pieces of sausage per package. PPCP was added to the mass of the sausages with other ingredients during the sausage mass preparation or directly into the packages to hurdle microbial growth after syneresis. The net weight of the sausages in the packages was used to calculate the volume of PPCP added into the packages. Sodium lactate FCC85 (Corbion, Purak, Brazil), added to the mass or on the sausages' surface, was included in the design to compare the efficacy of the PPCP with that of a reference widespread commercial preservative. The addition of sodium lactate was performed following the manufacturer's recommendation. Sausages without preservatives or with sterile deionized water, added to the mass or on the sausages' surface, were included as blank and control, respectively (Table 1). After manufacturing, the packages were immediately addressed to the laboratory.

Table 1. Formulation of pork sausage samples.

	Treatments										
		Sausage Surface					**Sausage Mass**				
Ingredients (%)	**Blank**	**Control (Water) 2.0%**	**Sodium Lactate 2.0%**	**PPCP [3] 1.0%**	**PPCP 2.0%**	**PPCP 3.0%**	**Control (Water) 2.0%**	**Sodium Lactate 2.0%**	**PPCP 1.0%**	**PPCP 2.0%**	**PPCP 3.0%**
Lean pork meat	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33
Pork fat	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Drinking water	10.00	10.00	10.00	10.00	10.00	10.00	8.00	8.00	9.00	8.00	7.00
Salt (sodium chloride)	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Seasoning [1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Sodium trypoliphosphate	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Sodium erythorbate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Curing salt [2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Sterile deionized water		2.00					2.00				
Sodium lactate FCC85			2.00					2.00			
PPCP				1.00	2.00	3.00			1.00	2.00	3.00

[1] Garlic powder, onion powder, black pepper, nutmeg, laurel powder, and celery powder; [2] sodium chloride (90%), sodium nitrite (6%), and sodium nitrate (4%); [3] potentially postbiotic-containing preservative.

2.5. Sample Characterization

2.5.1. Physicochemical Analyses

The analyses were carried out following the AOAC procedures [23]. Moisture content (%*w*/*v*) was determined by oven drying at 105 °C until constant weight. Ash content (%*w*/*v*) was determined by incinerating samples in a muffle furnace at 550 °C for 4 h.

Protein level (%*w*/*v*) was obtained by the Kjeldahl method. The Soxhlet extraction method with hexane was applied to determine the total fat content (%*w*/*v*). The total carbohydrate content was calculated as the difference between 100 and the sum of the percentages of moisture, ash, lipid, and protein. Total energy (kcal/100 g sample) was calculated according to the Atwater specific factor system (4.27 kcal/g for protein or carbohydrate and 9.02 kcal/g for fat).

2.5.2. Water Activity Measurement

Changes in the electrical conductivity of an electrolyte, in accordance with the method ISO 18787 (2017) [24], were used for water activity measurement in a AcquaLab Lite device (Decagon, Washington, USA) provided with a dielectric humidity sensor and infrared sample surface temperature. Before measuring, the equipment was calibrated with two standard solutions (K_2SO_4, *aw* 0.973 (CAS 7778–80–5) and KCl, *aw* 0,843 (CAS 7447–40–7)) provided by the manufacturer. A maximum error of ±0.005 was considered as accuracy. To obtain a uniform sample, a piece of sausage was ground in an electric meat grinder (Centrífuga 1000, Britânia, Brazil). Excessive milling, which could lead to heating of samples and affect measurements, was avoided. Immediately after grinding, the sample portion was taken as quickly as possible to minimize exposure to humidity in the laboratory. A sample dish with a capacity of 7 mL was $^1/_3$ filled with sample so that there was no empty space at the bottom. During the analytical series, the measurement stability was verified using standard solutions. A waiting time of approximately 15 min was established between each measurement after opening the equipment lid.

2.5.3. pH Values

Nondestructive measurement of pH was performed according to the method ISO 2917:1999(E) [25]. A portable meat pHmeter device (pH Classic, Akso, Brazil), equipped with a knife probe electrode (IP65, Akso, Brazil) and automatic compensation of temperature, was used. Sausages were randomly withdrawn from the packages, and the pH value was determined by direct sticking the electrode in 3 different positions of the sausage: the two ends and the central section of the pieces. Before measuring, the equipment was calibrated with buffer solutions, pH 4.00 and pH 6.88 at 20 °C. A maximum error of ±0.01 was considered as accuracy.

2.6. Durability Study

A predictive microbial method, named *MicroLab_ShelfLife*, was used to estimate the use-by date of vacuum-packaged cooked sausages at a chosen dynamic temperature profile (Figures S4 and S5 in Supplementary Material). The use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of ca. 9.3 log cfu/g. This is the borderline to determine when changes in sensory attributes related to the appearance of vacuum-packaged cooked sausages occur (Figures S2 and S3, Table S1). The horizontal method for enumeration of microorganisms (ISO 4833-1:2013) [26] was performed to determine the total microbial load, using plate count agar medium (HiMedia, Mumbai, India), at the zero time and after stimulating the microbial growth in the packages by pair incubation of samples at 7 and 36 °C, with counts on days 3 and 6 (7 °C) and on days 2 and 4 (36 °C) of incubation (Figure S4). The number of colonies obtained at each dilution level was imputed in the *MicroLab_ShelfLife* computational package to determine the parameters of the microbial growth and to plot the predictive microbial growth curve (Figure S5).

A dynamic temperature profile was entered in the predictive model based on the measurements published by the AccuWeather forecast during 2021. Latitude and longitude coordinates (22°54′13′′ South; 43°12′35′′ West; Rio de Janeiro, Brazil) were considered as the climatic location, indicating the place where the sausages would be sold. According to the Köppen–Geiger classification, the climate of Rio de Janeiro is a tropical monsoon climate (*Am*) [27]. The temperature data were grouped by season. The daily temperature

profile, representing each climate season, was hourly grouped to fit in the *MicroLab_ShelfLife* platform (Figure 1). This profile was used to mimic the temperature during the product storage and disposal for sale in markets.

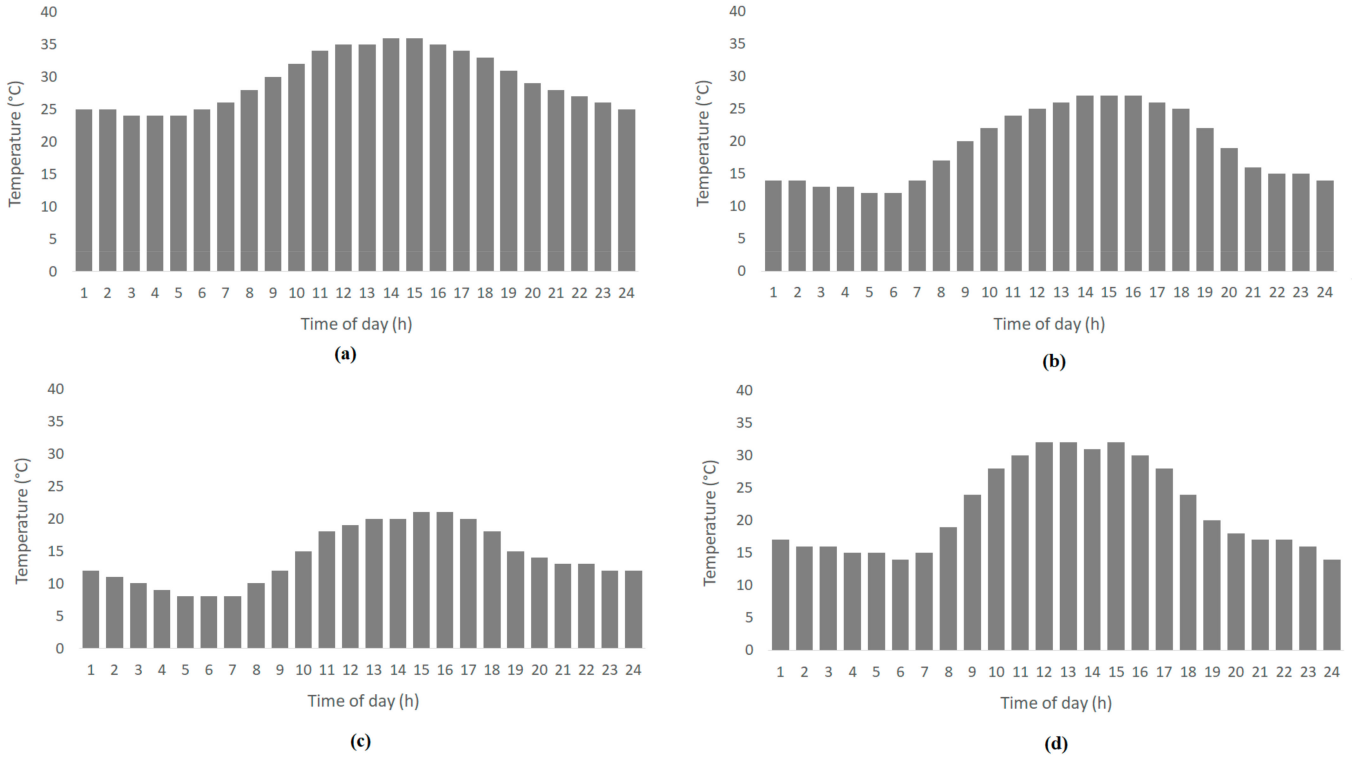

Figure 1. Temperature profile based on hourly variation during a one-day period to simulate the seasons: (**a**) summer, (**b**) autumn, (**c**) winter, (**d**) spring. They were determined based on the measurements published by AccuWeather (www.accuweather.com) for 2021. Latitude and longitude coordinates: 22°54′13″ South; 43°12′35″ West; Rio de Janeiro, Brazil, where a tropical monsoon climate (*Am*) has been reported (Köppen–Geiger climatic classification) [27].

