PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
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GABRIELA MAIA DANIELSKI

CARNOBACTERIUM MALTAROMATICUM COMO CULTURA BIOPROTETORA CONTRA PATÓGENOS ALIMENTARES DE INTERESSE EM PRODUTOS CÁRNEOS PERECÍVEIS

(*Carnobacterium maltaromaticum* as bioprotective culture against food pathogens of interest in perishable meat products)

CURITIBA
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Dissertação oriunda do programa institucional PIBIC Master *Combined Degree*, apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Saúde, Tecnologia e Produção Animal, da Escola de Ciências da Vida da Pontifícia Universidade Católica do Paraná, para obtenção do título de Mestre em Ciência Animal.
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ABREVIAÇÕES

BAL: Bactérias ácido láticas

BHI: Brain Heart Infusion

CDC: Centers for Disease Control and Prevention

CIM: Concentração inibitória mínima

DTAs: Doenças Transmitidas por Alimentos

ECDC: European Center for Disease Prevention and Control

GRAS: Generally recognized as safe

LAB: Lactic acid bacteria

MIC: Minimum inhibitory concentration

OMS: Organização Mundial da Saúde

PCA: Plate Counter Agar

WHO: World Health Organization

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FORMATO DA DISSERTAÇÃO

A presente dissertação é composta por capítulos. O *Capítulo 1* apresenta uma introdução geral, a contextualização do tema, o estado da arte e os objetivos da pesquisa. O *Capítulo 2* apresenta o artigo da dissertação que será submetido à revista científica de interesse a essa pesquisa, relatando os materiais e métodos, resultados e discussões do presente trabalho. O *Capítulo 3* finaliza esta dissertação com conclusões e considerações finais deste trabalho.

RESUMO GERAL

Biopreservação é um conceito que tem recebido a atenção da indústria alimentícia como um meio de controlar naturalmente a vida útil e a segurança dos alimentos. As bactérias láticas (BAL) apresentam significativo potencial para uso como biopreservativo em alimentos e rações. *Carnobacterium maltaromaticum* é uma espécie de bactéria lática que pode ser encontrada como microbiota natural de carne refrigerada, peixe e produtos lácteos, conhecida pela sua habilidade em sobreviver sob condições de refrigeração. Essa espécie bacteriana tem demonstrado capacidade de inibir microorganismos patogênicos e deteriorantes em diversas matrizes alimentares, exercendo seu efeito inibitório devido à competição por nutrientes e/ou produção de metabólicos como bacteriocinas. Neste sentido, seu uso como cultura bioprotetora natural em produtos cárneos, especialmente os refrigerados, pode trazer benefícios tanto à indústria quanto aos consumidores. Este estudo objetivou avaliar o efeito bioprotetor de *C. maltaromaticum* contra patógenos alimentares, *in vitro* e em presunto cozido. Três diferentes cepas de *C. maltaromaticum* (CM_B824, CM_B827 e CM_B829) e três patógenos alimentares, *Eschericia coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 19117 e *Salmonella* Typhimurium ATCC 14028 foram cultivados em co-cultura a uma concentração de 10^6 UFC/mL de cada cepa de *C. maltaromaticum* e 10^3 UFC/mL de cada patógeno. A incubação ocorreu a -1 °C durante 28 dias, 4 °C durante 14 dias e 25 °C durante 48 horas. A inibição dos patógenos foi verificada pela contagem em placas. A ação inibitória sobre os mesmos patógenos também foi avaliada em co-cultura, a 25 °C, com adição de EDTA a 1mM. Esse agente quelante foi adicionado visando meior interação com bactérias gram-negativas. O efeito antimicrobiano do sobrenadante livre de células (CFS) de *C. maltaromaticum* foi avaliado utilizando duas diferentes metodologias: difusão em poço de ágar e concentração inibitória mínima em tubos. O efeito bioprotetor das três cepas de *C. maltaromaticum* foi avaliado contra *Listeria innocua,* que foi utilizada como modelo para *L. monocytogenes* por questões de biossegurança e manipulação, em presunto cozido. Fatias de presunto foram inoculadas com *C. maltaromaticum* (10^6 UFC/mL) e um pool de *L. innocua* (33014, 33016 e HPB 568) (10^4 UFC/mL). As amostras foram mantidas sob refrigeração a 4 °C por 7 dias e avaliadas quanto ao pH, cor instrumental e contagem microbiológica nos dias 0, 2, 5 e 7. Nos testes *in vitro* a -1 °C e 4 °C, as cepas de *C. maltaromaticum* testadas mostraram efeito inibitório sobre *L. monocytogenes*. Uma leve inibição dos patógenos gram-negativos também foi verficada em co-cultura adicionada de EDTA. A atividade antimicrobiana do CFS não foi observada contra os patógenos nas condições estudadas. A adição de *C. maltaromaticum* não afetou a cor ou o pH do presunto, sendo verificada significativa redução na contagem de *L. innocua* a partir do segundo dia de estocagem. A combinação dessas duas barreiras, estudadas nesse projeto, (armazenamento sob refrigeração e adição de *C. maltaromaticum* como cultura bioprotetora) apresenta-se como estratégia promissora para o aumento da inocuidade de produtos cárneos.

Palavras-chave: atividade antilistéria, culturas bioprotetoras, biopreservação, bactérias ácido láticas.

ABSTRACT

Biopreservation has received the attention of food industry as a mean of naturally controlling the shelf life and safety of food. Lactic acid bacteria (LAB) have significant potential for use in biopreservation and have been traditionally used as natural biopreservatives of food and feed. Carnobacteria are ubiquitous LAB isolated from cold and temperate environments and can be found as natural microbiota of chilled meat, fish and dairy products, well known for its ability in survive under refrigeration. This bacterial species has demonstrated the capacity of inhibiting deteriorating and pathogenic microrganisms in various food matrices by their inhibitory effect due the competition of nutrients and/or production of metabolites such as bacteriocins. Taking these characteristics in account, its use as a natural bioprotective culture in meat products, especially those storage under refrigeration, can bring benefits to both industry and consumers. The aim of this study is then, to evaluate the bioprotective effect *of Carnobacterium maltaromaticum* against food pathogens *in vitro* and in cooked ham. Three different strains of *C. maltaromaticum* (CM_B824, CM_B827 and CM_B829) and three strains of pathogenic bacteria, *E. coli* O157:H7 ATCC 35150, *L. monocytogenes* ATCC 19117 and *Salmonella* Typhimurium ATCC 14028 were grown in co-culture at a concentration of 10^6 CFU/mL of each strain of *C. maltaromaticum* and 10^3 CFU/mL of each pathogen. The incubation happened at −1 °C for 28 days, 4 °C for 14 days and 25 °C for 48 hours respectively. The inhibition of the pathogens was verified by plate counting. The inhibitory action against the same pathogens was also measured in co-culture at 25°C, with the addition of EDTA. This chelating agente was added aiming a better interaction with gram-negative bacteria. The antimicrobial effect of the cell-free supernatant (CFS) of *C. maltaromaticum* was evaluated using two different approaches: agar well diffusion and minimum inhibitory concentration in tubes. The bioprotective effect of the three isolates of *C. maltaromaticum* was assessed against *Listeria innocua*, that was used as a surogate of *L. monocytogenes* in cooked ham. Ham slices were inoculated with *C. maltaromaticum* (10^6 CFU/mL) and a pool of *L. innocua* (33014, 33016 e HPB 568) (10^4 CFU/mL). The ham samples were storaged under refrigeration at 4°C for 7 days and the pH, instrumental color and microbial counting were evaluated at the days 0, 2, 5 and 7. *In vitro* tests at -1°C and 4°C showed inhibitory effect against *L. monocytogenes.* An inhibitory effect against the gram-negative pathogens was also verified at the co-cultures test added with EDTA. The antimicrobial effect of CFS was not observed against the pathogens under the studied conditions. The addition of *C. maltaromaticum* in cooked ham did not changed the color or the pH of the product, although a significant inhibition of the *L. innocua* counting already in the second day of storage was verified. The association of two hurdles, studied in this project (storage under refrigeration and addition of *C. maltaromaticum* as bioprotective culture) shows itself as a promising strategy for increasing the safety of meat products.

Keywords: antilisterial activity, bioprotective cultures, biopreservation, lactic acid bacteria.

CAPÍTULO 1

INTRODUÇÃO E CONTEXTUALIZAÇÃO GERAL

1.1. Doenças Transmitidas por Alimentos

As doenças transmitidas por alimentos (DTA) são doenças causadas pela ingestão de alimentos contaminados por um agente infeccioso e, ou pela toxina produzida por esse agente. Já os surtos alimentares são definidos como, um incidente em que duas ou mais pessoas apresentam sintomas semelhantes após a ingestão do mesmo alimento, ou água, que podem ter sido contaminados por diversos agentes como, bactérias, vírus, príons, produtos químicos, agroquímicos, toxinas, metais pesados e parasitas (Sousa, 2016).

A Organização Mundial da Saúde (OMS) reconhece os surtos alimentares como uma importante ameaça à saúde da população mundial pois pode causar sintomas como, diarreia, vômitos, náuseas e dores abdominais, acompanhadas ou não por febre (Salter, 2014). A diarreia causada por DTAs é considerada, principalmente em países subdesenvolvidos e em desenvolvimento, uma importante causa de morbidade e mortalidade em crianças (Tobias *et al.*, 2015), por isso o controle desses surtos é considerado de grande importância mundial.

De acordo com a OMS estima-se que todos os anos 580 milhões de pessoas sejam acometidas por esse tipo de doença mundialmente, com mais de 350 mil mortes ao ano (WHO, 2015). No Brasil, dados de 2007 a 2016 indicam que cerca de 118.000 pessoas ficaram doentes e 17.000 foram hospitalizadas devido aos surtos alimentares ocorridos durante esse período (Sousa, 2016). Na Austrália mais de 10 mil casos de surtos alimentares foram reportados durante os meses de junho a setembro de 2015. Dos pacientes acometidos pelas DTAs, 28% buscam assistência médica, gerando um custo de aproximadamente 1,2 bilhões de dólares ao ano para o governo do país (OzFoodNet, 2015). Nos Estados Unidos o número estimado de pacientes que contraem uma DTA por ano é de 48 milhões de pessoas (Roshanfekr *et al.*, 2014).

A globalização e o desenvolvimento do mercado e comércio internacionais aumentam o risco de surtos causados por alimentos e a rapidez de distribuição desses patógenos, seja pela venda de produtos contaminados ou pelo possível risco de

contaminação interna, por patógenos provenientes de origens externas. Esse risco é ainda favorecido pela diferença de fiscalização e padrões de segurança alimentar dentre países (Callejón *et al.*, 2015).

De acordo com o European Centre for Disease Control (ECDC), em 2013, a categoria de alimentos que mais causou surtos de infecções alimentares na Europa foi a de carnes e produtos cárneos, com 23,7% dos surtos (Hennekinne, 2015; Jayasena, 2013). Nos Estados Unidos esses mesmos produtos foram responsáveis por cerca de 20.000 surtos, no período de 1998 a 2016 (CDC, 2016).

