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CAROLINE MARIA DE ANDRADE CAVALARI

CARNOBACTERIUM MALTAROMATICUM AS BIOPROTECTIVE CULTURE AGAINST SPOILAGE BACTERIA IN MEAT PRODUCTS

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Aos vinte e seis dias do mês de junho do ano de dois mil e vinte, às 09:00 horas, por videoconferência realizou-se a sessão pública de defesa da dissertação da mestranda Caroline Maria de Andrade Cavalari, intitulada: **"*CARNOBACTERIUM MALTAROMATICUM* AS BIOPROTECTIVE CULTURE AGAINST SPOILAGE BACTERIA IN MEAT PRODUCTS"**. A mestranda concluiu os créditos exigidos para obtenção do título de Mestre em Ciência Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pela Professora orientadora e Presidente da banca, Dra. Renata Ernlund Freitas de Macedo (PUCPR), auxiliado pelos Professores Doutores Pedro Henrique Didimo Imazaki (Université de Toulouse) e Julia Arantes Galvão (UFPR). Procedeu-se à exposição da Dissertação, seguida de sua arguição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a Dissertação, que nos termos do Artigo 53 do Regulamento deste Programa de Pós-Graduação, foi considerada **APROVADA.**

Profa. Dra. Renata Ernlund Freitas de Macedo (Presidente)

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Prof. Dr. Pedro Henrique Didimo Imazaki (Université de Toulouse)

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Proclamado o resultado, o Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Aline Francielle Bueno Retzlaff, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

Pontifícia Universidade Católica do Paraná
Escola de Ciências da Vida
Programa de Pós-Graduação em Ciência Animal

Curitiba, 26 de junho de 2020.

Aline Francielle Bueno Retzlaff
Secretária do Programa de Pós-Graduação em Ciência Animal

Profa. Dra. Renata Ernlund Freitas de Macedo
Coordenadora do Programa de Pós-Graduação em Ciência Animal

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ABSTRACT

The consumption of meat and meat products has increased over the years. In 2019, the world meat production was 335.2 million tons (carcass weight equivalent), and beef and pork production represented 72.2 and 110.5 million tons, respectively. Meat contains a high amount of nutrients, which along with the moderate pH and the high-water content favors microbial growth. Food industry and retailers estimate that 40% of the meat produced is lost due to microbial spoilage, leading to economic, social and environmental impacts. Therefore, methods to preserve meat and extend the product shelf life play a crucial role for both consumers and industry. Biopreservation consists of using a microorganism naturally present or artificially inoculated on the food in concentrations high enough to inhibit the growth of other spoilage and pathogenic microorganisms. This preservation method is an interesting alternative to preserve meat products due to the minimal impacts on the nutritional and sensorial parameters of the product. Lactic acid bacteria are strongly used for this method. *Carnobacterium maltaromaticum* shows desirable characteristics as a bioprotective culture. The genus Carnobacterium can grow under adverse environmental conditions, such as low temperature, high salt concentration and moderate pH range. Bacteria from this genus are naturally found in several foods and environments. Some representatives, *C. maltaromaticum* and *Carnobacterium divergens* have inhibitory effects against spoilage and pathogenic microorganisms. Thus, this study aimed to evaluate the potential inhibitory effect of *C. maltaromaticum* previously isolated from vacuum packaged Australian beef (*longissimus dorsi*) with 140 d of shelf life at -1 °C. Three strains of *C. maltaromaticum* (CM_B824, CM_B827 and CM_B829) and the pool of these strains (CM) were tested against *Pseudomonas fluorescens* ATCC®1355™ (PF), a pool of three strains of *Brochothrix thermosphacta* (ATCC®11509™, s109 and s153) (BT) and *Listeria monocytogenes* ATCC®19117™ (LM) *in vitro*. The bacteria were grown in BHI broth for 24 h at 25 °C (spoilers) and at 37 °C (pathogen), from which 7 log CFU/mL of Carnobacteria and 3 log CFU/mL of target bacteria were cocultured in BHI broth for 72 h at 15 °C in aerobic and anaerobic conditions. All treatments containing Carnobacteria showed a high inhibitory effect against BT and LM, the performance varied according to the atmosphere. The inhibition of Listeria corroborates with previous studies conducted with the same Carnobacteria strains in other temperatures. The PF growth was more efficiently inhibited in anaerobiosis. Carnobacteria reached high concentrations independently of the presence of the competitor under the conditions tested. Moreover, the bioprotective effect of the pool of Carnobacteria was tested in two food matrices (raw ground beef and sliced cooked ham) against *P. fluorescens* ATCC®1355™ and *B. thermosphacta* (ATCC®11509™, s109 and s153). The ground beef was inoculated with the concentrations of 6.76 (CM), 3.49 (BT) and 3.48 (PF) log CFU/mL and the sliced cooked ham with 6.37 (CM), 3.99 (BT) and 3.79 (PF). The ground beef was stored under modified atmosphere with high oxygen content (MAP - 66% O_2, 4% N_2 and 30% CO_2) for 7 d (initially at 4 °C for 3 d and after at 8°C for 4 d); and the sliced cooked ham was stored under MAP with low oxygen content (70% N_2 and 30% CO_2) for 28 d (initially at 4 °C for 10 d and after at 8°C for 18 d). The microbiological and physicochemical analyses (pH, instrumental colors and headspace gas composition) were performed at the 0, 3 and 7 d (ground beef) and at 0, 5, 10, 19 and 28 d (ham) of storage. Results for ground

beef: the pool of Carnobacteria significantly reduced the counts of BT and PF inoculated populations and indigenous *Brochothrix spp*. and *Pseudomonas spp.* The final counts of CM were not influenced by BT presence. CM treatments showed slight pH values decrease and increase in CO_2 package concentration. The quality characteristics of the ground beef were practically not affected by Carnobacteria. Results for sliced cooked ham: both spoilage bacteria were inhibited. The CM growth was not influenced by the spoilage bacteria. The presence of CM weakly reduced the pH values and increased the CO_2 concentration in package. CM maintained the color of the product and color differences during storage were below theoretical decline of the product by the costumer. The results corroborate with the abilities of Carnobacteria to inhibit spoilage bacteria; to grow and maintain its population at a high number until the end of storage and has low impact on the physical-chemical characteristics of the meat products. Thus, the use of *C. maltaromaticum* can be considered a natural alternative to the bioprotection of meat products.

Key words: bioprotection, lactic acid bacteria, ground beef, cooked ham, spoilage.