2.7. Statistical Analyses

Results related to PPCP efficacy against the growth of natural microbiota and physico-chemical characterization of vacuum-packaged cooked sausages were obtained in triplicate. Linear regression was applied in the turbidity method regarding the incubation time with representative microbial growth, comprehended from 6 to 30 h of incubation at 36 °C, according to Equations (1)–(3). Angular coefficients from regressions (mean ± standard error) were evaluated by analysis of variance (ANOVA) followed by Fisher's (LSD) test ($p < 0.05$):

$$\dot{Y} \pm \frac{t\alpha}{2} \times SE \times \sqrt{hi} \quad (1)$$

$$\dot{Y} \pm t\alpha/2 \times SE \times \sqrt{1 + hi} \quad (2)$$

$$hi = 1/n + (xi - x)^2 / \sum (xi - x)^2 \quad (3)$$

$\dot{Y}$ is the estimated value, $t\alpha/2$ is the value of Student's t distribution, n is the number of observations, xi is the value of the sample, and x is the mean.

A computational predictive modeling package, *MicroLab_ShelfLife*, was developed in the present study and used to predict the use-by date of vacuum-packaged cooked sausages from five packages of each sample group (Supplementary Material). The method was also used to evaluate the effect of the temperature associated with preservatives in the shelf life of the products and to estimate the initial microbial load of vacuum-packaged cooked sausages to achieve the proposed shelf-life period of 90 days.

3. Results

In vitro trials revealed that PPCP addition at concentrations up to 0.5% did not inhibit microbial growth. In samples containing 1.0–3.0% of PPCP, microbial inhibition was partially achieved. Although the efficacy was directly proportional to the added concentration of PPCP, similar results were obtained by adding 1.0 or 1.5% of PPCP ($p > 0.05$). Total inhibition was achieved at concentrations above 3.0% (Table 2, Figures 2 and 3).

Table 2. Linear regression parameters of microbial growth.

	(%) of Potentially Postbiotic-Containing Preservative (PPCP)									
Coefficients	**0.0**	**0.1**	**0.3**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**
xi	0.044 ^{a}	0.044 ^{a}	0.044 ^{a}	0.044 ^{a}	0.035 ^{a}	0.031 ^{a}	0.025 ^{b}	0.016 ^{c}	0.004 ^{d}	0.004 ^{d}
yi	−0.299	−0.294	−0.298	−0.305	−0.309	−0.281	−0.242	−0.137	−0.018	−0.034
R^2	0.978	0.981	0.979	0.977	0.959	0.955	0.921	0.961	0.881	0.088
SE	0.111	0.109	0.110	0.111	0.088	0.079	0.065	0.040	0.012	0.011
SQ	1890	1890	1890	1890	1890	1890	1890	1890	1890	1890
n	18	18	18	18	18	18	18	18	18	18
DF ($n - 2$)	16	16	16	16	16	16	16	16	16	16
tα/2	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729
Confidence Interval	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95

xi—angular coefficient; *yi*—linear coefficient; R^2—coefficient of determination; SE—Standard error; SQ—sum of squares; n–number of observations; DF—degrees of freedom; tα/2—value of Student's t distribution. Different capital letters indicate significant differences by Fisher's (LSD) test ($p < 0.05$).

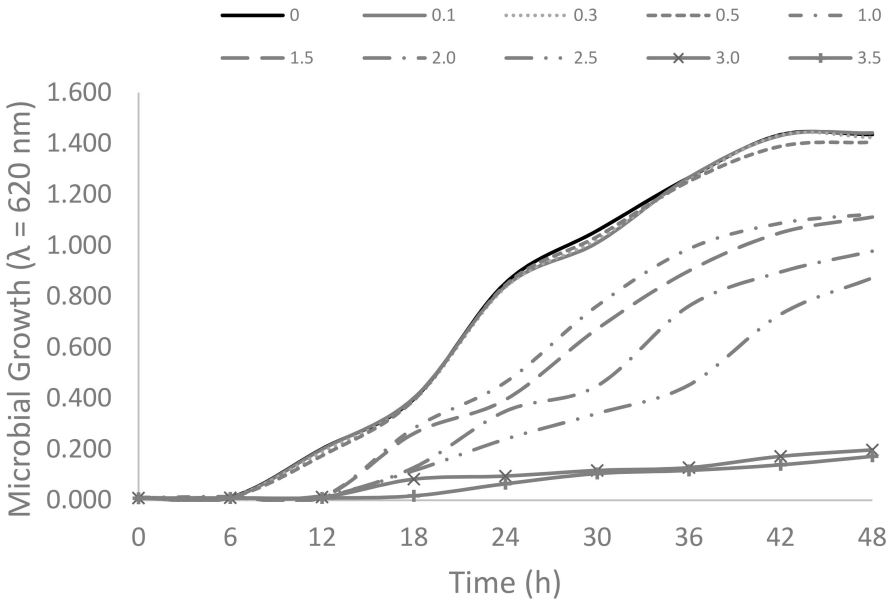

Figure 2. In vitro efficacy of PPCP against the growth of natural microbiota in vacuum-packaged cooked sausages. The inoculum was adjusted to ca. 5.5 log cfu/g before testing, and the turbidity method was used to evaluate the efficacy.

Table 3 shows the physicochemical characterization, water activity measurements, and pH values of sausages.

PPCP and FCC85 can reduce the growth of natural microbiota in vacuum-packaged cooked sausages and extend the shelf-life period. However, the strategy of addition must be carefully designed. The superficial treatments with 1.0% of PPCP and 2.0% of FCC85 should be discouraged, since these treatments did not present effective results compared with blank and control. In the sausages' mass, the addition of 1.0% PPCP was as effective as the addition of 2.0% of FCC85, indicating a potential natural alternative for product preservation (Table 4).