No Brasil, os produtos cárneos corresponderam a cerca de 5% dos alimentos veiculadores de doenças transmitidas por alimentos nos anos de 2000 a 2016. Outros produtos de origem animal, como leite e seus derivados e ovos, também aparecem dentre os mais importantes veiculadores de patógenos. Levando em consideração todos os produtos de origem animal, a porcentagem de disseminação de DTA sobe para 12% (Sousa, 2016). Porém esse valor pode ser maior do que o reportado às autoridades públicas. Alguns patógenos causam sintomas brandos, não havendo necessidade, pelo ponto de vista do paciente, de procurar atendimento médico ou ajuda especializada, o que dificulta a vigilância dessas doenças (de Oliveira, et.al., 2010).

Dentre os mais importantes agentes infecciosos causadores de surtos alimentares no Brasil do período de 2000 a 2015, encontram-se as bactérias, identificadas como causadoras de 90% dos casos. Logo em seguida, causando cerca de 7% do número de casos de DTA, encontram-se os vírus. Dentre as principais bactérias responsáveis por esse tipo de surto, aponta-se a *Salmonella* sp.*, Escherichia coli* e *Staphylococcus aureus* (Sousa, 2016). Na União Européia os principais patógenos foram *Campylobacter, Salmonella* sp. e *Yersisnia,* no ultimo levantamento, do ano de 2016. (Food e Authority, 2017). Nos Estados Unidos os principais agentes patogênicos no ano de 2015 foram *Salmonella* sp.*, Clostridium perfringens, Escherichia coli* e *Campylobacter* (Dewey-Mattia *et al.*, 2017).

Salmonella sp. foi causadora de 22,5% dos casos de infecções associadas a produtos cárneos em todo o mundo no ano de 2013. Outras bactérias, como *Listeria monocytogenes* também apresentam grande importância nesse tipo de produto. Essa espécie bacteriana, foi responsável pelo maior número de hospitalizações e mortes causadas por infecções alimentares na Europa no ano de 2016 (Food Safety Authority,

2017). *Escherichia coli* O157:H7 é outra espécie que causou grandes perdas para a indústria alimentícia nos Estados Unidos no ano de 2013, e assim como *L. monocytogenes* é frequentemente isolada de carne e produtos cárneos, devido principalmente à contaminação cruzada (Hennekinne, 2015; Jayasena, 2013, Ozbey, 2013; Sheen, 2010). Juntos, os 3 patógenos citados acima foram responsáveis por aproximadamente 75% das mortes associadas a bactérias por ano, nos Estados Unidos.

1.2. Patógenos de alta importância nas DTAs

1.2.1 *Escherichia coli*

Escherichia coli é uma bactéria gram-negativa, em forma de bacilo, aeróbia e anaeróbia facultativa, móvel e não formadora de esporos da família *Enterobacteriaceae* (Jang *et al.*, 2017). É uma bactéria comensal, encontrada como um dos principais componentes da microflora intestinal de mamíferos (Kaper, Nataro e Mobley, 2004).

Como são comensais, vivem em equilíbrio com o hospedeiro, em mutualismo, pois podem auxiliar no processo de absorção de vitaminas e ocupar a mucosa intestinal, impedindo a fixação de bactérias maléficas. (Kaper, Nataro e Mobley, 2004). Porém dentre os 4 grupos filogenéticos (A, B1, B2 E D) dessa espécie, dois são considerados patogênicos para humanos (B2 e D), devido a presença de fatores de virulência nesses grupos. Eles estão relacionados com doenças intestinais, bem como extra intestinais. As cepas pertencentes a esses grupos são capazes de sintetizar toxinas como a, toxina citoletal distensiva e o fator necrosante citotóxico (Bonnet *et al.*, 2014).

O grupo de *E. coli* classificada como diarreiogênica (DEC) é capaz de causar diarreia, principalmente pela contaminação por alimentos e água. Esse grupo, é subdivido em patotipos: *E. coli* enterotoxigênica (ETEC), que produz enterotoxinas sem penetrar nas células da mucosa intestinal, *E. coli* produtora de shiga-toxina (STEC) , *E. coli* enteroinvasiva (EIEC) produtora de toxinas, que consegue penetrar nas células da mucosa intestinal, *E.coli* enteropatogenica (EPEC) que atua na redução do potencial de absorção do intestino e na concentração de eletrólitos nesse ambiente, causando diarréia e *E. coli* enteroagregativa (EaggEC) (Jensen *et al.*, 2014 Carnevale *et al*., 2015). Diferentemente, o grupo denominado como extraintestinal

(EXPEC), que é subdividido em, *E. coli* uropatogênica (UPEC) e *E. coli* de meningite neonatal (NMEC), são responsáveis por infecções no trato urinário, meningite e septicemia (Lopes *et al.*, 2016).

Além dessa divisão, essa espécie pode ser classificada em serogrupos, de acordo com os antígenos exibidos por cada cepa, como o antígeno O e o antígeno H. O primeiro está presente na camada de lipopolissacarideos (LPS) de bactérias Gram negativas, e pode apresentar alta variabilidade mesmo dentro da mesma espécie. Dentro da espécie *E. coli* já foram caracterizadas 184 diferentes serogrupos do antígeno O. Dentre eles os serogrupos O157, O26, O103 e O111 são muito importantes, sendo o primeiro grupo o mais relacionado a doenças enterohemorrágicas (Iguchi *et al.*, 2015). O antígeno H é responsável pela variedade flagelar encontrada nessa espécie, com 53 diferentes tipos relatados (Chui *et al.*, 2015).

O grupo STEC é o mais importante quanto se trata de surtos alimentares em humanos, e pode evoluir rapidamente de uma enterite para uma colite hemorrágica, causando diarreia sanguinolenta, dores abdominais, náuseas e vômitos. Se esses sintomas se alongarem durante mais de 7 dias, o paciente pode vir a apresentar síndrome hemolitica uremica (SHU) que pode causar falha renal, anemia hemolítica microangiopática e trombocitopenia (Ardissino *et al.*, 2015; Imdad *et al.*, 2018). Nesses casos a taxa de mortalidade pode chegar a até 5%, com números mais altos quando essa doença se manifesta em grupos de risco, como crianças e idosos (Humphries e Linscott, 2015).

Escherichia coli está presente no trato gastrointestinal de bovinos e ruminantes em geral, além de outros animais (Humphries e Linscott, 2015). Mas, também pode ser encontrada em amostras de solo (Ahlstrom, 2017) adubos orgânicos (Berry *et al.*, 2017; Erickson *et al.*, 2015; Ongeng *et al.*, 2015), plantas e sementes (Martinez *et al.*, 2015; Ongeng *et al.*, 2015), fezes (Berry *et al.*, 2017; Guber *et al.*, 2015) e água (Probert, Miller e Ledin, 2017; Truchado *et al.*, 2016, 2018).

1.2.2 *Listeria monocytogenes*

O gênero *Listeria* é composto por 17 espécies, sendo que duas dessas possuem importância como patógenos humanos, *Listeria monocytogenes* e *Listeria ivanovii* sendo a primeira de maior incidência e causadora de listeriose em humanos (Orsi e Wiedmann, 2016).

Listeria monocytogenes é uma bactéria Gram-positiva, bacilar, aeróbia e anaeróbia facultativa, móvel, patógena intracelular facultativa, pertencente a família *Listeriaceae*. Ela é capaz de crescer sob amplos espectros de variações ambientais como, temperatura, de -0,4 a 45°C e pH, de 4,0 a 9,6. Também são capazes de formar biofilme, conferindo maior tolerância a condições ambientais adversas (Orsi e Wiedmann, 2016; Välimaa, Tilsala-Timisjärvi e Virtanen, 2015). Essa espécie pode ser diferenciada em 13 serotipos. Os mais importantes, 1/2a, 1/2b, e 4b são os mais relacionados a casos de listeriose humana (Hernandez-Milian e Payeras-Cifre, 2014).

A listeriose pode ocorrer em toda população, mas em grau mais importante e mais grave em grupos de risco como, mulheres grávidas, idosos, pessoas imunossuprimidas, fetos e recém-nascidos. Nesses grupos, a listeriose pode causar sintomas severos como, sepse, meningite, encefalite, aborto, nascimento prematuro e, em casos mais extremos, óbito. Pessoas saudáveis e fora dos grupos citados desenvolvem sintomas normais de uma gastroenterite (Aguilar-Bultet *et al.*, 2018; Noordhout, de *et al.*, 2014).

L. monocytogenes, na listeriose, uma vez dentro da célula hospedeira, produz proteínas chamadas de listeriolisinas, que irão lisar a membrana vacuolar, impedindo o funcionamento do mecanismo de defesa intracelular. Dessa forma, a bactéria está livre para realizar o movimento de célula a célula, sem passar para o ambiente extracelular, conferindo maior proteção contra o sistema imune do hospedeiro (Hernandez-Milian e Payeras-Cifre, 2014). Esse microrganismo pode ultrapassar a barreira epitelial intestinal e atingir outros tecidos do organismo hospedeiro, como o sistema nervoso central, causando meningite, o sangue, podendo evoluir para sepse, e o feto, onde pode causar aborto. Apesar de ser uma contaminação rara, é de alto risco, por ter uma taxa de mortalidade dentre 20 a 30% (Humphries e Linscott, 2015).

As espécies desse gênero são consideradas ubíquas do ambiente e estão distribuídas em fezes (Cao *et al.*, 2018), solo (Locatelli *et al.*, 2013; Vivant *et al.*, 2015), água (Stea *et al.*, 2015), vegetais (Medeiros Barbosa, de *et al.*, 2016), animais (Aguilar-Bultet *et al.*, 2018) e instalações e equipamentos da indústria alimentícia (Buchanan *et al.*, 2017). A alta taxa de reincidência e a difícil descontaminação dessa bactéria nas indústrias alimentícias se deve à constante recontaminação por fontes ambientais e matérias primas e pela deficiência nos processos de higiene (Linke *et al.*, 2014).

Dentro da indústria alimentícia, produtos prontos para consumo, derivados de carne e frutos do mar são os mais afetados pela contaminação por *Listeria* spp. (Lomonaco, Nucera e Filipello, 2015; Montero *et al.*, 2015). A contaminação desses produtos é problemática devido à mudança do comportamento de consumo da população, que prefere produtos prontos devido à maior praticidade, aumentando a procura por esse tipo de artigo alimentício. Outro agravante é a não necessidade de cozimento do produto antes do consumo (Ahmed *et al.*, 2015). Além dessa categoria de mercadoria, outros produtos associados a surtos causados por *L. monocytogenes* são aqueles manipulados após o processamento, como por exemplo, produtos fatiados. Essa contaminação ocorre pela colonização dos equipamentos, utensílios, caixas ou ambientes de estocagem (Brasileiro *et al.*, 2016; Lahou e Uyttendaele, 2017).

1.2.3 *Salmonella* sp.

Salmonella é uma bactéria Gram-negativa, em forma de bacilo, aeróbia e anaeróbia facultativa, não formadora de esporos, móvel e pertencente à família *Enterobacteriaceae*. Esse gênero possui duas espécies, *Salmonella bongori* e *Salmonella enterica* (Lee *et al.*, 2015), sendo que *Salmonella enterica* é dividida em dois grandes grupos, *Salmonella* tifoide e *Salmonella* não tifoide, seis subespécies e em mais de 200 sorotipos (Crump *et al.*, 2015). Já a *Salmonella bongori* é dividida em 22 serotipos (Lamas *et al.*, 2018). Cada sorotipo desse gênero possui uma combinação de antígenos O (antígeno membranar), H (antígeno flagelar) e Vi (antígeno capsular) diferente (Cosby *et al.*, 2015).