RESUMO GERAL

O consumo médio de produtos cárneos e carne está aumentando gradativamente ao longo dos anos. Em 2019, A produção mundial de carne é de 335,2 milhões de toneladas (peso equivalente em carcaça). A produção de carne bovina e suína representam 72,2 e 110,5 milhões de toneladas, respectivamente. A carne é um alimento rico em nutrientes, que em conjunto com pH moderado e alta atividade de água, favorece o crescimento microbiano. A indústria alimentícia e vendedores estimam que 40% da carne produzida é perdida por causa da deterioração microbiana, acarretando impactos econômicos, sociais e ambientais. Portanto, métodos de preservação da carne e prolongamento da sua vida de prateleira são de suma importância para a indústria e consumidores. A técnica de biopreservação consiste no uso de uma bactéria artificialmente ou naturalmente presente no alimento, inoculada em concentrações suficientes para inibir o crescimento de outras bactérias patogênicas e deteriorantes. Essa técnica é uma opção atrativa para preservar os produtos cárneos em razão dos mínimos impactos nos parâmetros nutricionais e sensoriais do produto. Bactérias ácido lácticas (LAB – *lactic acid bacteria*) são fortemente empregadas com essa finalidade. A *Carnobacterium maltaromaticum* apresenta características desejáveis de uma cultura bioprotetora. O gênero Carnobacterium é capaz de crescer em condições ambientais adversas como baixas temperatura usadas na refrigeração de alimentos, altas concentrações de sal e moderada variação de pH. Bactérias desse gênero são naturalmente encontradas em vários alimentos e ambientes. Alguns representantes, como *C. maltaromaticum* e *Carnobacterium divergens*, apresentam espectro inibitório de bactérias deteriorantes e patogênicas. Dessa forma, este estudo objetivou avaliar o potencial efeito inibitório de *C. maltaromaticum* previamente isolada de bife australiano (*longissimus dorsi*) armazenado a vácuo com 140 d de vida de prateleira a -1 °C Três cepas de *C. maltaromaticum* (CM_B824, CM_B827 e CM_B829) e o pool dessas cepas (CM) foram testadas contra *Pseudomonas fluorescens* ATCC®1355™ (PF), pool de três cepas de *Brochothrix thermosphacta* (BT- ATCC®11509™, s109 e s153) e *Listeria monocytogenes* ATCC®19117™ (LM) *in vitro*. As bactérias foram crescidas em caldo BHI por 24 h a 25 °C (deteriorantes) e a 37 °C (patógeno), dos quais 7 log UFC/mL de Carnobacteria e 3 log UFC/mL de bactéria alvo foram crescidos em co-cultura em BHI por 72 h a 15 °C em condições aeróbia e anaeróbia. Todos os tratamentos contendo Carnobacteria tiveram um efeito inibitório elevado contra BT e LM, com diferentes desempenhos de acordo com a atmosfera. A inibição da Listeria corrobora com estudos anteriores feitos com as mesmas cepas de Carnobacteria em outras temperaturas. O crescimento da PF foi inibido com maior eficácia em anaerobiose. A Carnobacteria atingiu altas concentrações independente da presença do competidor nas condições testadas. O efeito bioprotetor do pool de Carnobacteria foi testado em duas matrizes alimentares (carne moída crua e presunto cozido fatiado) contra *P. fluorescens* ATCC®1355™ e *B. thermosphacta* (ATCC®11509™, s109 e s153). A carne moída foi inoculada com as concentrações de 6,76 (CM), 3,49 (BT) e 3,48 (PF) log UFC/mL e o presunto com 6,37 (CM), 3,99 (BT) e 3,79 (PF) UFC/m. A carne moída foi armazenado em atmosfera modificada com elevada concentração de oxigênio (MAP - 66% O_2, 4% N_2 e 30% CO_2) por 7 d

(inicialmente a 4 °C por 3 d e depois a 8°C por 4 d); e o presunto foi armazenado em MAP com baixa concentração de oxigênio (70% N_2 e 30% CO_2) por 28 d (inicialmente a 4 °C por 10 d e depois a 8°C por 18 d). As análises microbiológicas e físico químicas (pH, cor instrumental e composição gasosa) foram desenvolvidas para 0, 3 e 7 d (carne moída) e 0, 5, 10, 19 e 28 d (presunto) de armazenamento. Considerando a carne moída, o pool de Carnobacteria reduziu a contagem da população de BT e PF inoculadas e *Brochothrix spp*. e *Pseudomonas spp.* naturalmente presentes no alimento. A contagem final de CM não foi afetada pela BT. Tratamentos com CM tiveram leve caimento de pH e elevada concentração de CO_2. As características de qualidade da carne moída praticamente não foram afetadas. Considerando o presunto, ambas bactérias deteriorantes foram inibidas. O crescimento de CM não foi afetado por nenhuma das bactérias deteriorantes. A presença da CM reduziu fracamente o pH e elevou a concentração de CO_2. CM manteve as características de cor instrumental preservadas e abaixo da rejeição teórica do produto pelo consumidor. Os resultados se justificam nas habilidades da Carnobacteria: inibir bactérias deteriorantes; crescer e manter uma população elevada e estável até o final da vida de prateleira; causar impactos mínimos nas características físico-químicas dos produtos cárneos. Dessa forma, o uso da *C. maltaromaticum* pode ser considerado como uma alternativa natural para a bioproteção de produtos cárneos.

Palavras chaves: bioproteção, bactéria ácido lática, carne moída, presunto cozido, deterioração.

CAPÍTULO 1

INTRODUCTION AND CONTEXTUALIZATION

Meat and meat products are economically important and take part of a considerate segment of the food chain system. The consumption, production and trade rate of meat is increasing worldwide. A higher increase is observed to bovine and poultry meat, followed by pig and sheep meat (FAO, 2018). Thus, the consumption of fresh meat and meat products such cooked, slicez9zd and processed products is also increasing (VERCAMMEN et al., 2011; GODFRAY et al., 2018). This is mainly justified by the search for a desirable eating experience and the intake of food with high nutritional value (protein, essential amino acids, lipids, iron, zinc and vitamin B_{12}) in daily basis (WYNESS, 2011; SMITH; GOTOH; GREENWOOD, 2018). Globally, the average yearly consumption of meat increased from 23 to 43 kg/year since 1961 to 2013 (LUNDSTRÖM, 2019). Meat production/year from 30 million tons, in 1970, reached 335.2 million tons (carcass weight equivalent) in 2019. From which, 72.2 and 110.5 million tons are represented by bovine and pig meat, respectively (FAO, 1995; 2019). Moreover, by the year of 2027, it is estimated that global meat consumption increases 35.4 kg (retail weight equivalent) per capita (FAO, 2018).