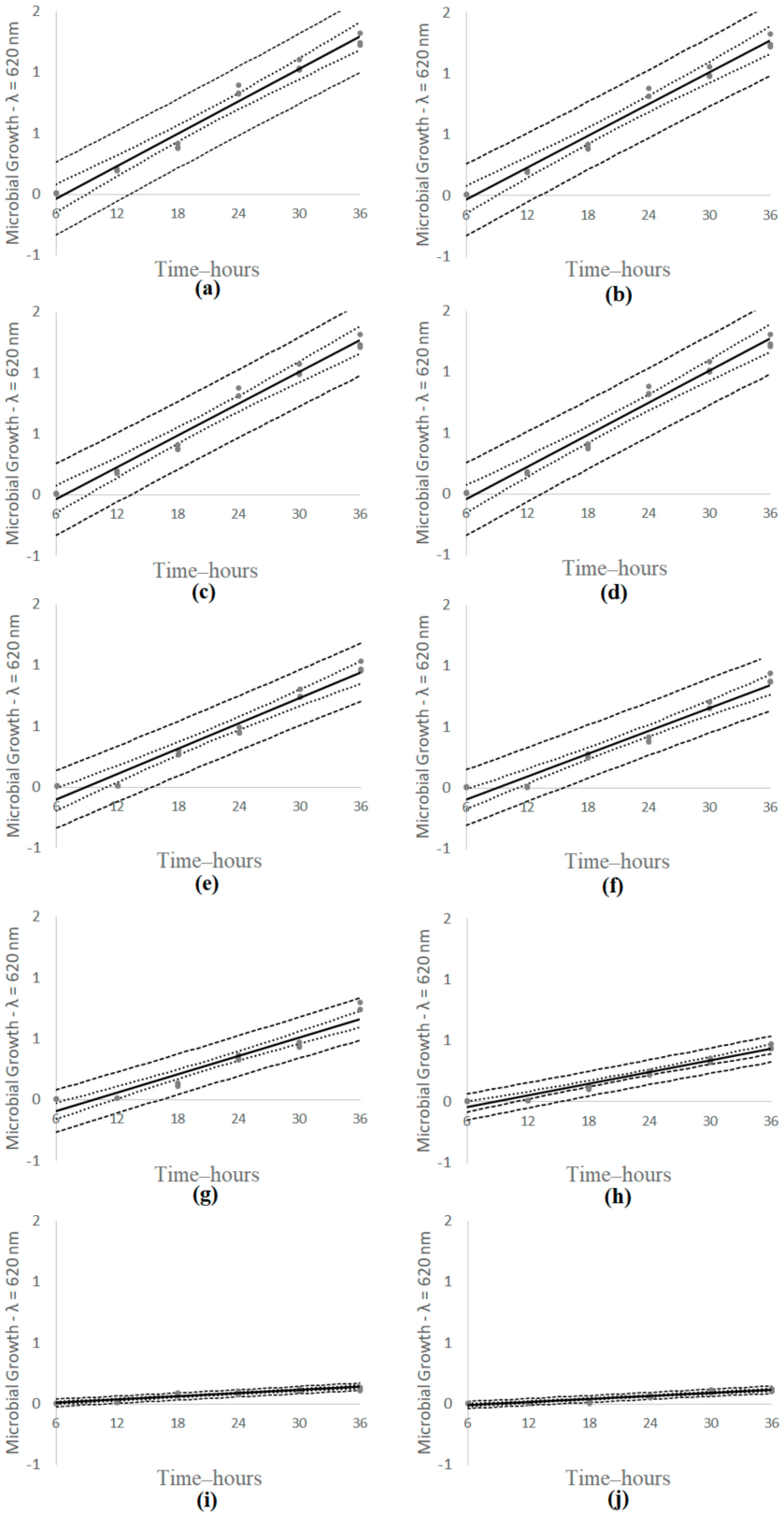

Figure 3. Linear regression (—-), confidence interval for the mean (. . . .), and prediction interval for the sample (—) of the period with microbial growth (from 6 to 36 h of incubation at 36 °C) at different concentrations of potentially postbiotic-containing preservative (PPCP): (**a**) 0.0%; (**b**) 0.1%; (**c**) 0.3%; (**d**) 0.5%; (**e**) 1.0%; (**f**) 1.5%; (**g**) 2.0%; (**h**) 2.5%; (**i**) 3.0%; (**j**) 3.5%.

Table 3. Physicochemical characterization of vacuum-packaged cooked sausages (n = 3).

Parameter	Mean ± Standard Error
Moisture (%)	56.663 ± 0.160
Protein (%)	14.434 ± 0.288
Fat (%)	23.550 ± 0.122
Ash (%)	3.550 ± 0.387
Carbohydrates [1] (%)	1.803 ± 0.627
Total energy (kcal/100 g sample)	281.749 ± 1.714
Potential of hydrogen (pH)	6.878 ± 0.004
Water activity (*Aw*)	0.964 ± 0.002

[1] Calculated according to the Atwater specific factor system (4.27 kcal/g for protein or carbohydrate and 9.02 kcal/g for fat).

Table 4. Durability study of vacuum-packaged cooked sausage samples.

			Treatments										
Sample Incubation				Sausage Surface					Sausage Mass				
	Temperature (°C)	Time (days)	Blank	Control (water) 2.0%	Sodium lactate 2.0%	PPCP [4] 1.0%	PPCP 2.0%	PPCP 3.0%	Control (water) 2.0%	Sodium lactate 2.0%	PPCP 1.0%	PPCP 2.0%	PPCP 3.0%
Laboratorial data (log cfu/g)		0	5.77	5.80	5.71	5.88	5.79	5.80	5.66	5.76	5.81	5.83	5.87
	7	3	6.01	5.99	5.89	5.99	5.90	5.81	5.95	6.01	6.02	5.92	6.01
		6	6.49	6.48	6.37	6.45	6.23	5.98	6.42	6.14	6.09	6.00	6.32
	36	2	6.69	6.72	6.70	6.80	6.64	6.14	6.59	6.66	6.61	6.33	6.26
		4	8.50	8.48	8.49	8.61	8.45	6.87	8.42	7.12	7.10	6.97	6.94
Specific maximum growth rate (log cfu/g/day)	7	L phase	0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0608
		D phase	0.0287	0.0253	0.0244	0.0189	0.0158	0.0048	0.0320	0.0210	0.0167	0.0084	0.0174
	36	L phase	0.5713	0.5650	0.5950	0.5713	0.5450	0.2188	0.5775	0.3950	0.3613	0.2675	0.2313
		D phase	0.1637	0.1619	0.1705	0.1637	0.1562	0.0627	0.1655	0.1132	0.1035	0.0767	0.0663
		Season											
Ngrowth (log cfu/g/day) [1]		Summer	0.4649	0.4575	0.4800	0.4572	0.4345	0.1732	0.4724	0.3224	0.2929	0.2137	0.1928
		Autumn	0.2970	0.2876	0.2982	0.2771	0.2599	0.1012	0.3064	0.2078	0.1850	0.1288	0.1321
		Winter	0.2158	0.2054	0.2103	0.1900	0.1754	0.0663	0.2261	0.1524	0.1328	0.0877	0.0608
		Spring	0.3383	0.3294	0.3429	0.3214	0.3028	0.1189	0.3473	0.2360	0.2115	0.1497	0.1470
Ndeceleration (log cfu/g/day) [2]		Summer	0.1332	0.1311	0.1375	0.1310	0.1245	0.0496	0.1354	0.0924	0.0839	0.0613	0.0553
		Autumn	0.1151	0.1115	0.1156	0.1074	0.1007	0.0392	0.1188	0.0805	0.0717	0.0499	0.0512
		Winter	0.1008	0.0960	0.0983	0.0888	0.0820	0.0310	0.1057	0.0712	0.0620	0.0410	0.0284
		Spring	0.1207	0.1175	0.1223	0.1146	0.1080	0.0424	0.1239	0.0842	0.0754	0.0534	0.0524
Use-by date (days) [3]		Summer	12	12	12	12	14	34	12	19	20	27	30
		Autumn	17	18	17	18	20	50	17	24	27	38	37
		Winter	22	22	22	24	26	70	21	31	35	52	45
		Spring	15	16	15	16	17	43	15	23	25	35	35

[1] *Ngrowth*— daily microbial population growth (log cfu/g) in the microbial growth (log) phase; [2] *Ndeceleration*— daily microbial population growth (log cfu/g) in the microbial deceleration phase; [3] the use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of ca. 9.3 log cfu/g. [4] PPCP—potentially postbiotic-containing preservative.

The temperature profile entered in the predictive model influenced the growth of the natural microbiota in vacuum-packaged cooked sausages. In summer, the values of the *Ngrowth* and *Ndeceleration* parameters of the predictive model, which represent the kinetics of the microbial growth in the growth (log) and deceleration phases, respectively, were higher than the values obtained during the other seasons. Thus, a shorter shelf life was observed during summer, with an early achievement of the predictive borderline limit, which can result in changes in sensory attributes related to sausages' appearance. As expected, microbial growth was reduced in winter. The correlation variable factor $FT(n)$, which describes specific growth rates between log and deceleration phases, can also be used to indicate the impact of the temperature profile on microbial growth, highlighting that the critical period for sausage preservation was summer ($FT(n)$ = 3.4894), followed by autumn ($FT(n)$ = 2.8038), spring ($FT(n)$ = 2.5801), and winter ($FT(n)$ = 2.1401)

4. Discussion

Over the past decade, novel terms have been used to represent the beneficial effects of microorganisms. Postbiotics, or paraprobiotics or metabiotics, represent structural components of probiotic microorganisms and/or formulation of signaling molecules with a known chemical structure that can optimize host-specific physiological functions and regulate metabolic and/or behavior reactions related to the activity of host natural microbiota [28–30].

Hill et al. (2014) proposed that a more grammatically correct definition of probiotics would be 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'. Thus, FAO and WHO definition of probiotics was reinforced as relevant and adaptable for current and further applications [31,32]. The development of metabolic by-products, dead microorganisms, or other microbial-based, nonviable products has potential; however, these do not fall under the probiotic construct'.

Once the viability of *Lacticaseibacillus* is reduced by cooking, postbiotic compounds can be a suitable alternative for the development of functional cooked foods. Moreover, precultured medium by LAB has been reported in the literature as a promising natural technology for food preservation [33].

Poor-quality raw material and inadequate handling can anticipate sausage spoilage. In vacuum-packaged cooked sausages, changes in the sensory attributes related to the sausages' appearance, which can be a decisive factor for consumer appraisal, occur when the microbial population achieves the stationary phase in the microbial growth curve (ca. 9.3 log cfu/g) (Figures S2 and S3, Table S1). The use of preservatives may reduce the activity of the natural microbiota, impacting the cell viability.