Causadora da salmonelose em humanos, a *Salmonella* é capaz de crescer sob condições ambientais adversas. pHs menores que 4,6 e baixas temperaturas não são capazes de matar esses microrganismos. Os sintomas da salmonelose são os de uma gastroenterite, vômitos, diarreia, nauseas e dores abdominais. Em grupos de risco como, crianças, idosos e pessoas imunossuprimidas o caso pode se agravar para sepse e raros casos de óbito. Os serotipos mais comuns associados a essa doença são *Salmonella* Enteritidis e *Salmonella* Typhimurium, pertencentes ao grupo *Salmonella* não tifoide (Bell *et al.*, 2016; Cosby *et al.*, 2015; Crump *et al.*, 2015).

Salmonella é uma patógena intracelular facultativa e a capacidade de infectar humanos e causar salmonelose se dá primeiramente pela habilidade desse microrganismo de se ligar e colozinar as células do epitélio intestinal, se multiplicando

no interior dessas células e também em macrófagos. O patógeno então se dissemina e causa uma infecção sistêmica (Cosby *et al.*, 2015; Lamas *et al.*, 2018). Essa bactéria pode causar diarréia por meio da produção de D-mio-inositol-1,4,5,6,- tetraquisfosfato e da indução de liberação de íons cloreto pelas células infectadas (Howlader *et al.*, 2016). A severidade da salmonelose depende dos fatores de virulência que a bactéria apresenta, fatores esses que dependem dos fatores do ambiente e do hospedeiro (Jaiswal *et al.*, 2016).

Salmonella pode ser encontrada em carnes e produtos de origem animal, principalmente ovos (Flockhart *et al.*, 2017; Guran, Mann e Alali, 2017; Keerthirathne *et al.*, 2016), fezes (Hanlon *et al.*, 2018; Park *et al.*, 2017), água (Jokinen *et al.*, 2015), além de ser considerada uma bacteria ubíqua de intestino de mamíferos (Nowakiewicz *et al.*, 2016), aves (Silva *et al.*, 2015), repteis, anfíbios e peixes (Zajac *et al.*, 2013). Na cadeia de produção de alimentos a contaminação por esse microrganismo é recorrente, por estar presente na microbiota intestinal de muitos animais. Devido á essa característica manter as boas práticas de fabricação e higiene são muito importantes para evitar a contaminação (Mhugasefsaeuropaeu e Beloeil, 2014).

1.3. Produtos de origem animal

Animais e seus produtos derivados são importantes fontes de nutrientes, como proteínas, vitaminas, principalmente do complexo B, sais minerais, ferro e zinco, além de outros micronutrientes, para a dieta humana. Porém, essa característica, os tornam mais susceptíveis a deterioração química e, principalmente, microbiológica (Sánchez-Ortega *et al.*, 2014).

Além da maior susceptibilidade, esses alimentos abrigam naturalmente muitos patógenos, provenientes dos animais, e que podem ser transferidos a humanos por produtos alimentícios. Fontes de contaminação também são frequentemente encontradas na superfície de equipamentos industriais e dos trabalhadores que entram em contato com as matérias primas durante seu processamento. A falta de higiene nos ambientes de processamento e armazenamento e a adição de ingredientes previamente contaminados, também são importantes fontes de contaminação de alimentos (Boqvist, 2015; Stellato, 2016; Masoumbeigi, 2017).

Nos produtos lácteos encontram-se altas taxas de contaminação por *Listeria monocytogenes, Salmonella* spp. *Staphylococcus aureus* e *Escherichia coli* (Arqués,

2015). Dentre as carnes e os produtos cárneos podem-se encontrar contaminações principalmente por *Salmonella* spp. *Campylobacter jejuni, Escherichia coli* O157:H7, *Clostridium perfringens, Clostridium botulinium, Listeria monocytogenes, Staphylococcus aureus* e *Yersinia enterocolitica* (Woraprayote *et al.*, 2016).

Os produtos derivados da carne são obtidos de cortes frescos, que não são consumidos *in natura,* e devem passar por algum tipo de processamento prévio como, cozimento, salga, adição de temperos, defumação, embutimento, cura, fermentação ou desidratação. Esses processos não modificam de forma significativa, os valores nutricionais da matéria prima, porém alteram características como cor, sabor e aroma de acordo com cada processo. Os produtos cozidos incluem os perecíveis curados ou não curados submetidos a tratamento térmico. Eles são comercializados como produtos prontos para consumo e podem ser fabricados a partir de pedaços inteiros de carne (presuntos, peito de peru) ou com carne picada, gordura e ou sangue (Castro *et al.*, 2015). A diversidade de produtos manufaturados a partir da carne beneficia a industrialização, a agregação de valor à carne e redução de problemas de perecibilidade.

Para assegurar a segurança dos alimentos, estes podem passar por diferentes processos físicos como pasteurização, ultrasonicação, irradiação, receber embalagens bioativas e de atmosfera modificada (Hultman *et al.*, 2015) e também, a adição de substâncias conservantes, buscando manter a qualidade dos alimentos durante maior tempo. Após o processamento, algumas outras barreiras são submetidas aos produtos para evitar o deterioramento dos mesmos. São as mais comuns, a conservação em baixas temperaturas, em baixo pH, baixos níveis de atividade de água, adição de solutos em altas concentrações e adição de antimicrobianos (Singh e Shalini, 2016).

A indústria alimentícia utiliza substâncias, como os aditivos, para evitar a contaminação dos produtos e aumentar sua vida de prateleira. O aditivo alimentar é definido, de acordo com (Ministério da Saúde, 2015) como: “Qualquer ingrediente adicionado intencionalmente aos alimentos, sem propósito de nutrir, com o objetivo de modificar características químicas, físicas, biológicas e sensoriais, durante a fabricação, processamento, preparação, tratamento, embalagem, acondicionamento, armazenagem, transporte ou manipulação de um alimento”.

Apesar de possuir um importante papel na conservação dos alimentos, os aditivos químicos, já preconizados e utilizados pela indústria alimentícia, começaram a ser questionados pelos consumidores sobre possíveis riscos à saúde humana, por serem relacionados a doenças autoimunes e cardiovasculares (Lerner e Eakin, 2011). A indústria alimentícia vem, então, sofrendo grande pressão para substituir tais conservantes, de preferência por outros compostos mais eficazes, naturais e orgânicos (Carocho, Morales e Ferreira, 2015) como óleos essenciais, extratos vegetais (Abdollahzadeh, Rezaei e Hosseini, 2014; Calo *et al.*, 2015; Vergis *et al.*, 2015), bacteriocinas (Field, Ross e Hill, 2018; Kaškonienė *et al.*, 2017), peptídeos (Castro, de e Sato, 2015), polissacarídeos (Hamed, Özogul e Regenstein, 2016) e culturas bioprotetoras (Li *et al.*, 2016).

1.4. Bactérias Ácido Láticas

As bactérias ácido láticas são um grupo fenotipicamente diverso de bactérias Gram-positivas, não formadoras de esporos, aerotolerantes, não móveis, caracterizadas por pontos em comum em seu metabolismo, morfologia, fisiologia e filogenética. Esse grupo incluiu bactérias em forma de cocos ou bacilos e seu maior produto durante a fermentação de carboidratos é o ácido lático (Khalisanni Khalid, 2011)

A taxonomia recente classifica as bactérias ácido láticas nos seguintes gêneros: *Streptococcus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc, Lactobacillus, Carnobacterium, Aerococcus, Alloiococcus, Dolosigranulum, Globicatella, Tetragenococcus, Vagococcus, Oenococcus* e *Weisella* (Khalid, 2011).

Essas bactérias estão presentes em ambientes variados e nutricionalmente ricos como, alimentos (leite, carne, vegetais), intestino, boca e vagina de mamíferos (Khalid, 2011). Nos alimentos as BAL (bactérias ácido láticas) possuem aplicações industriais importantes, de caráter tecnológico, na produção de fermentados, e de caráter sensorial, atribuindo características desejadas de aroma, sabor e textura. Além disso, podem atuar como preservantes nos alimentos (Evivie *et al.*, 2017).

A capacidade de preservar os alimentos se deve a capacidade bacteriostática ou bactericida das BAL devido a queda do pH no ambiente, ou pela formação de compostos que possuem capacidade de afetar patógenos ou bactérias deteriorantes, como o peróxido de hidrogênio, enzimas e bacteriocinas (Ho *et al.*, 2018).

1.5. Carnobacterium maltaromaticum

O gênero *Carnobacterium,* da família *Carnobacteriaceae*, faz parte do grupo de bactérias ácido láticas (BAL), e foi proposto para diferir bactérias heterofermentativas atípicas, incapazes de crescer na presença de acetato, que pertenciam originalmente ao gênero *Lactobacillus.* São bactérias Gram-positivas, bacilares, aeróbias e anaeróbias facultativas, mesófilas e ou psicrotróficas, alcalifílicas, não móveis, não formadoras de esporos. Apesar de serem do grupo das BAL, esse gênero não produz grandes quantidade de ácido lático (Rahman *et al.*, 2014).

Carnobacterium atualmente engloba 11 espécies que apresentam características diversas e grande variabilidade genética (Iskandar *et al.*, 2017). Essas bactérias são isoladas de predominantemente dois hábitats: produtos de origem animal e ambientes frios (Afzal, 2010).

Duas espécies, *Carnobacterium maltaromaticum* e *Carnobacterium divergens,* são frequentemente isoladas de fontes alimentares, como carnes, peixes e leite e seus derivados. Em produtos do mar, *Carnobacterium* spp., pode, frequentemente, ser encontrada como população dominante, principalmente em produtos embalados sob atmosfera modificada e, ou, altas pressões. Nos produtos cárneos, bem como em queijos, a carnobacteria também pode ser encontrada como população predominante, principalmente naqueles que são estocados em temperaturas entre 4 e -1,5°C (Leisner *et al.*, 2007).

Além de ser frequentemente isolada, e ser bem adaptada a crescer em condições de armazenamento de alimentos, a *Carnobacterium* é reconhecida por sintetizar metabólitos antimicrobianos. Ademais, esse gênero não produz ácido lático de maneira significativa não interferindo no pH de matrizes alimentares. Isso atraiu o interesse dos pesquisadores a estudarem sua relação com alimentos e possível aplicação como cultura bioprotetora. (Leisner, 2007; Afzal *et al.*, 2010).