The rich nutritious composition (essential amino acids, lipids, vitamins and minerals) with high water activity and moderate pH of the meat create an appropriate environment to microbes to develop; thus, meat products are extremely perishable (DAVE; GHALY, 2011; WYNESS, 2011; SMITH; GOTOH; GREENWOOD, 2018). The consumption of meat nutrients results in the production of undesirable metabolites that characterize the spoilage (ERCOLINI et al., 2009). Thus, the product shelf life is determined based on the period that the food still retains its qualitative parameters and safeness (IULIETTO et al., 2015). The loss of these characteristics leads to consumers rejection of the products, consequently, resulting in food waste and economic losses. Globally, the food waste counts to 1.3 billion tons a year, from which 21% is represented by meat and meat products losses in Europe and Northern America (HÖLL; BEHR; VOGEL, 2016; GODFRAY et al., 2018). Moreover, meat industry and retail market losses 40% of the production due to spoiled meat (SPERBER, 2009). Additionally, meat is product with high value added. In May 2020, beef prices (USD/

ton), accounts for $ 5895/ ton in the United States of America, $ 4390/ ton in Brazil and $ 4980/ ton in Australia. While pork prices were $ 2548/ ton in the United States of America, $ 2409/ ton in Brazil and $ 1836/ ton in Germany (FAO, 2020). Thus, it is more than an economic and credibility losses, the spoilage causes lack of edible food with significant added value.

The increase of the demand of beef is related to seven factors: price, health, social aspect, sustainability, nutrition, and more importantly, product quality and food safety. Thus, for meat marketing the industry heavily relies on the food safety, product quality, nutritional value and healthfulness (FLOWERS et al., 2019). Consequently, the use of natural biopreservatives to extend the product shelf life, to keep safeness and the food quality, is of the industry's best interest; which also works as a label to attract consumers avoiding meat waste and incrementing the sales.

The spoilage, consequently, results from strong off-odors, gross discoloration, gas production, unattractive flavors and slime production. What changes the quality parameters of the meat, shortening the shelf life and leading to the rejection by the consumers (ERCOLINI et al., 2006; NYCHAS et al., 2008; IULIETTO et al., 2015). This cause meat waste and economic and credibility losses to meat industry (NYCHAS et al., 2008; MOHAREB et al., 2015; STANBOROUGH et al., 2016; LORENZO et al., 2018). The spoilage process is deeply related to initial contaminations, which may vary according to such factors: slaughtering houses of origin, transportation, processing and storage of the product in the markets (NYCHAS et al., 2008). The microbial growth alters the protein and lipidic content with the consumption of sugar and free amino acids present in the food matrix, secreting volatile compounds (DRAGOEV et al., 2014; ERCOLINI, 2006). The spoilage can be delayed by controlling conditions such as contamination, temperature, gas composition and by adding chemical or natural preservatives (ZHOU; XU; LIU, 2010). Although, psychrotrophic microorganisms such as *Brochothrix thermosphacta*, *Pseudomonas spp*., *Enterobacterium*, *Leuconostoc* and *Carnobacterium* can grow to spoilage levels throughout storage time for several packaging conditions (air, vacuum and modified atmosphere packaging - MAP) (JAY; VILAI; HUGHES, 2003; RUSSO et al., 2006; PENNACCHIA; ERCOLINI; VILLANI, 2011; HÖLL; BEHR; VOGEL, 2016). These bacteria can grow to loads of 10^7 CFU cm^{-2}, which are connected to fruity, cheesy and buttery odors; when bacterial concentration reaches 10^9 CFU/ cm^{-2} the odors turn putrid (ERCOLINI et al., 2006).

Pseudomonas spp*.* and *B. thermosphacta* along with other spoilage bacteria, such as Enterobacteria, can produce sulfur-containing compounds and metabolites such as acetoin, diacetyl and 3-methylbutanol, causing sulfuric and cheesy odors in meat (CASABURI et al., 2015; MANSUR et al., 2019).

Pseudomonas spp. are gram-negative bacteria and abundant spoilage agents of aerobically refrigerated meat, such as beef (ERCOLINI et al., 2006, 2009). Strains belonging to the species *Pseudomonas fragi* and *Pseudomonas fluorescens* are commonly isolated from deteriorated meat and dairy products (BOUCHARD, et al., 2006; ERCOLINI et. al, 2009; MARTIN et. al, 2011; STELLATO et. al, 2017). *P. fluorescens* is highly capable of contaminating and colonizing the matrix, due to the formation of biofilms which are resistant to cleaning procedures of industrial equipment surfaces (STELLATO et al., 2017; WANG et al., 2018). This *bacterium* as a spoiler cause meat discoloration (CHAN et al., 1998; CIRCELLA et al., 2020), affecting the market. The color of the red meat is essential for the market and the discoloration is the first reason for consumer's rejection (ZAGOREC; CHAMPOMIER-VERGÈS, 2017). This species also produces undesirable flavors, off-odors and green pigments as a result from strong proteolytic and lipolytic activities (PENNACCHIA; ERCOLINI; VILLANI, 2011; STANBOROUGH et al., 2016). Although *P. fluorescens* is an aerobic spoilage bacterium, it can also grow slowly in meat preserved with low oxygen and high CO_2 content (TAN; GILL, 1982; HENDRICKS; HOTCHKISS, 1997; STOOPS et al., 2012). *P. fluorescens* can easily adhere to surfaces and produce biosurfactants, compounds that degrade the meat matrix, release fat and nutrients enabling the faster bacteria growth over the others. This results in intensified decomposition (MELLOR; BENTLEY; DYKES, 2011). These bacteria can survive under the stressful environment present in MAP packaging (LIU et al., 2018). *Pseudomonas spp.* was found in spoiled ground beef stored aerobically, in MAP_1 (60%O_2 and 40%CO_2), MAP_2 (20%O_2, 40%CO_2 and 40%N_2) (ERCOLINI et al., 2006) and MAP_3 (70%O_2 and 30%CO_2) (CHAILLOU et al., 2014).

The genus *Brochothrix* belongs to the family Listeriaceae. The most important species causing meat spoilage are *Brochothrix thermosphacta* and *Brochothrix campestris* (ZAGOREC; CHAMPOMIER-VERGÈS, 2017). *B. thermosphacta* is a natural inhabitant of the meat environment that can grow in either aerobic or anaerobic conditions (PIN; FERNANDO; ORDÓÑEZ, 2002). Its development usually is a problem to chilled meat products packaged in modified atmosphere (MAP) and vacuum

(PENNACCHIA; ERCOLINI; VILLANI, 2011; STANBOROUGH et al., 2016; RAIMONDI et al., 2018). These *bacteria* are responsible for the production of gas, slime, putrefaction odors and discoloration in meat (CASABURI et al., 2015), which changes the texture of the food and leads to rejection by consumers (ZAGOREC; CHAMPOMIER-VERGÈS, 2017). Moreover, *B. thermosphacta* has tolerance to high salt concentrations and low pH values (GONÇALVES et al., 2017).