None of the treatments maintained the microbial load below the predicted model's borderline during the 90 days of shelf life indicated by the meat industry. Therefore, additional hurdles, such as cold storage, should be used combined with preservatives. When the cold storage temperature profile (7 °C) was entered in the predictive model to estimate the use-by date of the sausages, adding 3.0% of PPCP on the surface or adding 2.0% or more to the mass extended the use-by date by more than 90 days (Table 5).

Table 5. Durability study of vacuum-packaged cooked sausages stored at 7 °C.

Sample Incubation			Sausage Surface						Sausage Mass				
	Temperature (°C)	Time (days)	Blank	Control (water) 2.0%	Sodium lactate 2.0%	PPCP [4] 1.0%	PPCP 2.0%	PPCP 3.0%	Control (water) 2.0%	Sodium lactate 2.0%	PPCP 1.0%	PPCP 2.0%	PPCP 3.0%
Laboratorial data (log cfu/g)		0	5.77	5.80	5.71	5.88	5.79	5.80	5.66	5.76	5.81	5.83	5.87
	7	3	6.01	5.99	5.89	5.99	5.90	5.81	5.95	6.01	6.02	5.92	6.01
		6	6.49	6.48	6.37	6.45	6.23	5.98	6.42	6.14	6.09	6	6.32
	36	2	6.69	6.72	6.70	6.80	6.64	6.14	6.59	6.66	6.61	6.33	6.26
		4	8.50	8.48	8.49	8.61	8.45	6.87	8.42	7.12	7.10	6.97	6.94
Specific maximum growth rate (log cfu/g/day)	7	L phase	0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0608
		D phase	0.0287	0.0253	0.0244	0.0189	0.0158	0.0048	0.0320	0.0210	0.0167	0.0084	0.0174
	36	L phase	0.5713	0.5650	0.5950	0.5713	0.5450	0.2188	0.5775	0.3950	0.3613	0.2675	0.2313
		D phase	0.1637	0.1619	0.1705	0.1637	0.1562	0.0627	0.1655	0.1132	0.1035	0.0767	0.0663
Ngrowth (log cfu/g/day) [1]			0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0250
Ndeceleration (log cfu/g/day) [2]			0.0661	0.0584	0.0562	0.0435	0.0363	0.0110	0.0738	0.0485	0.0386	0.0193	0.0165
Use-by date (days) [3]			41	46	49	60	73	240	38	56	68	136	158

[1] *Ngrowth*—daily microbial population growth (log cfu/g) in the microbial growth (log) phase; [2] *Ndeceleration*—daily microbial population growth (log cfu/g) in the microbial deceleration phase; [3] the use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of ca. 9.3 log cfu/g. [4] PPCP—potentially postbiotic-containing preservative.

The addition of 2.0% of FCC85 on the surface or to the mass of the sausages little increased the use-by date. However, this is close to the maximum concentration permitted by the regulatory agency for the use of sodium lactate in heat-treated meat products [34]. This fact casts doubt on the efficacy of sodium lactate in increasing the use-by date of

vacuum-packaged cooked sausages. Although PPCP showed advantages compared with FCC85 regarding the extension of the use-by date of the sausages, it did not maintain the microbial load below the predictive model's borderline over 90 days either. However, there is no prescribed limit on the use of natural substances in sausages. Moreover, co-use of preservatives and proper management of the cold chain are suitable strategies to achieve a use-by date higher than 90 days.

Cold chain management of meat products, including raw material supply, processing, distribution, and retail, is a crucial factor to prevent spoilage [12]. The specific maximum growth rate obtained at 36 °C was expressively higher than the value determined at 7 °C (Table 2), showing the influence of the temperature on sausage spoilage. Indeed, the temperature profile during distribution, storage, and disposal in the market plays a role in the durability of meat products.

The addition of 3.0% of PPCP on the surface or 2.0% or more of PPCP to the mass, combined with management of the cold chain, resulted in a use-by date higher than 90 days (Table 5).

These results highlighted the potential use of PPCP on the surface of sausages. However, the concentration to achieve total inhibition of the microbiota, determined in vitro, should be respected. Thus, regarding the addition of PPCP to the mass of the sausages, the concentrations used to achieve partial inhibition of the microbiota can be used.

After packaging, syneresis may be induced during the storage and distribution of sausages, resulting in the accumulation of water, nutrients, and microorganisms inside the package. Preservatives are usually added to the mass with other ingredients during meat products preparation. However, there are no barriers to prevent microbial growth in the liquid accumulated inside the package after syneresis. Even when effective preservatives are added to the mass, this strategy may fail after syneresis because of the partial migration of these additives to the liquid phase. It can be of great concern if the storage temperature allows microbial activity.

The initial microbial load of the sausages may contribute to shortening the use-by date. By fixing the values of predictive model's parameters (*Ngrowth*, *Ndeceleration*, and *Ft*(*n*)) for each treatment, a use-by date of 90 days was achieved with the predicted initial microbial loads presented in Table 6.

Table 6. Estimated initial microbial load of vacuum-packaged cooked sausages to achieve the predictive model's borderline of 90 days.

Treatments		Presumed Initial Microbial Load (log cfu/g)				
		Summer	**Autumn**	**Winter**	**Spring**	**Cold Storage**
	Blank	−20.00	−13.05	−9.03	−14.90	0.87
Sausage surface	2.0% of water (control)	−19.17	−12.22	−8.20	−14.07	1.90
	2.0% of sodium lactate	−19.46	−12.51	−8.49	−14.36	2.21
	1.0% of PPCP [1]	−17.59	−10.64	−6.62	−12.49	3.88
	2.0% of PPCP	−16.38	−9.43	−5.41	−11.28	4.84
	3.0% of PPCP	−6.57	0.38	4.40	−1.47	8.30
Sausage mass	2.0% of water (control)	−20.91	−13.96	−9.94	−15.81	−0.24
	2.0% of sodium lactate	−14.21	−7.26	−3.24	−9.11	3.26
	1.0% of PPCP	−12.46	−5.51	−1.49	−7.36	4.56
	2.0% of PPCP	−7.62	−0.67	3.35	−2.52	7.18
	3.0% of PPCP	−6.27	0.68	4.70	−1.17	7.57

[1] PPCP—potentially postbiotic-containing preservative.

Only the treatments with 3.0% of PPCP on the surface or 2.0% or more of PPCP in the mass of the sausages during the winter achieved the proposed use-by date. This result highlights the importance of considering additional factors to hurdle microbial growth in the sausages. During summer and spring, sausage preservation during the proposed use-by date was elusive for any treatment. With the co-use of preservatives and the management

of cold chain, the meat industry may reduce the initial microbial load to the levels presented in Table 6.

Satisfactory results regarding the extension of the shelf life of meat products can be achieved by reducing the initial microbial load, as well as by improving the product formulation to prevent syneresis [35]. Indeed, the microbial growth and durability of sausages are greatly influenced by the initial microbial load and the use of effective hurdles [36]. However, sporulated bacteria groups cannot be eliminated by cooking processes and hurdlers. This fact highlights the importance of avoiding the presence of these microorganisms in products by applying microbiological quality control in the meat supply chain [37].

Handlers, utensils, equipment, and microbial load of the raw material are the main microbial vehicles during production [38–40]. The environment is also a factor in meat spoilage [41], and it depends on the region; climate; microclimate, season; and anomalous environmental events such as forest fires, deforestation, rainwater excess, etc. [42].