Carnobactérias são conhecidas pela capacidade de produzir bacteriocinas. Esse metabólito é produzido quando uma BAL compete por nutrientes com um microrganismo antagônico, a fim de garantir sua sobrevivência (Reis, dos *et al.*, 2011). Alguns autores já caracterizaram alguns desses peptídeos, dentre os quais pode-se citar a bacteriocina U149, produzida pela cepa *C. maltaromaticum* U149 isolada por Stoffles *et.al.,* (1992). Outros estudos como o de Yamazaki *et.al.,* (2005) mostraram que uma cepa de *C. maltaromaticum* produzia uma bacteriocina nomeada de

piscicolina CS526, e Martin-Visscher *et.al.,* (2011) também verificaram a produção de carnociclina A pela cepa UAL307 de *C. maltaromaticum.*

Por isso, as bacteriocinas possuem um grande potencial de uso como aditivo natural para aumentar a estabilidade microbiana em alimentos, substituindo aditivos químicos sintéticos. Essas moléculas não apresentam toxicidade a células eucarióticas, são inativadas por proteases no trato digestório do organismo consumidor (dos Reis *et.al.*, 2011), não possuem cor, odor ou sabor, (Pérez *et al.*, 2013) são reconhecidas como seguras (GRAS) e apresentarem um modo de ação bactericida, agindo na membrana plasmática de procariotos.

Esses polipeptídeos são sintetizados ribossonalmente e liberados ao meio extracelular. São capazes de interagir com a parede celular de outras bactérias, formarem poros, causar a lise e, consequente, a morte celular ou então de inibir o crescimento de bactérias alvo (Martin-Visscher *et al.,* 2011) Essas moléculas podem apresentar um amplo ou restrito espectro de ação antibacteriana. Elas são resistentes a largas faixas de variação de pH, calor, frio, congelamento, ácidos orgânicos, altas concentrações de sal e também resistentes a algumas enzimas (Ünlü, Nielsen e Ionita, 2015).

Dentre as bacteriocinas produzidas por BAL, pode-se encontrar dois principais grupos: classe I, classe II de acordo com a classificação proposta por Cotter *et.al.,* (2005). Os peptídeos pertencentes a classe I são chamados de lantibióticos e sofrem modificações pós traducionais, com adição de aminoácidos como, metionina, matilantanina e desidrolanina. As bacteriocinas desse grupo atuam na síntese da parede celular de bactérias e promovem a formação de poros nas mesmas. A classe II abriga bacteriocinas termorresistentes, não modificadas pós-traducionalmente e menores que 10k Da. Atuam na permeabilização da membrana de bactérias alvo. São divididas em 4 grupos: IIa, bacteriocinas pequenas similares a pediocina, IIb, bacteriocinas compostas por dois peptídeos, IIc, bacteriocinas cíclicas e IId, que agrupa bacteriocinas que não seguem as características mencionadas acima (Arqués *et al.*, 2015). Dentro do gênero *Carnobacterium* percebe-se, principalmente, a produção de bacteriocinas que pertecem ao grupo IIa (Leisner *et al.*, 2007).

O uso de cepas de *Carnobacterium* produtoras de bacteriocinas pode prevenir o crescimento de patógenos durante fases críticas do processamento de uma grande variedade de alimentos refrigerados. Porém, cepas não produtoras de bacteriocinas

também demonstram grande potencial para uso como bioprotetores, pela competição por nutrientes e produção de ácidos orgânicos ou outros compostos antimicrobianos (Chanos e Mygind, 2016; Yang et al., 2018). Diferentemente de outras BAL típicas, esse gênero apresenta uma importante e desejada característica, é capaz de crescer em ambientes extremos, com baixas temperaturas, alta concentração de sais e alta pressão.

Esse estudo teve como objetivo determinar a capacidade bioprotetora de três cepas de *Carnobacterium maltaromaticum* contra três importantes patógenos alimentares, *Escherichia coli* O157:H7, *Listeria monocytogenes* e *Salmonella*, sob diferentes condições *in vitro* e em matriz cárnea.

CAPÍTULO 2

Carnobacterium maltaromaticum as bioprotective culture *in vitro* and in cooked ham

ABSTRACT

This study aimed to evaluate the bioprotective effect of *Carnobacterium maltaromaticum* (CM) against food pathogens *in vitro* and in sliced cooked ham. Strains of CM were tested *in vitro* against *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* Typhimurium. *In vitro* tests consisted of co-cultures of CM and pathogenic bacteria in BHI with or without EDTA at −1, 4, and 25° C for 28 days, 14 days, and 48 h, respectively, agar well diffusion of CM cell-free supernatant using pathogenic bacteria as test microorganisms, and minimum inhibitory concentration determination. After that, their bioprotective effect against *Listeria innocua* was tested in cooked ham, as well as their effect on pH and color after 7 days of storage at 4° C. In co-cultures at −1° C and 4° C, all CM isolates inhibited *L. monocytogenes.* A slight inhibition was observed against the Gram-negative bacteria with addition of EDTA. The cell-free supernatant from CM culture did not show any inhibitory effect under the studied conditions. On ham, CM did not affect pH and color parameters of the product. Significant inhibition of *L. innocua* was observed during storage. The combination of two hurdles (refrigerated storage and bioprotective culture) shows potential to improve food quality and safety.

Key words: antilisterial activity, bioprotective cultures, meat product, lactic acid bacteria.

1. Introduction

Foodborne disease outbreaks are caused by the ingestion of contaminated food with pathogenic microorganisms. The World Health Organization (WHO)

considers that foodborne outbreaks are critical threats to global health (WHO, 2015). The high rate of infections caused by human pathogens including *Salmonella* spp., enterohemorrhagic *Escherichia coli* O157:H7 and *Listeria monocytogenes* is a frequent reminder of the complex food web that links humans, animals and microbial population (Wolfe, Dunavan and Diamond, 2007). Poor handling, incorrect storage, and consumption of raw meat are the leading causes of foodborne diseases (Jayasena and Jo, 2013; Ozbey *et al.*, 2013). *Salmonella* spp. is reported as the cause of 22.5% of the foodborne diseases associated with the consumption of meat and meat products worldwide (Hennekinne *et al.*, 2015). Other bacteria play an essential role in these outbreaks including *E. coli* O157:H7, which has a high level of incidence in meat products mainly by cross-contamination (Jayasena and Jo, 2013), and *L. monocytogenes,* which has the highest level of deaths and hospitalization for foodborne outbreaks (10 and 97%, respectively).

Meat products characteristics such as high protein content, low acidity, and high-water activity make them susceptible to microbial growth (Sánchez-Ortega *et al.*, 2014). The microbial contamination of meat products can happen during manufacturing due to a lack of hygiene of equipment, workers, and the environment of production (Stellato *et al.*, 2016). Other possible sources of contamination are the addition of contaminated ingredients, including spices, and cross contamination (Masoumbeigi *et al.*, 2017). According to the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (2017), in 2015, meat products were linked with the most critical cases of foodborne outbreaks in the European Union. Moreover, in Brazil, these products were responsible for 5% of the outbreaks in the period from 2000 to 2015 (Sousa, 2016). However, these numbers may be higher than those that are reported because of the lack of surveillance and because mild symptoms do not force patients to seek medical assistance (de Oliveira *et al.*, 2010). Finally, in the United States of America (USA), meat and meat products were responsible for approximately 20,000 outbreaks in the period from 1998 to 2016 (Dewey-Mattia *et al.*, 2017).

Biopreservation has received the attention of the food industry as a mean of naturally controlling the shelf life and safety of food. Lactic acid bacteria (LAB) have significant potential for use in biopreservation and have been traditionally used as natural biopreservatives of food and feed. Carnobacteria are ubiquitous LAB isolated

from cold and temperate environments and can be found as natural microbiota of chilled meat, fish and dairy products. Among the 11 species of *Carnobacterium*, two species, *Carnobacterium divergens* and *Carnobacterium maltaromaticum,* are frequently isolated from food and showed the ability to inhibit pathogenic and spoilage microorganisms in diverse food matrices, exerting their inhibitory effect by the competition for nutrients and the production of bacteriocins (Leisner *et al*., 2007). Thus, their use as bioprotective cultures in food has been considered (Iskandar *et al.*, 2017; Leisner *et al.*, 2007; Orihuel *et al.*, 2018). The antimicrobial properties of *Carnobacterium* spp. have been studied *in vitro* (Hammi *et al.*, 2016; Tulini *et al.*, 2014), in cold-smoked salmon (Brillet-Viel *et al.*, 2016; Brillet-Viel *et al.*, 2005), ricotta (Spanu *et al.*, 2018), cooked and peeled shrimp, and as a feed additive for rabbits to improve meat microbial quality and safety (Koné *et al.*, 2018). The use of *Carnobacterium* spp. has also been investigated as a probiotic culture in broiler chickens (Smialek, Burchardt and Koncicki, 2018). However, the effect of *C. maltaromaticum* as a bioprotective culture in meat products, especially ready-to-eat products, has not been assessed.

In this way, this study aimed to evaluate the antimicrobial effect of *C. maltaromaticum* towards different food pathogenic bacteria *in vitro* and to verify the potential of its use as a bioprotective culture in cold-stored sliced cooked ham.

2. Material and methods

2.1 *In vitro* experiments

2.1.1 Bacterial strains, media, and growth conditions

Three different strains of *C. maltaromaticum* (CM_B824, CM_B827, and CM_B829), obtained from Australian vacuum packaged chilled beef (*longissimus thoracis et lumborum*) with an extremely long shelf life (140 days at −1° C) were used in this study (Imazaki *et al.*, 2015). These isolates were selected among 11 CM isolates after the sequencing of their genome, which revealed the existence of three main phylogenetic groups. Therefore, one isolate of each group was selected to be used in the present research.

Foodborne pathogenic bacteria, *E. coli* O157:H7 ATCC®35150™ (EC), *L. monocytogenes* ATCC®19117™ (LM) and *Salmonella* Typhimurium ATCC®14028™ (ST) were used for the *in vitro* tests, and a pool of *Listeria innocua* (LI33314, LI33016 and LIHPB586, PUCPR strain collection) was used in the experiments with sliced cooked ham.

CM isolates were grown in brain heart infusion (BHI) (Kasvi, São José dos Pinhais, Brazil) at 25° C for 48 h, and pathogenic and LI strains at 37° C for 24 h in the same medium. Growth was verified by optical density at 540 nm for the pathogenic bacteria and *L. innocua* and 620 nm for CM (Gutiérrez *et al.*, 2016).

2.1.2 Evaluation of the antimicrobial effect of *C. maltaromaticum* in co-culture

The antimicrobial effect of CM in co-culture was determined inoculating Falcon flasks with 30 mL of BHI broth with each isolate of CM at 6.0 log CFU/mL and one of the pathogenic strains (LM, EC, ST) at 3.0 log CFU/mL. Flasks with BHI were also inoculated with each pathogenic strain (LM, EC, ST) at 3.0 log CFU/mL as positive controls. Flasks were incubated at −1 °C for 28 days, 4 °C for 14 days and 25 °C for 48 h in a shaker (model Rotamax 120, Heidolph, Germany) at 150 rpm. Pathogenic bacteria counts were performed using specific chromogenic media: RAPID'*L.mono*, RAPID'*Salmonella* and RAPID'*E.coli* 2 (BioRad, Marnes, France). CM concentration was estimated as the difference between counts on plate count agar (PCA) (BioRad, Marnes, France) and chromogenic media.