Listeria monocytogenes is a gram-positive pathogen found in several environments: soil, water, sewer; as part of human fecal flora and domestic animals, and more importantly, in both fresh and deteriorated fruits, vegetables (IVANEK; GRÖHN; WIEDMANN, 2006; HOLMØY; LIPPE; LEEGAARD, 2017; ZHU; GOONERATNE; HUSSAIN, 2017) meat and dairy products (ZAULET et al., 2016; HOLMØY; LIPPE; LEEGAARD, 2017). According to Barros et al. (2007), the contamination of the meat product comes from equipment, installations and from the product on itself. When ingested this pathogen causes listeriosis, and the infection can be deadly. Symptoms such as vomit, fever, fatigue and muscular pain are characteristics. It can also lead to other invasive infections, as meningitis, bacteremia, gastroenteritis and systemic infections (DREVETS; BRONZE, 2008). It shows a risk to elders, immunologically suppressed patients and newborn (PÉREZ-TRALLERO et al., 2014). Listeria can survive in adverse grow conditions used to preserve the meat, the develop occurs also during transport and storage of the product, reaching concentrations high enough to cause listeriosis when ingested (BORTOLUSSI, 2008; ZHU; GOONERATNE; HUSSAIN, 2017). The contamination of food by this pathogen is a concern of public health and food industry, with expenses for treatment and loss of productivity (SOUSA, 2008; THOMAS et al., 2015).

The food can be conserved using several methods: low temperature, freezing, sterilization, irradiation, MAP, salts and chemical additives. One of the most common methods to preserve meat is refrigeration, which is based on low temperatures to reduce the metabolism of microorganisms and extend the shelf life (MARCINKOWSKA-LESIAK; POŁAWSKA; WIERZBICKA, 2017). Notwithstanding, some microorganisms (classified as psychotropic) can grow in such conditions and induce spoilage. Moreover, not all these methods can be applied to all products neither attend to consumers demands for more naturally tasting foods, safer, additive-free and fresher are increasing (THERON; LUES, 2007). This is especially applied to ready-to-eat and fresh meat products (QUINTAVALLA; VICINI, 2002; OLASUPO et al., 2003).

Thus, the biopreservation method is advantageous over the others. The extension of the shelf life of products is obtained with minimal impact on the quality characteristics (organoleptic, hygienic and nutritional) (SINGH, 2018). This method relies on the use of bacteria, naturally or artificially present, to inhibit pathogenic or spoilage microorganisms as a result from competition for nutrients or release of antagonist compounds, such as bacteriocins.

Carnobacteria is one of most predominant lactic acid bacteria (LAB) in refrigerated, modified atmosphere-packed and vacuum stored meat microbiota (LAURSEN et al., 2005; ZHANG; BARANYI; TAMPLIN, 2015). The most common representatives of this group are *Carnobacterium maltaromaticum* and *Carnobacterium divergens* which tends to predominate in cheese and meat stored between -1.5 and 4 °C (LEISNER et al., 2007). These two species are frequently found in abundance as mesophilic and psychrophilic populations in beef (ERCOLINI et al., 2009). *Carnobacterium* species are isolated from several ecological niches, including food (meat, fish, dairy products) (LEISNER et al., 2007; AFZAL et al., 2010) and can survive adverse conditions (pH, temperature, osmose) (ZHANG; BARANYI; TAMPLIN, 2015). Studies demonstrate these bacteria growth in moderate pH (5.00-9.50) and wide temperature range (from -1.5 to 37 °C); high NaCl concentrations (5% (w/v) (LEISNER et al., 2007; EDIMA et al., 2008; WAYNE et al., 2012; ZHU et al., 2018); high pressure (200 – 400 MPa for 15-20 min) (DALGAARD et al., 2006); low pressure such as 0.0007 MPa (WAYNE et al., 2012); and packaging conditions such as vacuum (DALGAARD et al., 2006), MAP (LAURSEN et al., 2006; IMAZAKI, 2018) and air (DANIELSKI et al., 2020). Other studies indicate that Carnobacteria can both be related to spoilage (proteolytic activity, production of aldehydes and sulfur compounds) (ERCOLINI et al., 2009; CASABURI et al., 2011), biopreservation (BUCHANAN; BAGI,1997; GHANBARI et al, 2013; ZHANG et al., 2017; IMAZAKI, 2018; DANIELSKI et al., 2020) and can be used as a probiotic (NILSSON, 2005; PILCHOVÁ et al., 2016; KONÉ et al., 2018). *Carnobacterium* can display a better protective effect than most LAB in reason to its survival skills. This genus outcompetes both pathogens and spoilage, which likely results from several competition skills: production of bacteriocins (YOUSSEF et al., 2014; ZHANG et al., 2017); production of antimicrobial compounds such as organic acids, H_2O_2, diacetyl and CO_2 (KASRA-KERMANSHAHI; MOBARAK-QAMSARI, 2015; ZHANG et al., 2017; SAID et al., 2019); fast growth rate (NILSSON et al., 2005).

Thus, the following study intended to investigate the potential use of *C. maltaromaticum* as an innovation method for the preservation of chilled meat products, such as raw beef and sliced cooked ham. The goal was to assess the the antimicrobial effect of the protective culture in inhibiting spoilage bacteria (*B. thermosphacta* and *P. fluorescens*) and foodborne bacteria (*Listeria monocytogenes*) *in vitro*. Additionally, the evaluation of the potential of *C. maltaromaticum* to increase the microbiological stability of meat products by inhibiting spoilage bacteria was also carried out.

CAPÍTULO 2

Antimicrobial effect of *Carnobacterium maltaromaticum* against spoilage and pathogenic bacteria *in vitro*

Abstract

This study aimed to evaluate the antimicrobial effect of *C. maltaromaticum* strains, individually and in pool, towards pathogenic, *Listeria monocytogenes*, and food spoilage bacteria, *Pseudomonas fluorescens* and *Brochothrix thermosphacta in vitro*. Coculture tubes contained 7 log CFU/mL of *C. maltaromaticum* and 3 log CFU/mL of the target strains in 10 mL of BHI broth. Control tubes were prepared for each bacteria treatment. All tubes were incubated at 15 °C for 72 h in aerobic and anaerobic conditions. All *C. maltaromaticum* treatments showed strong inhibitory effect against *B. thermosphacta* and *L. monocytogenes*. *Pseudomonas fluorescens* was slightly, but significantly, inhibited in aerobiosis. Overall, among *C. maltaromaticum* strains, CM_B824 showed the greatest inhibition of spoilage bacteria and antilisterial effect. In summary, *C. maltaromaticum* seems to present interesting bioprotective potential for further tests on food matrix as natural meat biopreservative.