5. Conclusions

PPCP produced by an axenic fermentation system with *L. paracasei* DTA 83 was as effective as the reference widespread commercial preservative FCC85 in preserving vacuum-packaged cooked sausages. Thus, it can be highlighted as a promising alternative concerning the use of natural technologies to preserve and produce functional cooked sausages. These results also revealed a logical relation regarding in vitro and in situ tests to evaluate sausage preservation. The concentration needed to achieve total inhibition of the microbiota, determined by an in vitro trial, should be respected when adding PPCP on sausages' surface. When adding PPCP to the mass of the sausages, the concentration that showed a partial inhibition in vitro could also be applied in situ. However, proper chain management during distribution and disposal of products in the market are pivotal to achieve the desired use-by date. Although this study presented a potential postbiotic alternative by adding PPCP to sausages, a robust in vivo trial must be further designed to evaluate effects in the host.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8030106/s1, Figure S1: (a) Cluster analysis of RADP-PCR profiles obtained of 35 *Lacticaseibacillus* isolates from stool samples of infants aged between 7 to 21 days. The amplification patterns were analyzed using the software Gel Compar 4.1 (Applied Maths) [1], and (b) potential of *Lacticaseibacillus* to acidify the pasteurized deMan, Rogosa and Sharp broth medium.; Figure S2: The relative abundance (a and b), Krona plot (c), and dendrogram of similarities and discrepancies of high-throughput sequencing of bacterial phyla of vacuum-packaged cooked sausages; Figure S3: Microbial growth curves at 4 °C (a), 12 °C (b), 24 °C (**c**), and 36 °C (**d**). They were plotted regarding the natural microbiota of vacuum-packaged cooked sausages (–●– sample #1\; –◆– sample #2\; –△– sample #3). Drop-plate technique was used to count total bacteria. Baranyi's mathematical model was applied to model the microbial growth at each temperature. The initial population was ca. 2.8 log cfu/g. The growth (log) phase started suddenly after incubation at 24 and 36 °C, and extended up to 8.2 log cfu/g. Stationary phase started after the population had reached ca. 9.3 log cfu/g. The period between the log and the stationary phases was considered the deceleration phase; Figure S4. Sample incubation design. Microbial count at time zero must be below 8.2 log cfu/g to validate the test. Besides the time zero, there is no predefined time for microbial counting once the computational predictive modeling can process any time; however, microbial growth (log) phase must be included at least in one of the counts. Laboratories can determine the incubation temperatures; however, lower and higher temperatures between 4 and 20 °C, 25 and 36 °C, respectively, must be used; Figure S5. Illustration of the biological growth curve by predictive modeling. A—adaptation and acceleration growth phase; L—microbial growth (log) phase; D—deceleration phase; S—stationary phase. Correlations between specific growth rate in L and D phases were performed based on the correlation factor FT(n) value, according to the chosen temperature profile of the test; Table S1. Instrumental color measurement (on the unopened packaged sausages and after withdrawing the sausages from the packages and cleaning up their surfaces) and slime formation detection [43,44].

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8 CONCLUSÕES GERAIS

Múltiplos fatores podem impactar na eficácia de um conservante. Desta forma, é essencial a realização de testes em condições extremas, simulando condições de processos industriais e cobrindo as faixas de flutuação dos fatores intrínsecos e extrínsecos relacionados ao crescimento microbiano no alimento alvo.

Os bioconservantes BCPP_SP e BCPP_YE foram igualmente eficientes em inibir o crescimento de *Lm in vitro* ($p > 0,05$), apresentando CIM e CLM na concentração de 1%. Além disso, mostraram forte estabilidade ao calor e não foram afetados pelo tratamento com tripsina. Entretanto, a neutralização total e parcial dos ácidos orgânicos resultou na ausência de ação antilisterial em concentrações de até 10%.

Os testes *in vitro* demostraram que pH teve influência direta sobre a eficácia dos conservantes e bioconservantes. De igual modo, os testes *in situ* e as simulações *in silico* revelaram que a temperatura de armazenamento e a forma de aplicação dos bioconservantes nas LCEV impactaram nos resultados de vida de prateleira e na ação antilisterial.

Quando comparado aos controles, os BCPP_SP e BCPP_YE foram capazes de estender a vida de prateleira de LCEV em todas as simulações, entretanto, não foram eficientes para garantir a estabilidade microbiológica das LCEV armazenadas fora da refrigeração. A temperatura de refrigeração foi a principal barreira para impedir o crescimento de *Lm* e deteriorante nas LCEV.

BCPP_SP e BCPP_YE são alternativas promissoras para utilização em produtos cárneos prontos para o consumo, e sua utilização numa estratégia multiobstáculos pode contribuir para melhorar a robustez dos programas de segurança e qualidade na indústria da carne.

A adoção das boas práticas de fabricação, implantação de controle de matéria prima e processos, aliado a gestão da cadeia de frio durante toda a vida útil de um alimento é essencial para atingir o prazo de validade desejado e evitar riscos à saúde do consumidor.

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10 ANEXOS

Comparação dos tratamentos com BCPP_YE 5% e BCPP_YE 1%.

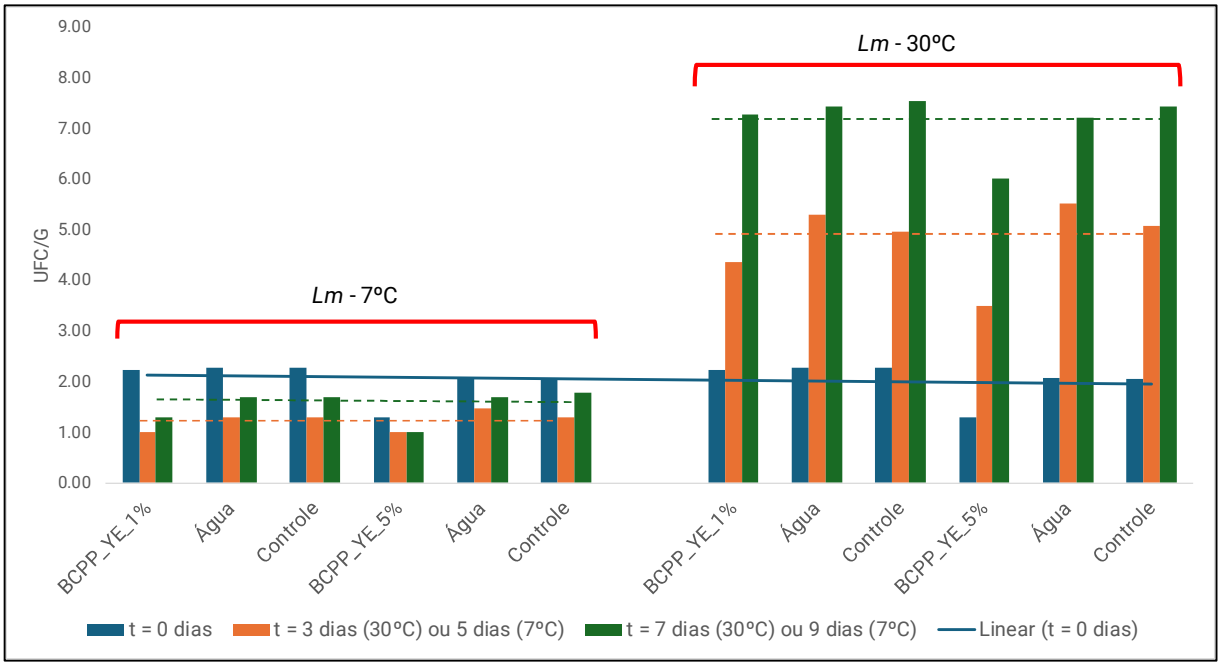


Figura A1. Contagem de *Listeria monocytogenes* (*Lm*) no tempo t = 0 dias e nos tempos t = 3 e 7 dias para as amostras incubadas a 30ºC, e nos tempos t = 5 e 9 dias para as amostras incubadas a 7ºC. Tratamentos: imersão em BCPP_YE 5% e BCPP_YE 1%, com seus respectivos controles (imersão em água destilada estéril e controle sem imersão).