2.1.3 Evaluation of the antimicrobial effect of *C. maltaromaticum* co-cultures with the addition of ethylenediaminetetraacetic acid

The influence of the addition of ethylenediaminetetraacetic acid (EDTA) (VWR, Radnor, USA) on the antimicrobial effect of CM on pathogens was investigated. Co-cultures were carried out in Falcon flasks containing 30 mL of BHI with EDTA 1.0 mM, incubated at 25 °C for 48 h, in a shaker (model Rotamax 120, Heidolph, Germany) at 150 rpm. Bacterial counts were performed following the same procedure described above. To find the concentration of EDTA that would not interfere in the bacterial growth by itself, a previous experiment was conducted. Serial concentrations of EDTA

(1, 5, 10, 20 and 40 mM) were added to the broth medium with each strain of pathogen. The concentration that showed no difference in growth compared to negative control (1.0 mM), inoculated broth without the addition of EDTA, was selected.

To determine if the CM isolates were able to produce bacteriocins, two approaches of cell-free supernatant (CFS) experiments were tested, as described below.

2.1.4 Evaluation of the antimicrobial effect of the CFS of CM by agar well diffusion

To check if the antimicrobial effect of CM was mediated by the production of antimicrobial molecules in the culture supernatant, tests were conducted as follows. Three tubes per isolate were prepared with 10 mL of BHI broth and one colony of each CM isolate. The tubes were incubated at 25° C for 48 h and centrifuged (Model Eppendorf Centrifuge 5804, Hamburg, Germany) at 16,000 g for 10 min. The pH of the supernatant was corrected to 6.5 with sodium hydroxide (NaOH) 1 M (VWR, Radnor, USA). Finally, the supernatant of the last tube was filtered through 0.2 µm sterile Minisart syringe filters (Sartorius, Germany). The treated supernatants were inoculated in wells made in three PCA plates previously spread with 100 µL each of the pathogenic bacteria cited above (6.0 log CFU/mL). Four treatments were applied on each plate: (1) sterile BHI broth (blank), (2) centrifuged supernatant, (3) centrifuged supernatant treated with NaOH, and (4) centrifuged supernatant treated with NaOH and filtered. The halo of inhibition was measured after 48 h of incubation at 37° C, and all treatments were performed in triplicate.

2.1.5 Determination of the minimum inhibitory concentration (MIC) of the CFS of CM towards LM

CM_B824, CM_B827 and CM_B829 at 6.0 log CFU/mL were incubated at two different growth conditions (at 25° C for 48 h and 4° C for 14 days) in co-culture with LM or not. The combination of the different parameters resulted in 12 treatments: (1) CM_B824 at 25° C (2) CM_B824 + LM at 25° C; (3) CM_B824 at 4° C, (4) CM_B824 + LM at 4° C, (5) CM_B827 at 25° C, (6) CM_B827 + LM at 25° C; (7) CM_B827 at 4° C, (8) CM_B827 + LM at 4° C, (9) CM_B829 at 25° C, (10) CM_B829 + LM at 25° C;

(11) CM_B829 at 4° C, (12) CM_B829 + LM at 4° C. CFS of the different co-culture treatments were obtained by centrifugation, as described previously.

LM at 6.0 log CFU/mL and the CFS were inoculated in 24-well plates at the different concentrations: 62.5, 50, 37.5 and 18.75 %, that were composed by the combination v/v of CFS and sterile BHI broth. Plates were incubated at 37° C for 24 h at 150 rpm, in triplicate. The growth of LM was verified visually. The minimum inhibitory concentration (MIC) was considered as the lowest concentration of the supernatant were no visible growth was observed (CLSI, 2013).

2.2 Cooked ham experiment

2.2.1 Evaluation of the effect of CM against LI in sliced cooked ham

Cooked ham slices (0,03 x 10,5 x 11,5 cm) from the same batch, with a shelf-life of 10 days were purchased packed in expanded polystyrene trays and covered with low density polyvinyl chloride film, from a local supermarket. After reception, they were sterilized at 121° C for 15 min, and chilled overnight at 4° C. LI cold adaptation was performed as follows: strains were activated at 37° C until stationary phase, subcultured in BHI broth at 4° C until stationary phase which was confirmed by colony count in PCA agar plates. Equal volumes of each LI strain were mixed in a sterile flask. From the mixture adequate serial decimal dilution were prepared in peptone water (0,1%) to adjust the concentration to 10^4 CFU/g. Then, the slices were distributed in eight treatments: (1) negative control (NC, no bacterial inoculation); (2) CM_B824; (3) CM_B827; (4) CM_B829; (5) LI (pool); (6) CM_B824 + LI (pool); (7) CM_B827 + LI (pool); and (8) CM_B829 + LI (pool).

Except for NC samples, each side of ham slices was inoculated with 50 µL of LI at 4.0 log CFU per gram of the product, which was spread evenly with a sterile Drigalski spatula. To allow the absorption of the inoculum, the slices were kept in a sterile cabinet for 1 h at room temperature. Then, the slices were inoculated with 50 µL of each isolate of CM at 6.0 log CFU/g (except the LI-treated ham). After 1 h, the slices were packed in sterile expanded polystyrene trays and covered with polyvinyl chloride film oxygen permeability 1400 cm^3/ m^2/ 24 h/ 22,8° C. The trays were stored in an

incubator BOD (Biochemical Oxygen Demand) (Fanem 347 CD, São Paulo, Brazil) at 4° C for 7 days. Samples were withdrawn at 0, 2, 5 and 7 days of storage for the determination of pH and instrumental color and bacterial counts.

2.2.2 Determination of instrumental color

The instrumental color was evaluated using a portable colorimeter (Model CR 410, Konica Minolta, Tokyo, Japan). The color was measured at three different points on the surface of ham slices. Measurement parameters were: color space – CIE *L*a*b**, light source – D65, opening diameter – 50 to 53 mm, and angle of observation – 2°. The hue value [h = ARCTAN (b/a)] and chroma ($C^* = \sqrt{a^2 + b^2}$), which indicate intensity of discoloration and color saturation, respectively, were calculated.

2.2.3 Determination of pH

For pH determination, 3 g of each sample were homogenized in 30 mL of deionized water for 1 min in a stomacher blender (Model Masticator Basic 2000, IUL, Barcelona, Spain). The pH value of the suspension was determined in triplicates using a pH-meter (Model HI 99163, Hanna, Póvoa de Varzim, Portugal).

2.2.4 Microbiological analysis

For microbiological analysis, 10 g of each sample were homogenized with 90 mL of sterilized peptone-water (0.1% w/v) (Kasvi, São José dos Pinhais, Brazil) for 2 min using a stomacher blender (Model Masticator Basic 2000, IUL, Barcelona, Spain). Serial tenfold dilutions until 10^{-6} were performed, and 100 µL of the selected dilution was inoculated on PCA for CM and PCA overlaid with 10 mL of melted PALCAM agar (Sigma Aldrich, MI) for LI. CM population was estimated as the difference between counts on plate count agar (PCA) and PCA overlaid with PALCAM agar. Moreover, where CM and LI colonies were differentially counted in PCA agar since the size of CM colonies were smaller. The experiment was carried in duplicate. The plates were incubated at 37° C for 48 h (LI) and at 25° C for 48 h (CM).

2.3 Statistical analysis

The effect of CM on the growth of EC, LM and ST for each incubation temperature (−1 °C, 4 °C and 25 °C) and treatment with EDTA at 25° C was analyzed by one-way ANOVA. When necessary, Tukey's test was used to assess differences between test groups. ANOVA was performed using VassarStats online.

Microbiological and physicochemical data obtained from cooked ham experiment were analyzed using a random block design, considering a mixed linear model including treatment and storage time as fixed effects and replication as a random effect. Means were compared by Tukey test ($P < 0.05$). Microbiological and physicochemical assays were performed two times independently and differences between replicates were not significant ($P < 0.05$). The microbiological assays were conducted in duplicate ($n = 4$) and physicochemical analysis in triplicate ($n = 6$). For statistical analysis, bacterial counts with values below the limit of detection were considered as 1.0 log CFU/g.

The analysis was performed using the software Statgraphics® Centurion XVI version 16.1.11 (Statpoint Technologies, Warrenton, Virginia, USA)

3. Results

3.1 In vitro tests

3.1.1 Evaluation of the antimicrobial effect of *C. maltaromaticum* in co-culture

When in co-culture at −1° C, CM isolates were able to reduce the population of LM from 6.6 (control) to < 1.0 (CM_B824), 2.3 (CM_B827), and 1.7 (CM_B829) log CFU/ mL (Figure 1). However, EC and ST were not inhibited when CM strains co-cultured at −1° C were used (data not shown). At 4° C, all CM isolates inhibited the growth of LM ($P < 0.05$), showing a count reduction of a least 5.5 log CFU/ mL as compared to LM alone (Figure 2). Nevertheless, CM did not show any inhibition effect against EC and ST at 4° C. Finally, CM_B824 and CM_B827 showed a weak but significant inhibition effect towards LM ($P < 0.05$) when in co-cultures at 25° C (Figure 3 C). CM did not show any inhibitory effect when grown in co-culture with EC and ST at 25° C (Figures 3 A and 3 E). However, when EDTA was added in the co-culture

broths, all CM isolates reduced the growth of EC ($P < 0.05$) (Figure 3 B), and CM_B824 and CM_B827 inhibited the growth of LM and ST ($P < 0.05$) (Figures 3 D and 3 F).

3.1.2 Evaluation of antimicrobial effect of cell-free supernatant

The evaluation of the antimicrobial activity of cell-free supernatant from CM culture by agar well diffusion assay did not highlight any inhibition effect against the pathogens. Similarly, in MIC determination test, the different cell-free supernatant treatments were not able to inhibit LM at any of the concentrations tested (data not shown).

3.2 Cooked ham experiment

3.2.1 Color and pH

There were significant interactions between ‘treatment’ and ‘storage time’ for all the parameters of instrumental color and pH of cooked ham ($P < 0.05$) (Tables 1 and 2).

A slight increase in luminosity (L*) and a slight decrease in pH was observed in ham from all treatments during storage.

The addition of CM isolates in cooked ham did not cause significant changes in the pH of the product, which remained similar to NC and LI during storage. After 7 days of storage, CM824+LI showed lower pH than the other treatments ($P < 0.05$), however, this condition did not affect the color parameters of the product, which presented similar values to the other treatments.

The addition of CM isolates to ham did not affect the instrumental color of the product. Small differences in instrumental color values observed during storage are likely due to intrinsic characteristics of the product itself than to the influence of the treatments.

3.2.2 Microbial analysis

There were significant interactions between ‘treatment’ and ‘storage time’ for the count of *C. maltaromaticum* in cooked ham ($P < 0.05$) (Table 3). For the count of *L. innocua*, there was no interaction between the fixed effects, but the differences for each effect were significant ($P < 0.05$) (Table 3).

During storage, the count of CM varied significantly, however, count differences were < 0.4 log CFU/g in all CM-treated hams from day 1 to day 7 of storage, showing a good adaptation of the *Carnobacterium* isolates to the cured cooked meat matrix. Conversely, the count of LI in the CM+LI treatments reduced significantly during storage, whereas the count of LI in the LI-treatment did not differ during storage ($P > 0.05$). At 2 days of storage, the count of LI in the CM treated ham decreased to non-detected values (< 1.0 log CFU/g). The addition of CM_B829 in ham caused a count reduction of LI > 2.0 log CFU/ g after 7 days of storage. CM_B824 and CM_B827 also caused a significant reduction of LI in ham during storage ($P < 0.05$).