Keywords: Biopreservation, meat spoiler, lactic acid bacteria; *in vitro*

1 Introduction

Meat products are highly susceptible to spoilage in reason to the nutritious components and elevated water activity. The microbiota and the storage conditions, such as temperature, gas composition and the use of chemical preservatives are fundamental tools to control the spoilage and determinate the shelf life (ZHOU; XU; LIU, 2010). The lack of control of these conditions may cause economic and credibility losses to meat in industry (NYCHAS et al., 2008; STANBOROUGH et al., 2016) also, waste of edible food (LORENZO et al., 2018) with high nutritional and economic value.

Cooked and sliced meat products are of great economic importance and are largely consumed world-wide (CASIRAGHI; ALAMPRESE; POMPEI, 2007; VERCAMMEN et al., 2011; KALSCHNE et al., 2015). These products are susceptible to spoilage even under refrigerated storage. The common spoilage bacteria in this type of product are *Pseudomonas*, *Brochothrix*, Enterobacteria and lactic acid bacteria (LAB) (PENNACCHIA; ERCOLINI; VILLANI, 2011).

Pseudomonas and *Brochothrix* take advantage of the selective combination of low temperature and long storage periods over other spoilage and pathogenic bacteria. Those genera represent an obstacle to the shelf life extension of refrigerated products. They contribute with spoilage by producing undesirable molecules that cause off-flavors, off-odors and changes in texture, main causes to consumer's rejection (ZAGOREC; CHAMPOMIER-VERGÈS, 2017).

Pseudomonas fluorescens is significant representative of meat spoiler, mainly aerobically stored and can adapt well to stressful environments (LIU et al., 2018). *Brochothrix thermosphacta*, facultative anaerobe, is a problem for meat quality, for both storage in modified atmosphere and vacuum (PENNACCHIA; ERCOLINI; VILLANI, 2011; CASABURI et al., 2015; STANBOROUGH et al., 2016; MANSUR et al., 2019).

Listeria monocytogenes is a foodborne pathogen found in several types of food (ZAULET et al., 2016; HOLMØY; LIPPE; LEEGAARD, 2017). Meat can get contaminated in slaughter or processing; or naturally contain levels of the pathogen (BARROS et al., 2007). This microorganism can grow in low temperatures used to storage meat and cause listeriosis when ingested. Thus, *L. monocytogenes* concerns food safeness (ZHU; GOONERATNE; HUSSAIN, 2017).

Carnobacterium spp., psychrotrophic LAB, can thrive and multiply under adverse conditions to other microorganisms, such as moderate pH (5.00-9.50) and wide temperature range (0 – 37 °C); high NaCl concentrations (5% (w/v) (EDIMA et al., 2008; WAYNE et al., 2012; ZHU et al., 2018); high pressure (200 – 400 MPa for 15-20 min) (DALGAARD et al., 2006); low pressure such as 0.0007 MPa (WAYNE et al., 2012); and packaging conditions such as vacuum (DALGAARD et al., 2006), modified atmosphere packaging (LAURSEN et al., 2006; IMAZAKI, 2018) and air (DANIELSKI et al., 2020) packaging. *Carnobacterium maltaromaticum* and *Carnobacterium divergens* are the main representatives of this group that are frequently found in meat products stored in diverse conditions (LAURSEN et al., 2005; LEISNER et al., 2007; ERCOLINI et al., 2009; ZHANG; BARANYI; TAMPLIN, 2015). Both species can show inhibitory effect with or without bacteriocin production. The bacteriocin production can be affected by several factors. When influenced by temperature, *C. maltaromaticum* shows to better produce bacteriocins at 15 °C (MATHIEU et al., 1994; GURSKY et al., 2006; BRILLET-VIEL et al., 2016). Moreover, studies have been exploring the abilities of strains of these of these two species, to inhibit animals (RINGØ et al., 2002), humans and food pathogens or spoilers *in vitro* (BUCHANAN; BAGI,1997; LAURSEN et al., 2005; NILSSON et al., 2005; PILCHOVÁ et al. 2016; ZHANG; BARANYI; TAMPLIN, 2015; ZHANG et al., 2017; IMAZAKI, 2018; DANIELSKI et al., 2020).

In this context, the use of naturally present bacteria can be beneficial as an alternative to extending the shelf life of refrigerated meat products. In order to reach this goal, firstly, the bioprotective effect of *Carnobacterium maltaromaticum* was evaluated *in vitro* against *Pseudomonas fluorescens*, *Brochothrix thermosphacta* and *Listeria monocytogenes.*

2 Materials and methods

2.1 Bacterial strains

The strains of *C. maltaromaticum* (CM_B824, CM_B827 and CM_B289) were isolated by Imazaki et al. (2018) from Australian beef (*longissimus dorsi*) with an extremely long shelf life (140 d) stored in vacuum packaging at -1 °C. These three strains were selected from 11 others, representing three different phylogenetic groups with carnobacteriocin BM1 and B2 (only CM_B824) genes.

The spoilage strains tested were *Pseudomonas fluorescens* (PF) (ATCC®1355™) and a pool of three stains of *Brochothrix thermosphacta* (BT) *(*ATCC®11509™, s109 and s153). The *B. thermosphacta* strain s109 was isolated from beef and strain s153 obtained from bone-in ham (*jambon a l'os*). As the pathogenic strain, *L. monocytogenes* (LM) (ATCC®19117™) was used.

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

CM and spoilage strains were grown in brain heart infusion (BHI) broth (OXOID) for 24 h at 25 °C and LM was grown in BHI broth for 24 h at 37 °C. Co-cultures (cell-to-cell contact) were carried out by inoculating the bacteria in Falcon® flasks containing 10 mL of BHI broth with CM pool or CM strains individually at 7.00 log CFU/mL and one of the target strain (PF, BT and LM) at 3.00 log CFU/mL, and incubated at 15 °C for 72 h in aerobic and anaerobic (anaerobic jar and AnaeroGen™ *sachet* generator - OXOID) conditions. AnaeroGen™ *sachet* can reduce O_2 levels to below 1% within 30 min and result in CO_2 levels of 9 and 13% of the jar volume. Negative controls were considered flasks with BHI inoculated with each target bacteria at ≈ 3 log CFU/mL and positive controls were considered flasks inoculated only with CM strains. Controls and treatments were conducted in triplicate.

PF was enumerated in *Pseudomonas* Agar Base added with CFC supplement (Cetrimide 10 mg/L; Fucidin 10 mg/L and Cephalosporin 50 mg/L) (OXOID) and BT in STAA agar base added with STAA supplement (Streptomycin sulphate 500 mg/L; Thallous acetate 50 mg/L and Cycloheximide 50 m/L) (OXOID), both incubated for 24 h at 25 °C. LM was counted using specific chromogenic media: RAPID'*L.mono* (BioRad, Marnes, France) for 24 h at 37 °C. CM population was estimated as the

difference between counts on Plate Count Agar (OXOID) and counts on specific agar used for target bacteria counting.