Descriptives

	Biopreservative/Concentration	T8	T16	T24	T32	T40	T48	T56	T64	T72	T80	T88	T96
Mean	BCPP_SP / 0.20%	0.0463	1.0743	1.0470	0.9867	0.9413	0.9137	0.8700	0.8090	0.7513	0.6780	0.6067	0.5550
	BCPP_SP / 0.40%	0.0177	0.8087	0.7883	0.7430	0.7247	0.7093	0.6997	0.6883	0.6827	0.6770	0.6560	0.6453
	BCPP_SP / 0.60%	0.0147	0.4017	0.4570	0.4493	0.4353	0.4273	0.4063	0.3907	0.3887	0.3833	0.3937	0.3987
	BCPP_SP / 0.80%	0.0090	0.0100	0.0120	0.0577	0.0900	0.1210	0.1573	0.1743	0.1957	0.2000	0.2043	0.2160
	BCPP_SP / 1.00%	0.0027	0.0010	0.0007	0.0047	0.0030	0.0003	0.0010	0.0003	0.0013	0.0017	0.0030	0.0020
	BCPP_SP / CN	0.0027	0.0003	0.0020	0.0027	0.0013	0.0020	0.0010	0.0007	0.0020	0.0010	0.0017	0.0010
	BCPP_SP / CP	0.0300	1.2600	1.2233	1.1647	1.0617	1.0113	0.9450	0.8437	0.7767	0.6503	0.5943	0.5393
	BCPP_YE / 0.20%	0.0247	1.0593	1.0283	0.9777	0.9430	0.9020	0.8597	0.8137	0.7713	0.7117	0.6430	0.6217
	BCPP_YE / 0.40%	0.0207	0.8030	0.7937	0.7597	0.7250	0.7070	0.7030	0.6937	0.6867	0.6733	0.6500	0.6427
	BCPP_YE / 0.60%	0.0170	0.4153	0.4563	0.4430	0.4067	0.3997	0.3907	0.3907	0.3843	0.3773	0.3810	0.3803
	BCPP_YE / 0.80%	0.0080	0.0080	0.0187	0.0383	0.0677	0.0917	0.1353	0.1477	0.1620	0.1660	0.1770	0.1963
	BCPP_YE / 1.00%	0.0017	0.0033	0.0003	0.0023	0.0007	-0.0003	-0.0003	0.0007	0.0017	0.0007	-0.0003	0.0013
	BCPP_YE / CN	0.0013	0.0033	0.0003	0.0000	0.0000	0.0007	-0.0003	-0.0007	-0.0003	0.0003	0.0017	0.0003
	BCPP_YE / CP	0.0267	1.2527	1.2190	1.1647	1.0477	0.9723	0.8697	0.8030	0.7233	0.6423	0.5820	0.5357
Standard deviation	BCPP_SP / 0.20%	0.0225	0.0060	0.0095	0.0111	0.0065	0.0060	0.0229	0.0213	0.0071	0.0193	0.0136	0.0210
	BCPP_SP / 0.40%	0.0100	0.0247	0.0228	0.0423	0.0283	0.0379	0.0329	0.0346	0.0319	0.0361	0.0342	0.0314
	BCPP_SP / 0.60%	0.0111	0.0015	0.0044	0.0078	0.0067	0.0070	0.0159	0.0164	0.0157	0.0136	0.0159	0.0127
	BCPP_SP / 0.80%	0.0026	0.0020	0.0026	0.0124	0.0056	0.0151	0.0101	0.0035	0.0050	0.0061	0.0136	0.0250
	BCPP_SP / 1.00%	0.0031	0.0010	0.0031	0.0064	0.0075	0.0021	0.0030	0.0015	0.0015	0.0021	0.0010	0.0010
	BCPP_SP / CN	0.0021	0.0015	0.0010	0.0025	0.0015	0.0010	0.0026	0.0031	0.0010	0.0020	0.0015	0.0026
	BCPP_SP / CP	0.0125	0.0078	0.0182	0.0107	0.0119	0.0360	0.0335	0.0323	0.0542	0.0481	0.0376	0.0119
	BCPP_YE / 0.20%	0.0116	0.0060	0.0070	0.0188	0.0079	0.0207	0.0229	0.0292	0.0085	0.0340	0.0085	0.0146
	BCPP_YE / 0.40%	0.0123	0.0128	0.0075	0.0140	0.0100	0.0261	0.0180	0.0162	0.0133	0.0137	0.0145	0.0167
	BCPP_YE / 0.60%	0.0070	0.0091	0.0090	0.0182	0.0083	0.0186	0.0140	0.0160	0.0160	0.0147	0.0113	0.0127
	BCPP_YE / 0.80%	0.0030	0.0030	0.0085	0.0068	0.0228	0.0294	0.0286	0.0206	0.0167	0.0156	0.0139	0.0205
	BCPP_YE / 1.00%	0.0025	0.0021	0.0023	0.0021	0.0032	0.0015	0.0025	0.0021	0.0021	0.0025	0.0025	0.0021
	BCPP_YE / CN	0.0015	0.0006	0.0015	0.0026	0.0010	0.0006	0.0012	0.0015	0.0021	0.0015	0.0025	0.0015
	BCPP_YE / CP	0.0031	0.0150	0.0494	0.0100	0.0070	0.0065	0.0617	0.0721	0.0933	0.0381	0.0183	0.0047
Minimum	BCPP_SP / 0.20%	0.0210	1.0680	1.0360	0.9750	0.9350	0.9080	0.8450	0.7860	0.7450	0.6570	0.5960	0.5400
	BCPP_SP / 0.40%	0.0100	0.7880	0.7680	0.7040	0.6990	0.6750	0.6700	0.6580	0.6540	0.6470	0.6310	0.6220
	BCPP_SP / 0.60%	0.0030	0.4000	0.4520	0.4430	0.4310	0.4200	0.3880	0.3720	0.3710	0.3690	0.3830	0.3890
	BCPP_SP / 0.80%	0.0060	0.0080	0.0100	0.0500	0.0850	0.1090	0.1510	0.1710	0.1910	0.1930	0.1890	0.1910
	BCPP_SP / 1.00%	0.0000	0.0000	-0.0020	0.0000	-0.0050	-0.0020	-0.0020	-0.0010	0.0000	0.0000	0.0020	0.0010
	BCPP_SP / CN	0.0010	-0.0010	0.0010	0.0000	0.0000	0.0010	-0.0010	-0.0020	0.0010	-0.0010	0.0000	-0.0010
	BCPP_SP / CP	0.0180	1.2550	1.2070	1.1530	1.0480	0.9760	0.9120	0.8140	0.7250	0.6040	0.5510	0.5260
	BCPP_YE / 0.20%	0.0140	1.0530	1.0210	0.9560	0.9340	0.8790	0.8340	0.7800	0.7630	0.6740	0.6340	0.6060
	BCPP_YE / 0.40%	0.0070	0.7920	0.7860	0.7460	0.7150	0.6820	0.6850	0.6790	0.6780	0.6610	0.6350	0.6240
	BCPP_YE / 0.60%	0.0100	0.4070	0.4460	0.4220	0.4000	0.3800	0.3770	0.3740	0.3690	0.3660	0.3680	0.3690
	BCPP_YE / 0.80%	0.0050	0.0050	0.0100	0.0330	0.0430	0.0600	0.1040	0.1280	0.1440	0.1560	0.1690	0.1760
	BCPP_YE / 1.00%	-0.0010	0.0010	-0.0010	0.0000	-0.0030	-0.0020	-0.0030	-0.0010	0.0000	-0.0020	-0.0030	-0.0010
	BCPP_YE / CN	0.0000	0.0030	-0.0010	-0.0020	-0.0010	0.0000	-0.0010	-0.0020	-0.0020	-0.0010	-0.0010	-0.0010
	BCPP_YE / CP	0.0240	1.2360	1.1620	1.1570	1.0410	0.9660	0.8140	0.7460	0.6490	0.6160	0.5660	0.5320
Maximum	BCPP_SP / 0.20%	0.0640	1.0800	1.0530	0.9970	0.9480	0.9200	0.8900	0.8280	0.7590	0.6950	0.6220	0.5790
	BCPP_SP / 0.40%	0.0290	0.8360	0.8130	0.7880	0.7550	0.7500	0.7350	0.7260	0.7170	0.7170	0.6950	0.6810
	BCPP_SP / 0.60%	0.0250	0.4030	0.4600	0.4580	0.4430	0.4340	0.4170	0.4030	0.4010	0.3960	0.4120	0.4130
	BCPP_SP / 0.80%	0.0110	0.0120	0.0150	0.0720	0.0960	0.1380	0.1690	0.1780	0.2010	0.2040	0.2150	0.2410
	BCPP_SP / 1.00%	0.0060	0.0020	0.0040	0.0120	0.0100	0.0020	0.0040	0.0020	0.0030	0.0040	0.0040	0.0030
	BCPP_SP / CN	0.0050	0.0020	0.0030	0.0050	0.0030	0.0030	0.0040	0.0040	0.0030	0.0030	0.0030	0.0040
	BCPP_SP / CP	0.0430	1.2690	1.2430	1.1740	1.0700	1.0480	0.9790	0.8780	0.8330	0.7000	0.6190	0.5490
	BCPP_YE / 0.20%	0.0370	1.0650	1.0350	0.9890	0.9490	0.9190	0.8780	0.8320	0.7800	0.7400	0.6510	0.6350
	BCPP_YE / 0.40%	0.0310	0.8170	0.8010	0.7740	0.7350	0.7340	0.7210	0.7110	0.7020	0.6880	0.6640	0.6560
	BCPP_YE / 0.60%	0.0240	0.4250	0.4620	0.4540	0.4160	0.4170	0.4050	0.4060	0.4010	0.3940	0.3880	0.3940
	BCPP_YE / 0.80%	0.0110	0.0110	0.0270	0.0460	0.0880	0.1180	0.1600	0.1690	0.1770	0.1840	0.1930	0.2170
	BCPP_YE / 1.00%	0.0040	0.0050	0.0030	0.0040	0.0030	0.0010	0.0020	0.0030	0.0040	0.0030	0.0020	0.0030
	BCPP_YE / CN	0.0030	0.0040	0.0020	0.0030	0.0010	0.0010	0.0010	0.0010	0.0020	0.0020	0.0040	0.0020
	BCPP_YE / CP	0.0300	1.2650	1.2500	1.1760	1.0550	0.9790	0.9360	0.8840	0.8280	0.6860	0.6020	0.5410
Shapiro-Wilk W	BCPP_SP / 0.20%	0.9129	0.9908	0.7940	0.9891	0.9980	0.9908	0.9643	0.9735	0.9735	0.9678	0.9119	0.8622
	BCPP_SP / 0.40%	0.8995	0.9453	0.9729	0.9849	0.9796	0.9791	0.9777	0.9663	0.9763	0.9423	0.8745	0.8841
	BCPP_SP / 0.60%	0.9891	0.9643	0.8421	0.9323	0.8120	0.9932	0.8267	0.8887	0.9134	0.9887	0.8267	0.8981
	BCPP_SP / 0.80%	0.8929	1.0000	0.8929	0.7840	0.9758	0.9181	0.7915	0.9932	0.9868	0.8176	0.9119	1.0000
	BCPP_SP / 1.00%	0.9643	1.0000	0.9643	0.8710	0.9868	0.9231	1.0000	0.9643	0.9643	0.9231	1.0000	1.0000
	BCPP_SP / CN	0.9231	0.9643	1.0000	0.9868	0.9643	1.0000	0.8929	0.9643	1.0000	1.0000	0.9643	0.8929
	BCPP_SP / CP	0.9952	0.8033	0.9749	0.9643	0.8501	0.9990	0.9993	0.9843	0.9944	0.9964	0.8156	0.9292
	BCPP_YE / 0.20%	0.9845	0.9908	0.9932	0.7727	0.8929	0.9368	0.9231	0.7931	0.9988	0.9434	0.9897	0.9809