4. Discussion

4.1 *In vitro* experiments

The food industry has largely investigated the potential use of LAB as biopreservatives. Their inhibitory effect against pathogenic and spoilage bacteria (Alves *et al.*, 2005; dos Reis, *et al.*, 2011; Hammi *et al.*, 2016; Ho *et al.*, 2018; Huang *et al.*, 2016; Rivas *et al.*, 2014) is an important indicator of their possible use as protective cultures in food matrices, where they could replace some synthetic conservatives (Engelhardt *et al.*, 2015; Huang *et al.*, 2016).

LAB can inhibit spoilage and pathogenic microorganisms by competitive growth and synthesis of antagonistic compounds such as organic acids and bacteriocins (Gómez-Sala *et al.*, 2016). In the present study, CM showed antilisterial activity in the co-cultures at the three temperatures tested (−1, 4, and 25° C). Other authors also demonstrated the antilisterial activity of LAB. Rivas *et al*. (2014) showed the antilisterial activity of *Lactobacillus curvatus* and its purified bacteriocin, sakacin Q. Ho et al (2018) found antilisterial activity in co-culture of *Lactococcus lactis, Lactococcus raffinolactis, Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides, Weissella soli*, and

Weiseella viridescens. Furthermore, Huang *et al.* (2016) demonstrated the inhibition of LM by *Enterococcus faecium* B1/B2 in co-culture. As *Carnobacterium* is closely phylogenetic related to other genera of LAB it is possible to compare these bacteria (Hammes e Hertel, 2006). Regarding the genus *Carnobacterium,* Alves *et al.* (2005) reported the antilisterial activity of a strain of CM (formerly *Carnobacterium piscicola*) isolated from Brazilian smoked fish. Dos Reis *et al*. (2011) also observed the antilisterial activity of *C. maltaromaticum* in fish models. Moreover, Hammi *et al*. (2016) demonstrated an anti-*Listeria* activity by a new class IIa bacteriocin, termed maltaricin CPN, produced by a CM strain isolated from mould-ripened cheese.

An inhibition effect against EC and ST was observed in the *in vitro* test with the addition of EDTA. Other authors also observed inhibition of Gram-negative pathogens when bacteriocins from Gram-positive bacteria were analyzed in the presence of EDTA (Camargo *et al.*, 2016; Field *et al.*, 2017; O'Connor *et al.*, 2015; Prudêncio, dos Santos and Vanetti, 2015). A bacteriocin produced by CM UAL307, termed carnocyclin A, showed an antimicrobial activity against Gram-negative bacteria including EC and *Pseudomonas aeruginosa*, when incubated with EDTA (Martin-Visscher *et al.*, 2011). Bacterial metabolites can become more effective biopreservatives when used in combination with other hurdles such as chelating agents. This combination is a strategy to increase the activity of the bacteriocins produced by Gram-positive bacteria. As the composition of membranes from Gram-positive bacteria, and Gram-negative bacteria are different, multiple approaches for increasing the activity of a bacteriocin or other bacterial metabolites are necessary (Hwanhlem *et al.*, 2017). The chelating capacity of EDTA, which acts by removing Mg^{2+} and Ca^{2+}, promotes the destabilization of the outer membrane from Gram-negative bacteria, allowing metabolites to access the cytoplasmic membrane (Field *et al.*, 2017; Mathur *et al.*, 2017).

Regarding the CFS experiments, there was no inhibition effect on LM growth at any of the tested conditions. Similarly, Schillinger and Holzapfel (1990) did not observe any antilisterial effect of CFS obtained from 37 isolates of *Carnobacterium* spp. Arena *et al.* (2016) also did not find any inhibitory effect of CFS obtained from 79 *Lactobacillus plantarum* isolates towards LM, EC, *Salmonella* sp. and *Staphylococcus aureus* using CFS in a well-diffusion assay. However, when these pathogens were challenged in the presence of the cells of *L. plantarum* an inhibitory effect was observed for 17 strains.

Based on these results, the isolates of CM used in this study are not likely to produce bacteriocins under the studied conditions. The bacteriocin production is related to the maximum cell growth and shows primary metabolic kinetics. So, the metabolization of bacteriocins are strictly related to optimal conditions of growth for the bacteriocinogenic strain, which depends on environmental conditions such as pH, temperature, media composition, aeration, salinity, agitation and incubation atmosphere (Elayaraja *et al.*, 2014; Malheiros *et al.*, 2015; Yang *et al.*, 2018). For *C. maltaromaticum*, the best temperatures to maximum production of bacteriocin were found to be between 19° C (Gursky *et al.*, 2006).

Thus, inhibition of pathogenic bacteria observed at the co-culture experiment at 25° C may be explained by the production of organic acids or other antibacterial metabolites, the competition of nutrients, the need of a more direct interaction between bacteria to activate the antimicrobial mechanisms or even, by the production of bacteriocins, although this production was not enough to cause a significant inhibition (Arena *et al.*, 2016; Chanos and Mygind, 2016; Yang *et al.*, 2018).

Each isolate of each species appears to have an exclusive optimal condition to produce bacteriocins, and these conditions should be determined for each parameter and producer isolate (Masuda *et al.*, 2016; Pérez *et al.*, 2013; Sidooski *et al.*, 2018).

4.2 Cooked ham experiments

The use of LAB as protective cultures to improve safety and prolong the shelf life of the meat products, including cooked meat products, is a new concept that has been suggested by many authors (Comi *et al.*, 2016; Metaxopoulos, Mataragas and Drosinos, 2002; Vermeiren, Devlieghere and Debevere, 2004). The biopreservative properties of *Lactobacillus* spp*.* and *Lactococcus* spp*.* in meat products have been reported in some studies. Gao *et al*. (2015) and Comi *et al*. (2016) reported that *Lactobacillus sakei* and *Lactococcus lactis* affected the growth of spoilage and *L. monocytogenes,* and did not negatively affect the physical chemical properties of sliced cooked ham and cooked bacon, respectively. On the other hand, the addition of C. *maltaromaticum* in meat products as a protective culture has not been assessed.

Regarding the physicochemical properties of cooked ham, *C. maltaromaticum* isolates used in this study did not affect the pH of the product during storage. Sliced

cooked ham samples from all treatments presented pH values within the normal range for this type of meat product, i.e., 5.6-6.2 (Arnau *et al.*, 1995).

Conversely to other aciduric LAB genera such as *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, *Carnobacterium* are not a strong acid producer. Even if carbohydrate catabolism by carnobacteria appears to result in a diverse number of metabolites, these have generally a limited their effect on the sensory attributes of foods (Leisner *et al*., 2007). Gao *et al*. (2015) found that the addition of *L. sakei* C2 in samples of vacuum packed sliced cooked ham decreased the pH value during the storage at refrigerated temperature.

Similar to the findings of the present study, Spanu *et al.* (2018) reported a limited variation of pH in modified atmosphere packaging (MAP) packed Ricotta cheese inoculated with a protective *Carnobacterium* strain, indicating low acidification properties of *Carnobacterium* spp.

The acceptability of cooked ham by the consumers is strongly related to the color of the product (Lloret *et al.*, 2016). The color parameters of cooked ham were not influenced by the addition of *C. maltaromaticum* isolates used in this study. Changes in the color of a meat product caused by *Carnobacterium* was reported only by Peirson, Guan and Holley (2003) observed that a strain of *C. viridans* induced greening in cured bologna. However, this effect was detected only after the vacuum packages were opened and took 2 days or less at 9° C and 3 days at 4° C. However, the authors state that representative strains of most known species of *Carnobacterium* failed to cause discoloration in this type of cooked meat product.

As *Carnobacterium* are frequently predominant members of the LAB microbiota of non-spoiled raw meat and processed meat products, including ham and bacon, irrespective of whether products have been stored aerobically, vacuum packaged, or subjected to modified atmospheres, they normally do not cause changes in the physicochemical and sensory characteristics of the products (Li *et al.*, 2018). Additionally, high concentrations of bacteria (> 10^6-10^7 CFU/g) in food are typically required before their activity is enough to influence the sensory properties of a product (Leisner *et al*., 2007).

C. maltaromaticum showed a significant inhibition of *L. innocua* growth in cooked ham, which may be attributed to the greater ability of *Carnobacterium* to grow

and to adapt to this food matrix and storage conditions in comparison to *Listeria* (Amézquita; Brashears, 2002; Brillet-Viel *et al*., 2005). This ability leads to a depletion of nutrients hindering the growth of the pathogen, as well as the occupation of the food matrix before the pathogen (Vermeiren; Devlieghere; Debevere, 2004; Nilsson *et al*., 2005).

Although the ability of *Carnobacterium* strains to produce bacteriocins under the studied conditions has not been proven, the bacteriocin production by LAB does not always lead to increased inhibitory activity towards pathogenic bacteria. Vermeinen *et al.* (2004) studied the antilisterial activity of LAB, able or not to produce bacteriocins, in cooked ham stored at 4 and 7°C and observed that strains that did not produce bacteriocin had a greater inhibition of the growth of *Listeria* sp. The occurrence of resistant *L. monocytogenes* target organisms has led to the suggestion that bacteriocin-negative LAB may be more suitable for practical use as bioprotective agents against *L. monocytogenes* in ready-to-eat foods (Nilsson *et al.*, 2005; Vermeiren *et al*., 2006). Indeed, *L. monocytogenes* is inhibited by carnobacteria that do not produce bacteriocins, and this is partly due to glucose depletion (Leisner *et al*., 2007).

Considering the inhibition of *L. innocua* in ham obtained by the addition of *C. maltaromaticum* isolates in the short storage time studied (7 d), the count reduction of *Listeria* was greater than that reported by other authors using LAB against spoilage and pathogenic bacteria in dairy and meat products. Spanu *et al*. (2018) observed a reduction of 1.3 log CFU/g and 0.8 log CFU/g in the count of *Pseudomonas* spp in Ricotta cheese inoculated with a protective strain of *Carnobaterium*, after 14 and 21 d of storage. Gao *et al*. (2015) found a reduction of 1 log CFU/ g in the count of *L. monocytogenes* in vacuum packed cooked ham inoculated with *L. sakei C2.* Greater reduction in *L. monocytogenes* count (2.7 log CFU/g) was reported by Ben Slima *et al*. (2017) in beef sausages inoculated with *L. plantarum* (7.0 log CFU/g) after 10 days of storage at 4 °C.

The *C. maltaromaticum* isolates used in this study present several properties that are desirable for biopreservative cultures: the isolates were able to growth and the count remained stable in the cooked ham during storage, the isolates did not cause

significant changes in pH and color of the product and the fast inhibition of *L. innocua,* at the second day of storage, is another advantage of these isolates.

As the artificial contamination of ham in this study extrapolated the natural contamination observed in the food industry by *Listeria,* the *C. maltaromaticum* could show more successful antimicrobial results in practical conditions. Despite the promising results observed in the present study, the possible impact of the addition of the *Carnobacterium* isolates as protective culture on sensory properties of the meat product should be further investigated.