2.3 Statistical analysis

The data were expressed in mean ± SE (standard error). The antimicrobial effect of CM towards each target bacteria in aerobic or anaerobic conditions was analyzed by Multi-Way ANOVA and means were compared by Tukey test ($P < 0.05$). The analyses were conducted using Statgraphics® Centurion XVI *version* 16.1.11 (Statpoint Technologies, Warrenton, Virginia, USA).

3 Results

3.1 Antimicrobial evaluation *in vitro*

BT growth was inhibited when in co-culture with CM for 72 h in all treatments tested (CM+BT, CM_B824+BT, CM_B827+BT and CM_B829+BT), in both atmospheres (Table 1). CM_B824 was the most effective CM strain in inhibiting BT compared to the others CM treatments in both atmospheres ($P < 0.05$).

CM_B824+BT and CM+BT showed higher BT growth inhibition in anaerobiosis than in aerobiosis ($P < 0.05$). There was no difference on the inhibitory effect of CM_B827 and CM_B829 towards BT in the atmospheres tested (Table 1).

BT grew similarly in both aerobic and anaerobic conditions ($P < 0.05$). Pin, Fernando and Ordonez (2002) also reported that gas composition had no effect on BT growth, but had effect on the metabolites production, affecting the spoilage activity of this bacterium (Table 1).

Table 1. Population of pool of *Brochothrix thermosphacta* alone and in co-culture with *Carnobacterium maltaromaticum* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+BT	$3.49 \pm 0.08^{Aa\psi}$	$3.69 \pm 0.02^{Aa\psi}$	$5.21 \pm 0.05^{Da\S}$	$4.12 \pm 0.12^{Db\S}$
CM_B827+BT	$3.58 \pm 0.03^{Aa\psi}$	$3.68 \pm 0.01^{Aa\psi}$	$6.49 \pm 0.08^{Ca\S}$	$6.51 \pm 0.16^{Ca\S}$
CM_B829+BT	$3.49 \pm 0.08^{Aa\psi}$	$3.69 \pm 0.02^{Aa\psi}$	$7.69 \pm 0.05^{Ba\S}$	$7.64 \pm 0.08^{Ba\S}$

CM+BT	3.58 ± 0.03$^{Aa\psi}$	3.71 ± 0.01$^{Aa\psi}$	7.89 ± 0.02^{Ba§}	7.49 ± 0.02^{Bb§}
BT	3.53 ± 0.06$^{Aa\psi}$	3.70 ± 0.02$^{Aa\psi}$	8.53 ± 0.07^{Aa§}	8.69 ± 0.02^{Aa§}

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Pool of *B. thermosphacta* (BT) = ATCC®11509™, s109 and s153.

Treatments for co-culture: CM_B824 + BT = *C. maltaromaticum* CM_B824 + pool of *B. thermosphacta*; CM_B827 + BT = *C. maltaromaticum* CM_B827 + pool of *B. thermosphacta*; CM_B829 + BT = *C. maltaromaticum* CM_B829 + pool of *B. thermosphacta*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*.

Negative control: BT = pool of *B. thermosphacta*.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions, at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

Regarding CM, in anaerobiosis CM growth in all CM treatments was not influenced by the presence of BT ($P < 0.05$). In aerobiosis, CM_B824 showed a slight lower count (Δ 0.3 log CFU/ mL) than CM pool, showing no difference to the other CM strains after 72 h (Table 2).

Table 2. Population of *Carnobacterium maltaromaticum* alone and in co-culture with pool of *Brochothrix thermosphacta* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+BT	7.24 ± 0.08$^{Aa\psi}$	7.22 ± 0.06$^{Aa\psi}$	9.11 ± 0.08^{Bb§}	9.36 ± 0.05^{Aa§}
CM_B827+BT	7.27 ± 0.04$^{Aa\psi}$	7.14 ± 0.06$^{Aa\psi}$	9.39 ± 0.03^{ABa§}	9.28 ± 0.03^{Ab§}
CM_B829+BT	7.23 ± 0.04$^{Aa\psi}$	7.10 ± 0.03$^{Aa\psi}$	9.37 ± 0.13^{ABa§}	9.18 ± 0.04^{Aa§}
CM+BT	7.14 ± 0.03$^{Aa\psi}$	7.32 ± 0.07$^{Aa\psi}$	9.45 ± 0.02^{Aa§}	9.27 ± 0.12^{Aa§}
CM_B824	7.24 ± 0.07$^{Aa\psi}$	7.26 ± 0.03$^{Aa\psi}$	9.24 ± 0.08^{ABa§}	9.17 ± 0.11^{Aa§}
CM_B827	7.27 ± 0.04$^{Aa\psi}$	7.11 ± 0.07$^{Aa\psi}$	9.27 ± 0.04^{ABa§}	9.08 ± 0.10^{Aa§}
CM_B829	7.23 ± 0.04$^{Aa\psi}$	7.14 ± 0.01$^{Aa\psi}$	9.23 ± 0.04^{ABa§}	8.99 ± 0.14^{Aa§}
CM	7.14 ± 0.03$^{Aa\psi}$	7.40 ± 0.06$^{Aa\psi}$	9.14 ± 0.03^{ABa§}	9.32 ± 0.07^{Aa§}

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Pool of *B. thermosphacta* (BT) = ATCC®11509™, s109 and s153.

Treatments for co-culture: CM_B824 + BT = *C. maltaromaticum* CM_B824 + pool of *B. thermosphacta*; CM_B827 + BT = *C. maltaromaticum* CM_B827 + pool of *B. thermosphacta*; CM_B829 + BT = *C. maltaromaticum* CM_B829 + pool of *B. thermosphacta*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*.

Positive controls: CM_B824 = *C. maltaromaticum* CM_B824*;* CM_B827 = *C. maltaromaticum* CM_B827; CM_B829 = *C. maltaromaticum* CM_B829; CM = pool of *C. maltaromaticum*.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions, at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

There was inhibition of PF growth by CM strains when cocultured in aerobiosis. In the presence of O_2, CM strains were more effective against PF individually (CM_B824+PF, CM_B827+PF and CM_B829+PF) than in pool (CM+PF) (Table 3). On the other hand, in anaerobiosis there was no inhibitory effect of PF by CM strains both individually and in pool.

As expected, PF population (PF) showed a great growth in aerobiosis. However, PF also grew under an anaerobic condition (Table 3). In fact, *P. fluorescens* still shows slight signs of growth in high concentrations of CO_2 (TAN; GILL, 1982; HENDRICKS; HOTCHKISS, 1997; STOOPS et al., 2012).