Descriptives

	Biopreservative/Concentration	T8	T16	T24	T32	T40	T48	T56	T64	T72	T80	T88	T96
	BCPP_YE / 0.40%	0.9453	0.9586	0.9985	0.9983	1.0000	0.9956	1.0000	0.9796	0.8120	0.9781	0.9964	0.9231
	BCPP_YE / 0.60%	1.0000	0.9838	0.7967	0.7734	0.9231	0.9882	0.9983	0.9948	0.9948	0.9018	0.7874	0.9745
	BCPP_YE / 0.80%	1.0000	1.0000	0.9988	0.9119	0.9729	0.9753	0.9592	0.9951	0.9758	0.8033	0.7500	0.9998
	BCPP_YE / 1.00%	0.9868	0.9231	0.7500	0.9231	0.8710	0.9643	0.9868	0.9231	0.9231	0.9868	0.9868	0.9231
	BCPP_YE / CN	0.9643	0.7500	0.9643	0.8929	1.0000	0.7500	0.7500	0.9643	0.9231	0.9643	0.9868	0.9643
	BCPP_YE / CP	0.9643	0.9372	0.7925	0.8995	0.9932	0.9980	0.9776	0.9168	0.9207	0.8446	0.9643	0.9067
Shapiro-Wilk p	BCPP_SP / 0.20%	0.428	0.817	0.100	0.800	0.915	0.817	0.637	0.688	0.688	0.656	0.424	0.274
	BCPP_SP / 0.40%	0.384	0.549	0.684	0.765	0.726	0.723	0.714	0.647	0.705	0.537	0.308	0.337
	BCPP_SP / 0.60%	0.800	0.637	0.220	0.497	0.144	0.843	0.180	0.350	0.430	0.797	0.180	0.380
	BCPP_SP / 0.80%	0.363	1.000	0.363	0.077	0.702	0.446	0.094	0.843	0.780	0.157	0.424	1.000
	BCPP_SP / 1.00%	0.637	1.000	0.637	0.298	0.780	0.463	1.000	0.637	0.637	0.463	1.000	1.000
	BCPP_SP / CN	0.463	0.637	1.000	0.780	0.637	1.000	0.363	0.637	1.000	1.000	0.637	0.363
	BCPP_SP / CP	0.868	0.122	0.696	0.637	0.241	0.939	0.951	0.760	0.857	0.885	0.152	0.485
	BCPP_YE / 0.20%	0.762	0.817	0.843	0.051	0.363	0.515	0.463	0.098	0.935	0.541	0.806	0.736
	BCPP_YE / 0.40%	0.549	0.609	0.927	0.921	1.000	0.873	1.000	0.726	0.144	0.716	0.886	0.463
	BCPP_YE / 0.60%	1.000	0.756	0.107	0.052	0.463	0.792	0.921	0.862	0.862	0.391	0.085	0.694
	BCPP_YE / 0.80%	1.000	1.000	0.935	0.424	0.684	0.698	0.612	0.866	0.702	0.122	< .001	0.973
	BCPP_YE / 1.00%	0.780	0.463	< .001	0.463	0.298	0.637	0.780	0.463	0.463	0.780	0.780	0.463
	BCPP_YE / CN	0.637	< .001	0.637	0.363	1.000	< .001	< .001	0.637	0.463	0.637	0.780	0.637
	BCPP_YE / CP	0.637	0.516	0.097	0.384	0.843	0.915	0.713	0.441	0.455	0.226	0.637	0.407

ANOVA

ANOVA - Absorbance

	Sum of Squares	df	Mean Square	F	p
Biopreservatives	0.0004	1	0.0004	2.0109	0.157
Concentration	57.3241	6	9.5540	43832.9180	< .001
Time	8.0106	11	0.7282	3341.0647	< .001
Biopreservatives ✻ Concentration	0.0012	6	0.0002	0.8938	0.500
Biopreservatives ✻ Time	0.0014	11	0.0001	0.5964	0.832
Concentration ✻ Time	9.4466	66	0.1431	656.6667	< .001
Biopreservatives ✻ Concentration ✻ Time	0.0091	66	0.0001	0.6333	0.987
Residuals	0.0732	336	0.0002		

Post Hoc Tests

Post Hoc Comparisons - Biopreservatives ✻ Concentration

Comparison									
Biopreservatives	**Concentration**		**Biopreservatives**	**Concentration**	**Mean Difference**	**SE**	**df**	**t**	**p_{tukey}**
BCPP_SP	0.20%	-	BCPP_SP	0.40%	0.1244	0.0035	336.0000	35.7538	< .001
		-	BCPP_SP	0.60%	0.3989	0.0035	336.0000	114.6373	< .001
		-	BCPP_SP	0.80%	0.6594	0.0035	336.0000	189.4975	< .001
		-	BCPP_SP	1.00%	0.7760	0.0035	336.0000	223.0002	< .001
		-	BCPP_SP	CN	0.7763	0.0035	336.0000	223.0801	< .001
		-	BCPP_SP	CP	-0.0583	0.0035	336.0000	-16.7474	< .001
		-	BCPP_YE	0.20%	-0.0016	0.0035	336.0000	-0.4550	1.000
		-	BCPP_YE	0.40%	0.1229	0.0035	336.0000	35.3307	< .001
		-	BCPP_YE	0.60%	0.4066	0.0035	336.0000	116.8324	< .001
		-	BCPP_YE	0.80%	0.6639	0.0035	336.0000	190.7747	< .001
		-	BCPP_YE	1.00%	0.7768	0.0035	336.0000	223.2397	< .001
		-	BCPP_YE	CN	0.7773	0.0035	336.0000	223.3674	< .001
		-	BCPP_YE	CP	-0.0561	0.0035	336.0000	-16.1167	< .001
	0.40%	-	BCPP_SP	0.60%	0.2745	0.0035	336.0000	78.8835	< .001
		-	BCPP_SP	0.80%	0.5350	0.0035	336.0000	153.7437	< .001
		-	BCPP_SP	1.00%	0.6516	0.0035	336.0000	187.2464	< .001
		-	BCPP_SP	CN	0.6519	0.0035	336.0000	187.3263	< .001
		-	BCPP_SP	CP	-0.1827	0.0035	336.0000	-52.5012	< .001
		-	BCPP_YE	0.20%	-0.1260	0.0035	336.0000	-36.2088	< .001
		-	BCPP_YE	0.40%	-0.0015	0.0035	336.0000	-0.4231	1.000
		-	BCPP_YE	0.60%	0.2821	0.0035	336.0000	81.0787	< .001
		-	BCPP_YE	0.80%	0.5394	0.0035	336.0000	155.0209	< .001
		-	BCPP_YE	1.00%	0.6524	0.0035	336.0000	187.4859	< .001
		-	BCPP_YE	CN	0.6529	0.0035	336.0000	187.6136	< .001
		-	BCPP_YE	CP	-0.1805	0.0035	336.0000	-51.8705	< .001
	0.60%	-	BCPP_SP	0.80%	0.2605	0.0035	336.0000	74.8603	< .001
		-	BCPP_SP	1.00%	0.3771	0.0035	336.0000	108.3630	< .001
		-	BCPP_SP	CN	0.3774	0.0035	336.0000	108.4428	< .001
		-	BCPP_SP	CP	-0.4572	0.0035	336.0000	-131.3846	< .001
		-	BCPP_YE	0.20%	-0.4005	0.0035	336.0000	-115.0923	< .001
		-	BCPP_YE	0.40%	-0.2760	0.0035	336.0000	-79.3065	< .001
		-	BCPP_YE	0.60%	0.0076	0.0035	336.0000	2.1952	0.633
		-	BCPP_YE	0.80%	0.2649	0.0035	336.0000	76.1375	< .001
		-	BCPP_YE	1.00%	0.3779	0.0035	336.0000	108.6025	< .001
		-	BCPP_YE	CN	0.3784	0.0035	336.0000	108.7302	< .001
		-	BCPP_YE	CP	-0.4550	0.0035	336.0000	-130.7540	< .001
	0.80%	-	BCPP_SP	1.00%	0.1166	0.0035	336.0000	33.5027	< .001
		-	BCPP_SP	CN	0.1169	0.0035	336.0000	33.5825	< .001
		-	BCPP_SP	CP	-0.7177	0.0035	336.0000	-206.2449	< .001
		-	BCPP_YE	0.20%	-0.6610	0.0035	336.0000	-189.9525	< .001
		-	BCPP_YE	0.40%	-0.5365	0.0035	336.0000	-154.1668	< .001
		-	BCPP_YE	0.60%	-0.2529	0.0035	336.0000	-72.6651	< .001
		-	BCPP_YE	0.80%	0.0044	0.0035	336.0000	1.2772	0.992
		-	BCPP_YE	1.00%	0.1174	0.0035	336.0000	33.7422	< .001
		-	BCPP_YE	CN	0.1179	0.0035	336.0000	33.8699	< .001
		-	BCPP_YE	CP	-0.7155	0.0035	336.0000	-205.6143	< .001
	1.00%	-	BCPP_SP	CN	0.0003	0.0035	336.0000	0.0798	1.000
		-	BCPP_SP	CP	-0.8343	0.0035	336.0000	-239.7476	< .001
		-	BCPP_YE	0.20%	-0.7776	0.0035	336.0000	-223.4552	< .001