5. Conclusion

In conclusion, the three *C. maltaromaticum* strains tested showed an antilisterial potential *in vitro*, which was more important at −1°C and 4°C than at 25°C.

When applied to a food matrix, in this case cooked ham, the antilisterial potential was confirmed. The approach of both methods, *in vitro* and in cooked ham, to determine the antimicrobial activity of *C. maltaromaticum* is an important aspect of this study. The combination of two hurdles, refrigerated storage and addition of bioprotective cultures, may provide novel solutions, that shows great potential to improve quality and food safety, for chilled foods such as processed meat products.

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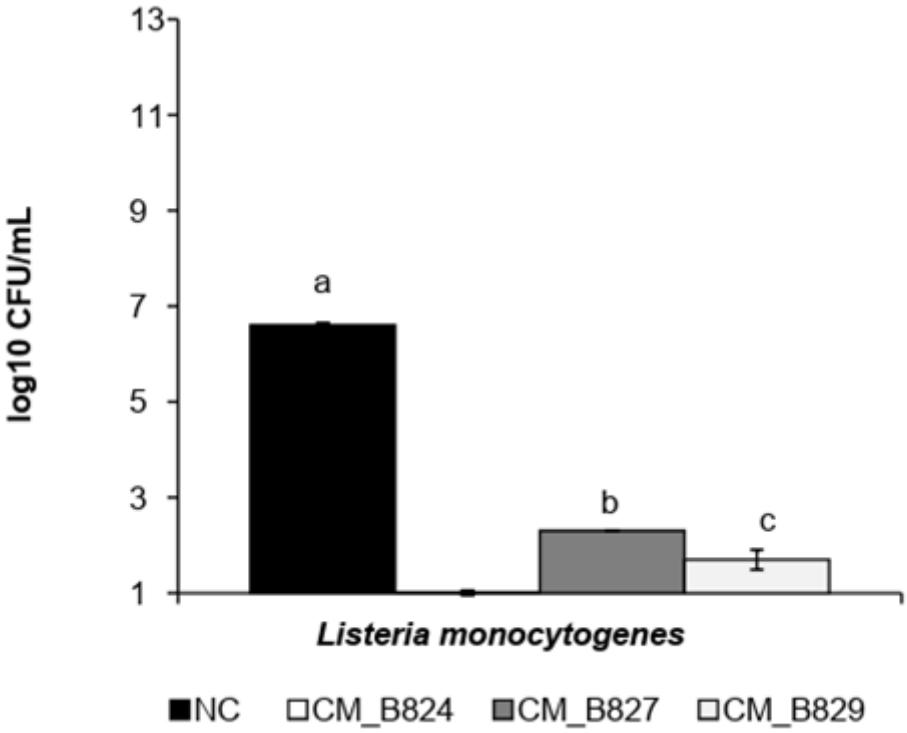
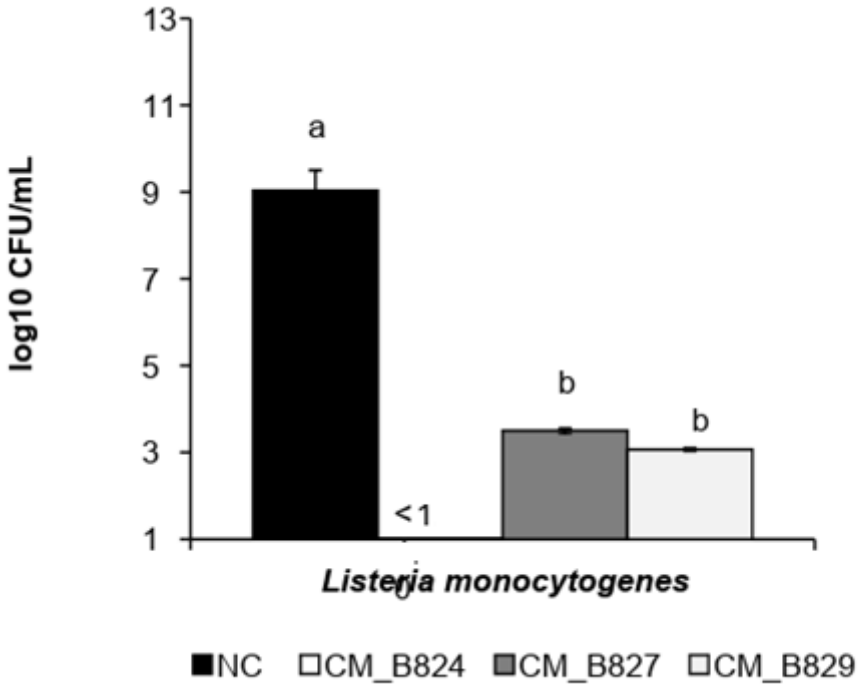


No common superscript indicates that there is a significant difference among treatments (P<0.05).

Figure 1. Count of *L. monocytogenes* at co-culture with *C. maltaromaticum* strains at 4 ºC for 14 d.

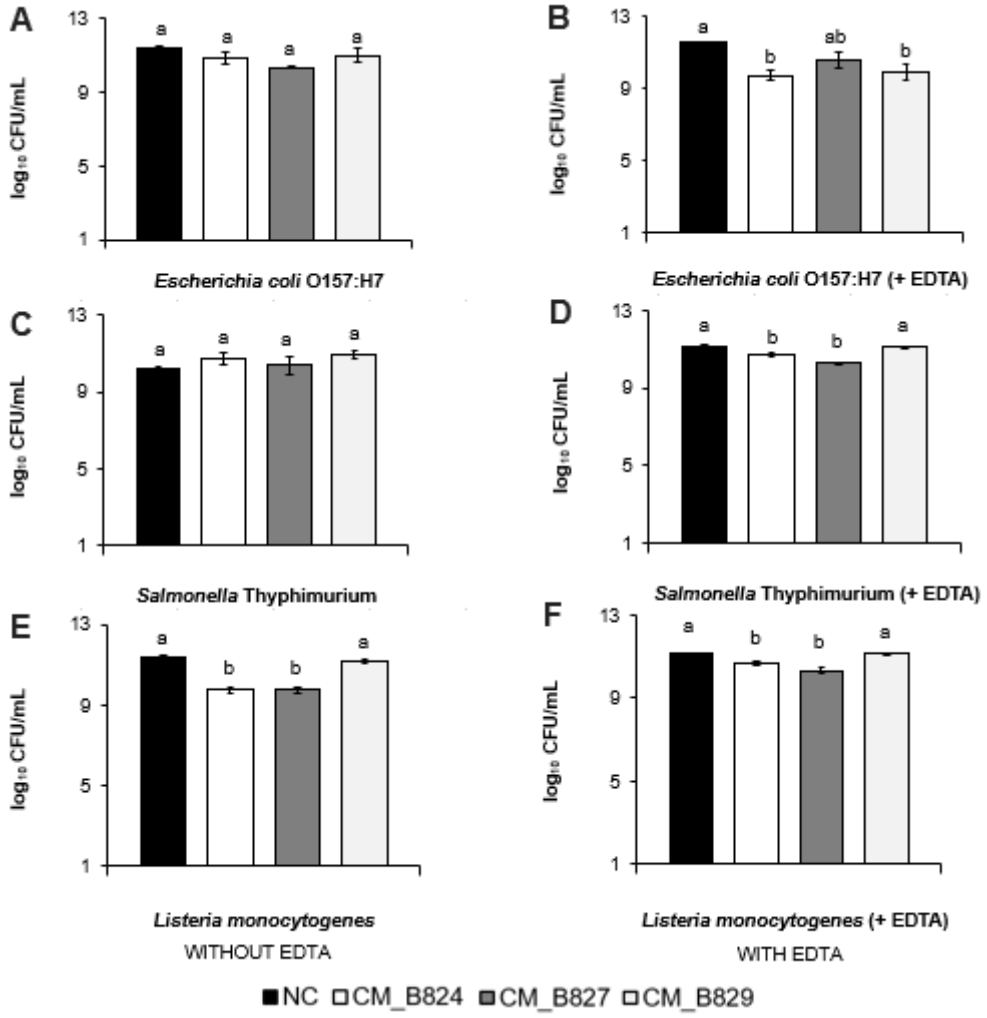
NC = negative control; CM = *Carnobacterium maltaromaticum*



No common superscript indicates that there is a significant difference among treatments (P<0.05).

Figure 2. Count of *L. monocytogenes* at co-culture with *C. maltaromaticum* strains at -1°C for 28 d.

NC = negative control; CM = *Carnobacterium maltaromaticum*



No common superscript indicates that there is a significant difference among treatments (P<0.05).

Figure 3. Count of pathogenic bacteria (*E. coli* O 157:H7 (A-B), *S*. Thyphimurium (C-D), *L. monocytogenes* (E-F) in co-culture with *C. maltaromaticum* strains at 25°C for 48 h, with and without EDTA.

NC = negative control; CM = *Carnobacterium maltaromaticum*

Table 1. Effect of the addition of three different strains of *C. maltaromaticum* and *L. innocua* on the instrumental color L*, a* and b* of cooked ham during storage at 4°C for 7 days.

Treament	Storage days				*P* value
	0	***2***	***5***	***7***	
			L*		
NC	60.10± 0.66^{abB}	63.05± 0.27^{abA}	63.26± 0.57^{aA}	62.91± 0.62^{aA}	0.0017
LI	60.28± 0.71^{abB}	63.65± 0.39^{aA}	64.20± 0.24^{aA}	65.35± 0.94^{aA}	0.0001
CM_824	59.90± 0.66^{abB}	63.56± 0.23^{aA}	63.83± 0.34^{aA}	64.55± 0.36^{aA}	0.0000
CM_827	61.04± 0.41^{abC}	62.60± 0.48^{abBC}	63.52± 0.58^{aAB}	64.70± 0.59^{aA}	0.0006
CM_829	59.18± 0.85^{bB}	62.07± 0.47^{abA}	62.83± 0.40^{aA}	64.13± 0.28^{aA}	0.0000
CM_824+LI	61.23± 0.70^{abA}	60.92± 0.59^{bcA}	63.10± 0.48^{aA}	62.97± 0.81^{aA}	0.0538
CM_827 + LI	61.99± 0.54^{abA}	59.71± 0.44^{cA}	61.03± 1.92^{aA}	63.17± 0.29^{aA}	0.1430
CM_829 + LI	62.32± 0.61^{aA}	63.11± 0.71^{aA}	61.20± 0.96^{aA}	63.98± 0.44^{aA}	0.4327
P value	0.0208	0.0000	0.1007	0.0454	
			a*		
NC	17.13± 1.01^{abA}	15.51± 0.32^{aA}	17.33± 0.32^{aA}	16.38± 0.56^{aA}	0.1822
LI	17.32± 0.64^{abA}	16.10± 0.54^{aA}	17.40± 0.30^{aA}	16.37± 0.56^{aA}	0.2248
CM_824	16.84± 0.57^{abA}	16.44± 0.42^{aA}	17.53± 0.42^{aA}	15.73± 0.88^{aA}	0.2321
CM_827	17.63± 0.57^{aA}	15.34± 0.15^{abB}	16.94± 0.26^{abA}	14.59± 0.27^{aB}	0.0000
CM_829	16.09± 0.62^{abAB}	16.73± 0.44^{aAB}	17.71± 0.43^{aA}	15.88± 0.18^{aB}	0.0374
CM_824+LI	15.17± 0.21^{abA}	13.25± 0.70^{bB}	16.15± 0.50^{abA}	16.43± 0.37^{aA}	0.0005