Table 3. Population of *Pseudomonas fluorescens* alone and in co-culture with Carnobacterium *maltaromaticum* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+PF	$3.52 \pm 0.02^{Aa\psi}$	$3.56 \pm 0.05^{Aa\psi}$	$7.90 \pm 0.03^{Ca\S}$	$6.27 \pm 0.02^{Ab\S}$
CM_B827+PF	$3.50 \pm 0.01^{Aa\psi}$	$3.52 \pm 0.02^{Aa\psi}$	$7.93 \pm 0.05^{Ca\S}$	$6.67 \pm 0.21^{Ab\S}$
CM_B829+PF	$3.52 \pm 0.02^{Aa\psi}$	$3.62 \pm 0.05^{Aa\psi}$	$7.84 \pm 0.07^{Ca\S}$	$6.71 \pm 0.16^{Ab\S}$
CM+PF	$3.50 \pm 0.00^{Aa\psi}$	$3.63 \pm 0.07^{Aa\psi}$	$8.80 \pm 0.04^{Aa\S}$	$6.33 \pm 0.01^{Ab\S}$
PF	$3.51 \pm 0.02^{Aa\psi}$	$3.62 \pm 0.05^{Aa\psi}$	$8.52 \pm 0.02^{Ba\S}$	$6.74 \pm 0.03^{Ab\S}$

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Treatments for co-culture: CM_B824 + PF = *C. maltaromaticum* CM_B824 + *P. fluorescens*; CM_B827 + PF = *C. maltaromaticum* CM_B827 + *P. fluorescens*; CM_829 + PF = *C. maltaromaticum* CM_B824 + *P. fluorescens*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Negative control: PF = *P. fluorescens* ATCC®1355™.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions, at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

In general, CM_B829 showed the lowest growth among the CM strains in the presence of PF in aerobiosis. In anaerobiosis, in a similar way as observed for BT, there was no difference in the growth of CM strains when in co-culture with PF ($P < 0.05$) (Table 4).

Table 4. Population of *Carnobacterium maltaromaticum* alone and in co-culture with *Pseudomonas fluorescens* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+PF	7.09 ± 0.02^{Abψ}	7.21 ± 0.03^{Aaψ}	9.31 ± 0.04^{ABa§}	9.37 ± 0.15^{Aa§}
CM_B827+PF	7.18 ± 0.02^{Aaψ}	7.13 ± 0.09^{Aaψ}	8.76 ± 0.23^{BCa§}	9.19 ± 0.16^{Aa§}
CM_B829+PF	7.13 ± 0.09^{Aaψ}	7.18 ± 0.02^{Aaψ}	8.62 ± 0.09^{Da§}	9.03 ± 0.12^{Aa§}
CM+PF	7.15 ± 0.04^{Aaψ}	7.15 ± 0.04^{Aaψ}	9.45 ± 0.02^{Aa§}	9.32 ± 0.07^{Aa§}
CM_B824	7.26 ± 0.01^{Aaψ}	7.21 ± 0.03^{Aaψ}	9.26 ± 0.01^{ABa§}	9.23 ± 0.03^{Aa§}
CM_B827	7.22 ± 0.03^{Aaψ}	7.13 ± 0.09^{Aaψ}	9.11 ± 0.08^{ABCa§}	8.97 ± 0.16^{Aa§}
CM_B829	7.18 ± 0.04^{Aaψ}	7.18 ± 0.01^{Aaψ}	8.82 ± 0.22^{BCa§}	8.87 ± 0.14^{Aa§}
CM	7.14 ± 0.04^{Aaψ}	7.14 ± 0.03^{Aaψ}	9.14 ± 0.04^{ABCa§}	9.27 ± 0.12^{Aa§}

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Treatments for co-culture: CM_B824 + PF = *C. maltaromaticum* CM_B824 + *P. fluorescens*; CM_B827 + PF = *C. maltaromaticum* CM_B827 + *P. fluorescens*; CM_B829 + PF = *C. maltaromaticum* CM_B829 + *P. fluorescens*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Positive controls: CM_B824 = *C. maltaromaticum* CM_B824*;* CM_B827 = *C. maltaromaticum* CM_B827; CM_B829 = *C. maltaromaticum* CM_B829; CM = pool of *C. maltaromaticum*.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions, at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

Noticeably, a high antilisterial effect was observed in all CM treatments, either in aerobiosis or in anaerobiosis (Table 5). In aerobiosis, CM_B824 showed the highest inhibitory effect between CM treatments, whereas in anaerobiosis, CM_B824 and CM_B827 showed the similar inhibitory effect**.**

Table 5. Population of *Listeria monocytogenes* alone and in co-culture with *Carnobacterium maltaromaticum* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+LM	4.08 ± 0.01$^{Aa\psi}$	4.12 ± 0.01$^{Aa\psi}$	4.69 ± 0.06^{Db§}	5.07 ± 0.05^{Ca§}
CM_B827+LM	4.02 ± 0.06$^{Aa\psi}$	4.10 ± 0.01$^{Aa\psi}$	5.43 ± 0.22^{Ca§}	5.40 ± 0.10^{Ca§}
CM_B829+LM	4.03 ± 0.06$^{Aa\psi}$	4.09 ± 0.01$^{Aa\psi}$	6.13 ± 0.07^{Ba§}	6.25 ± 0.14^{Ba§}
CM+LM	4.06 ± 0.03$^{Aa\psi}$	4.12 ± 0.02$^{Aa\psi}$	5.55 ± 0.02^{Cb§}	6.14 ± 0.03^{Ba§}
LM	4.08 ± 0.02$^{Aa\psi}$	4.11 ± 0.04$^{Aa\psi}$	9.08 ± 0.02^{Aa§}	9.12 ± 0.01^{Aa§}

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Treatments for co-culture: CM_B824 + LM = *C. maltaromaticum* CM_B824 + *L. monocytogenes*; CM_B827 + LM = *C. maltaromaticum* CM_B827 + *L. monocytogenes*; CM_829 + LM = *C. maltaromaticum* CM_B829 + *L. monocytogenes*; CM + LM = pool of *C. maltaromaticum* + *L. monocytogenes*.

Negative control: LM = *L. monocytogenes* ATCC®19117™.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions. at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

In general, inhibition of LM was higher in aerobiosis than in anaerobiosis. Regarding the growth of CM, there was no effect of the presence of LM on the CM population for all CM strains ($P < 0.05$) (Table 6).

Table 6. Population of *Carnobacterium maltaromaticum* alone and in co-culture with *Listeria monocytogenes* at 15 °C for 72 h in aerobic and anaerobic conditions.