Note. Comparisons are based on estimated marginal means

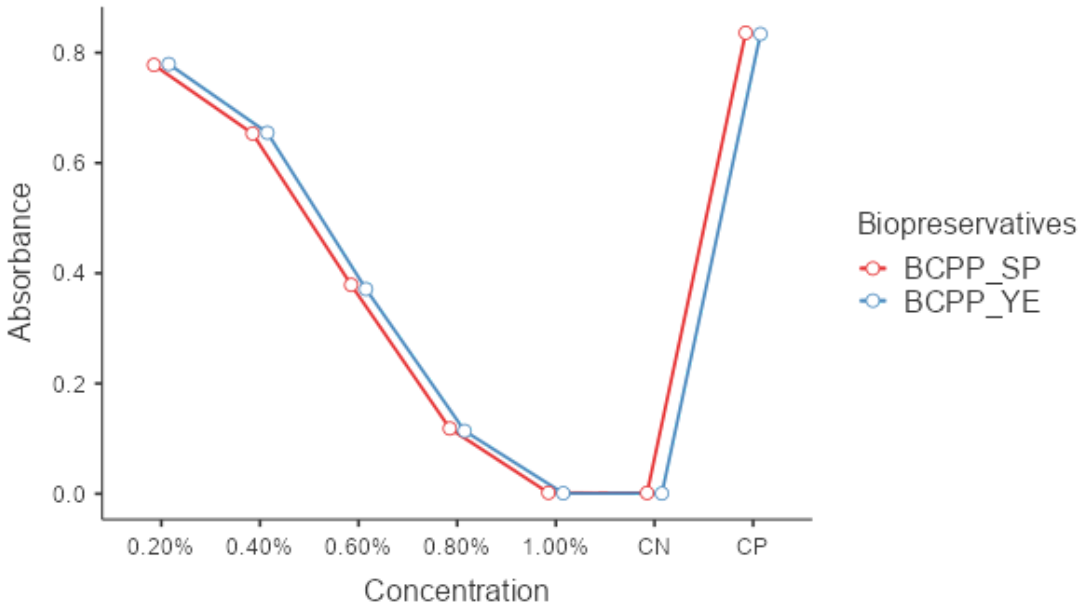
Post Hoc Comparisons - Biopreservatives ✻ Concentration

Comparison									
Biopreservatives	Concentration		Biopreservatives	Concentration	Mean Difference	SE	df	t	p_{tukey}
		-	BCPP_YE	0.40%	-0.6531	0.0035	336.0000	-187.6695	< .001
		-	BCPP_YE	0.60%	-0.3694	0.0035	336.0000	-106.1678	< .001
		-	BCPP_YE	0.80%	-0.1121	0.0035	336.0000	-32.2255	< .001
		-	BCPP_YE	1.00%	0.0008	0.0035	336.0000	0.2395	1.000
		-	BCPP_YE	CN	0.0013	0.0035	336.0000	0.3672	1.000
		-	BCPP_YE	CP	-0.8321	0.0035	336.0000	-239.1170	< .001
	CN	-	BCPP_SP	CP	-0.8346	0.0035	336.0000	-239.8274	< .001
		-	BCPP_YE	0.20%	-0.7779	0.0035	336.0000	-223.5351	< .001
		-	BCPP_YE	0.40%	-0.6533	0.0035	336.0000	-187.7493	< .001
		-	BCPP_YE	0.60%	-0.3697	0.0035	336.0000	-106.2476	< .001
		-	BCPP_YE	0.80%	-0.1124	0.0035	336.0000	-32.3053	< .001
		-	BCPP_YE	1.00%	0.0006	0.0035	336.0000	0.1597	1.000
		-	BCPP_YE	CN	0.0010	0.0035	336.0000	0.2874	1.000
		-	BCPP_YE	CP	-0.8324	0.0035	336.0000	-239.1968	< .001
	CP	-	BCPP_YE	0.20%	0.0567	0.0035	336.0000	16.2924	< .001
		-	BCPP_YE	0.40%	0.1812	0.0035	336.0000	52.0781	< .001
		-	BCPP_YE	0.60%	0.4648	0.0035	336.0000	133.5798	< .001
		-	BCPP_YE	0.80%	0.7221	0.0035	336.0000	207.5221	< .001
		-	BCPP_YE	1.00%	0.8351	0.0035	336.0000	239.9871	< .001
		-	BCPP_YE	CN	0.8356	0.0035	336.0000	240.1148	< .001
		-	BCPP_YE	CP	0.0022	0.0035	336.0000	0.6306	1.000
BCPP_YE	0.20%	-	BCPP_YE	0.40%	0.1245	0.0035	336.0000	35.7857	< .001
		-	BCPP_YE	0.60%	0.4081	0.0035	336.0000	117.2875	< .001
		-	BCPP_YE	0.80%	0.6654	0.0035	336.0000	191.2297	< .001
		-	BCPP_YE	1.00%	0.7784	0.0035	336.0000	223.6947	< .001
		-	BCPP_YE	CN	0.7789	0.0035	336.0000	223.8224	< .001
		-	BCPP_YE	CP	-0.0545	0.0035	336.0000	-15.6617	< .001
	0.40%	-	BCPP_YE	0.60%	0.2836	0.0035	336.0000	81.5017	< .001
		-	BCPP_YE	0.80%	0.5409	0.0035	336.0000	155.4440	< .001
		-	BCPP_YE	1.00%	0.6539	0.0035	336.0000	187.9090	< .001
		-	BCPP_YE	CN	0.6543	0.0035	336.0000	188.0367	< .001
		-	BCPP_YE	CP	-0.1790	0.0035	336.0000	-51.4475	< .001
	0.60%	-	BCPP_YE	0.80%	0.2573	0.0035	336.0000	73.9423	< .001
		-	BCPP_YE	1.00%	0.3703	0.0035	336.0000	106.4073	< .001
		-	BCPP_YE	CN	0.3707	0.0035	336.0000	106.5350	< .001
		-	BCPP_YE	CP	-0.4626	0.0035	336.0000	-132.9492	< .001
	0.80%	-	BCPP_YE	1.00%	0.1130	0.0035	336.0000	32.4650	< .001
		-	BCPP_YE	CN	0.1134	0.0035	336.0000	32.5927	< .001
		-	BCPP_YE	CP	-0.7199	0.0035	336.0000	-206.8915	< .001
	1.00%	-	BCPP_YE	CN	0.0004	0.0035	336.0000	0.1277	1.000
		-	BCPP_YE	CP	-0.8329	0.0035	336.0000	-239.3565	< .001
	CN	-	BCPP_YE	CP	-0.8334	0.0035	336.0000	-239.4842	< .001

Note. Comparisons are based on estimated marginal means

Estimated Marginal Means

Concentration ✻ Biopreservatives


Absorbance
0.8
0.6
0.4
0.2
0.0
0.20%
0.40%
0.60%
0.80%
1.00%
CN
CP
Concentration
Biopreservatives
BCPP_SP
BCPP_YE