CM_827 + LI	14.81± 0.59^{bBC}	13.27± 0.62^{bC}	16.79± 0.25^{abA}	15.96± 0.26^{aAB}	0.0002
CM_829 + LI	15.73± 0.63^{abA}	15.58± 0.53^{aA}	15.54± 0.45^{bA}	14.67± 0.31^{aA}	0.3356
P value	0.0159	0.0000	0.0024	0.0349	
			b*		
NC	12.31± 0.60^{bcA}	11.25± 0.40^{abA}	11.51± 0.61^{aA}	11.64± 0.34^{aA}	0.5059
LI	13.55± 0.65^{bcA}	11.50± 0.48^{abA}	11.92± 0.50^{aA}	12.73± 0.58^{aA}	0.0750
CM_824	10.94± 0.16^{cB}	11.75± 0.07^{aAB}	11.99± 0.62^{aAB}	12.71± 0.36^{aA}	0.0246
CM_827	14.56± 0.29^{abA}	11.33± 0.45^{abB}	11.44± 0.49^{aB}	12.04± 0.35^{aB}	0.0000
CM_829	17.19± 0.99^{aA}	11.59± 0.66^{abB}	11.13± 0.15^{aB}	12.30± 0.25^{aB}	0.0000
CM_824+LI	11.52± 0.46^{cAB}	10.50± 0.33^{abB}	12.60± 0.34^{aA}	12.40± 0.44^{aA}	0.0048
CM_827 + LI	11.76± 0.75^{cA}	9.89± 0.13^{bB}	12.37± 0.31^{aA}	11.61± 0.36^{aAB}	0.0052
CM_829 + LI	11.75± 0.44^{cA}	12.34± 0.39^{aA}	11.60± 0.49^{aA}	12.30± 0.20^{aA}	0.5228
P value	0.0000	0.0071	0.3624	0.2701	

NC = negative control; CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua*. Means ± standard error. Different lowercase letters in the same column show significant differences among treatments (*P*<0.05*).* Different uppercase letters in the same row show significant differences among storage time (*P*<0.05*)*.

Table 2. Effect of the addition of three different strains of *C. maltaromaticum* and *L. innocua* on the instrumental color C*, h and pH of cooked ham during storage at 4°C for 7 days.

	Storage days				
Treatment	0	2	5	7	*P value*

Chroma					
NC	21.15± 1.10^{bcdA}	19.18± 0.20^{abA}	20.85± 0.26^{aA}	20.17± 0.33^{abA}	0.1266
LI	22.02± 0.71^{abcA}	19.80± 0.51^{aB}	21.12± 0.36^{aAB}	19.80± 0.25^{abB}	0.0100
CM_824	20.08± 0.52^{cdA}	20.22± 0.34^{aA}	21.25± 0.65^{aA}	19.70± 0.35^{abA}	0.1630
CM_827	23.23± 0.50^{abA}	19.09± 0.31^{abB}	20.43± 0.47^{aB}	18.92± 0.29^{bB}	0.0000
CM_829	24.61± 0.71^{aA}	20.37± 0.71^{aB}	20.93± 0.34^{aB}	20.09± 0.15^{abB}	0.0000
CM_824+LI	18.76± 0.47^{dAB}	16.92± 0.69^{bcB}	20.51± 0.36^{aA}	20.44± 0.58B^{aA}	0.0003
CM_827 + LI	18.45± 0.26^{dB}	16.58± 0.45^{cC}	20.87± .026^{aA}	19.76± 0.18^{abA}	0.0000
CM_829 + LI	19.63± 0.56^{cdA}	19.82± 0.57^{aA}	20.00± .36^{aA}	19.15± 0.29^{abA}	0.6064
P value	0.0000	0.0000	0.4157	0.0335	
H					
NC	35.77± 1.11^{bcA}	35.96± 1.42^{aA}	33.55± 1.76^{abA}	35.01± 1.60^{aA}	0.6609
LI	38.02± 1.45^{bcA}	35.55± 1.50^{aA}	34.37± 1.25^{abA}	40.13± 1.90^{aA}	0.0675
CM_824	33.02± 0.85^{cB}	35.60± 0.74^{aAB}	33.75± 1.14^{abB}	40.11± 2.09^{aA}	0.0048
CM_827	40.50± 1.59^{abA}	36.39± 1.09^{aAB}	33.86± 0.88^{abB}	39.60± 1.07^{aA}	0.0028
CM_829	46.01± 1.33^{aA}	34.58± 1.06^{aBC}	32.20± 0.81^{bC}	37.77± 0.77^{aB}	0.0000
CM_824+LI	37.05± 0.92^{bcA}	38.56± 1.16^{aA}	38.02± 1.37^{aA}	37.00± 0.69^{aA}	0.6754
CM_827 + LI	37.18± 2.07^{bcA}	36.88± 1.55^{aA}	36.35± 0.85^{abA}	36.04± 1.11^{aA}	0.9463
CM_829 + LI	36.74± 0.74^{bcA}	38.22± 0.94^{aA}	37.54± 1.64^{abA}	39.99± 0.70^{aA}	0.2078
P value	0.0000	0.2915	0,0191	0.0461	
pH					

NC	6.42± 0.02^{aA}	5.92± 0.02^{aB}	5.70± 0.02^{bC}	5.88± 0.02^{aB}	0.0000
LI	6.33± 0.11^{aA}	5.61± 0.01^{cC}	5.72± 0.05^{bBC}	5.87± 0.02^{aB}	0.0000
CM_824	5.93± 0.01^{bA}	5.64± 0.02^{cB}	5.61± 0.02^{bB}	5.87± 0.02^{aA}	0.0000
CM_827	6.46± 0.02^{aA}	5.85± 0.04^{abB}	5.61± 0.01^{bC}	5.89± 0.03^{aB}	0.0000
CM_829	6.47± 0.02^{aA}	5.94± 0.03^{aB}	5.62± 0.01^{bC}	5.93± 0.03^{aB}	0.0000
CM_824+LI	5.83± 0.01^{bB}	5.93± 0.02^{aA}	5.89± 0.02^{aAB}	5.66± 0.02^{bC}	0.0000
CM_827 + LI	5.82± 0.01^{bAB}	5.94± 0.01^{aA}	5.90± 0.02^{aAB}	5.80± 0.06^{aB}	0.0196
CM_829 + LI	5.85± 0.03^{bA}	5.76± 0.07^{bcA}	5.90± 0.02^{aA}	5.91± 0.02^{aA}	0.0649
P	0.0000	0.0000	0.0000	0.0000	

NC = negative control; CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua*. Means ± standard error. Different lowercase letters in the same column show significant differences among treatments (*P*<0.05*).* Different uppercase letters in the same row show significant differences among storage time (*P*<0.05*)*.

Table 3. Count of the different strains of *C. maltaromaticum* and the pool of *L. innocua* in sliced cooked ham stored at 4 ºC for 7 days.

Treat.	Storage days				
	0	2	5	7	*P*
CM_B824	5.60± 0.02^{aA}	5.60± 0.02^{aAB}	5.26± 0.02^{abC}	5.33± 0.03^{aBC}	0.0120
CM_B827	5.42± 0.07^{aA}	5.41± 0.07^{aA}	5.08± 0.03^{bA}	5.40± 0.05^{aA}	0.0034
CM_B829	5.49± 0.05^{aA}	5.48± 0.05^{aA}	5.34± 0.08^{aA}	5.35± 0.03^{aA}	0.2163
CM_B824+LI	4.75± 0.00^{bA}	4.75± 0.00^{bA}	4.64± 0.01^{cB}	4.31± 0.01^{cC}	0.0000
CM_B827+LI	4.50± 0.14^{bA}	4.49± 0.13^{bA}	4.60± 0.01^{cA}	4.34± 0.02^{cA}	0.2174
CM_B829+LI	4.46± 0.03^{bB}	4.46± 0.03^{bB}	4.71± 0.05^{cA}	4.54± 0.03^{bAB}	0.0222

P	0.0000	0.0002	0.0056	0.0075	
LI	3.06± 0.02^{aA}	2.60± 0.11^{aA}	2.59± 0.50^{aA}	2.40± 0.09^{aA}	0.1190
CM_B824+LI	2.15± 0.15^{bA}	ND^{bB}	ND ^{bB}	1.350± 0.50^{bAB}	0.0355
CM_B827+LI	1.70± 0.50^{bA}	ND^{bB}	ND ^{bB}	ND^{bB}	0.0298
CM_B829+LI	2.15± 0.15^{bA}	ND^{bB}	1.50± 0.50^{bAB}	ND^{bB}	0.0079
P	0.0067	0.0008	0.0042	0.0133	

CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua*

ND = non-detected (limit of detection = 1 log CFU/g)

Means ± standard error. Different lowercase letters in the same column show significant differences among treatments (*P*<0.05*).* Different uppercase letters in the same row show significant differences among storage time (*P*<0.05).

CAPÍTULO 3

CONSIDERAÇÕES FINAIS

Com o objetivo de trazer soluções mais naturais ao consumidor para evitar casos de surtos e doenças transmitidas por alimentos, buscou-se determinar a capacidade de utilizar uma bactéria lática, *Carnobacterium maltaromaticum*, como cultura bioprotetora em alimentos. Por ser uma bactéria pertencente à microbiota de produtos de origem animal, um produto cárneo pronto para consumo foi escolhido para este estudo. Além disso, *C. maltaromaticum*, por possuir a habilidade de crescer em ambientes com baixas temperaturas, pode ser associada a outras tecnologias de segurança alimentar.

A partir dos testes *in vitro* observou-se um efeito antimicrobiano importante de *C. maltaromaticum* contra *Listeria monocytogenes*, patógeno alimentar de grande relevância para a indústria alimentícia, com especial importância em alimentos prontos para consumo. O mesmo resultado foi observado nos testes em matriz alimentar. *C. maltaromaticum* foi capaz de reduzir a população de *L. innocua* em presunto cozido, já no segundo dia de estocagem, mantendo essa redução durante os dias de análises subsequentes. A adição de *C. maltaromaticum* não causou grandes diferenças na cor e no pH do produto e se manteve estável durante todo o período de estocagem, evidenciando um potencial uso da cultura diretamente na matriz alimentar.

A fim de reiterar a atividade antilistérica dessas cepas, estudos futuros devem ser conduzidos sob diferentes condições ambientais, buscando atividade antimicrobiana ótima da *C. maltaromaticum*. Outra alternativa para buscar maior capacidade bioprotetora, seria a associação desses isolados a diferentes compostos ou métodos que atuem em sinergia no combate aos patógenos alimentares, garantindo maior segurança aos alimentos.

Pelas características desejáveis de *C. maltaromaticum,* como a estabilidade em matriz cárnea, capacidade de crescimento em ambientes com alta osmolaridade, alta pressão e baixas temperaturas, além da atividade antilistéria desse microrganismo, mostram que a utilização dessa espécie como cultura bioprotetora em ambientes industriais e comerciais apresenta grande potencial e relevância.

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