Treatment	Time			
	0 h		72 h	
	Atmosphere			
	A	AN	A	AN
CM_B824+LM	7.09 ± 0.05$^{Aa\psi}$	7.20 ± 0.06$^{Aa\psi}$	9.08 ± 0.02^{Aa§}	9.11 ± 0.01^{Aa§}
CM_B827+LM	7.16 ± 0.05$^{Aa\psi}$	6.85 ± 0.08$^{Bb\psi}$	9.08 ± 0.02^{Aa§}	9.12 ± 0.01^{Aa§}
CM_B829+LM	7.19 ± 0.07$^{Aa\psi}$	7.18 ± 0.04$^{Aa\psi}$	9.08 ± 0.02^{Aa§}	9.12 ± 0.01^{Aa§}
CM+LM	7.24 ± 0.03$^{Aa\psi}$	7.25 ± 0.05$^{Aa\psi}$	9.08 ± 0.02^{Aa§}	9.12 ± 0.02^{Aa§}
CM_B824	7.09 ± 0.05$^{Aa\psi}$	7.20 ± 0.06$^{Aa\psi}$	9.10± 0.05^{Aa§}	9.10 ± 0.05^{Aa§}
CM_B827	7.16 ± 0.05$^{Aa\psi}$	6.85 ± 0.07$^{Bb\psi}$	9.16 ± 0.05^{Aa§}	9.16 ± 0.05^{Aa§}
CM_B829	7.19 ± 0.08$^{Aa\psi}$	7.18 ± 0.04$^{Aa\psi}$	9.19 ± 0.08^{Aa§}	9.19 ± 0.08^{Aa§}
CM	7.23 ± 0.03$^{Aa\psi}$	7.25 ± 0.04$^{Aa\psi}$	9.24 ± 0.04^{Aa§}	9.25 ± 0.05^{Aa§}

Pool of *C. maltaromaticum* (CM) = CM_B824, CM_B827 and CM_B829.

Treatments for co-culture: CM_B824 + LM = *C. maltaromaticum* CM_B824 + *L. monocytogenes*; CM_B827 + LM = *C. maltaromaticum* CM_B827 + *L. monocytogenes*; CM_B829 + LM = *C. maltaromaticum* CM_B829 + *L. monocytogenes*; CM + LM = pool of *C. maltaromaticum* + *L. monocytogenes*.

Positive controls: CM_B824 = *C. maltaromaticum* CM_B824*;* CM_B827 = *C. maltaromaticum* CM_B827; CM_B829 = *C. maltaromaticum* CM_B829; CM = pool of *C. maltaromaticum*.

Treatments and controls were applied in triplicate for both aerobic (A) and anaerobic (AN) conditions. at time 0 and 72 h.

Values are expressed as Mean ± SE. Different lowercase letters in the same row indicate significant differences between atmospheres in the same incubation time ($P < 0.05$). Different uppercase letters in the same column indicate significant differences between treatments in the same atmosphere ($P < 0.05$). Different symbols in the same row indicate significant differences between storage time in the same atmosphere ($P < 0.05$).

4 Discussion

CM strains and pool showed to be competitive LAB, inhibiting both spoilage and pathogenic bacteria *in vitro* with different levels of efficiency. In general, CM_B824 was the most efficient treatment in both atmospheres tested. Overall, when comparing treatments to the controls, *B. thermosphacta* and *L. monocytogenes* inhibition was strong whereas *P. fluorescens* was slightly inhibited (BURT, 2004). This competitiveness may be due to different mechanisms such as (i) faster growth rate than other bacteria in the medium (ii) nutrients competition which leads to nutrients depletion for the other bacteria; (iii) production of antagonist compounds such as lactic acid, H_2O_2, diacetyl, CO_2 and bacteriocins (VERMEIREN; DEVLIEGHERE; DEBEVERE, 2004; NILSSON et al., 2005; KASRA-KERMANSHAHI; MOBARAK-QAMSARI, 2015; SAID et al., 2019). Advantageously, *C. maltaromaticum* started with higher concentration over the competitor for the coculture treatments.

The atmosphere condition slightly affected each Carnobacteria treatment effect on *B. thermosphacta* and *L. monocytogenes* inhibition. The growth of these target bacteria was not affected by the atmosphere as the negative controls show. This suggests that the inhibitory effect may result from the antibacterial compounds and competition of Carnobacteria which respond according to the atmosphere conditions. This antagonist effect against *B. thermosphacta* was also reported by other authors *in vitro*: Russo et al. (2006) showed that LAB isolated from spoiled raw beef were able to compete with *B. thermosphacta* populations as a result of pH decrease and substrate competition without necessarily producing bacteriocins; Zhang, Baranyi and Tamplin (2015) observed that *C. maltaromaticum* isolates slightly inhibited *B. thermosphacta* in aerobic conditions. Moreover, in a previous study, an antilisterial effect was observed in coculture carried out at 25 °C and at extremely low temperatures (-1 °C and 4 °C) (DANIELSKI et al., 2020). In the present study, the antilisterial effect of *C. maltaromaticum* is also observed at intermediate temperature (15 °C). This is an optimal temperature condition to induce bacteriocin production by *C. maltaromaticum* (GURSKY et al., 2006; BRILLET-VIEL et al., 2016). Accordingly, *C. maltaromaticum* reveals interesting bioprotective characteristics and may be suggested as potential bioprotective culture for food.

P. fluorescens counts were affected by anerobic condition as *Pseudomonas sp.* is sensitive to CO_2 concentrations (AMÉZQUITA; BRASHEARS, 2002; BRILLET-VIEL

et al., 2005). Although, residual levels of O_2 as low as 0.1% in the culture media still allow the bacteria to grow. *P. fluorescens* growth can also be detected in products packaged in the absence of oxygen such as modified atmosphere packaging containing 100%N_2; 30/70% CO_2/N_2; 100%CO_2 (CLARK; BURKI, 1972; SEYDIM et al., 2006; STOOPS et al., 2012). Despite that, the anaerobiosis displayed an importance role on slowing *P. fluorescens* growth along with the bioprotective effect. On the other hand, Carnobacteria strains showed a slight significant inhibition of this spoilage bacteria in aerobiosis. This effect may be a result of the antagonist compounds produced by *Carnobacterium spp.* such as lactic, formic and acetic acids (ZHANG; GÄNZLE; YANG, 2019). Hence, the combination of *C. maltaromaticum* and packaging conditions with low oxygen content may be a good strategy to reduce the counts of *P. fluorescens* in meat. Zhang, Baranyi and Tamplin (2015) demonstrated *in vitro* (cell-to-cell on agar and cell free supernatant assay in broth) that *C. maltaromaticum* and *C. divergens*, showed wider range of inhibition of *P. fluorescens* and *B. thermosphacta*, than others LAB. Moreover, Zhang et al. (2017) found that *C. maltaromaticum* cell free supernatant (CFS) was a strong inhibitor of *Pseudomonas spp.*, with higher efficiency in aerobiosis than anaerobiosis. This might suggest that not only *C. maltaromaticum* cells have an inhibitory effect, but also the molecules released by it in the CFS. Moreover, this effect may be influenced by the atmosphere condition.

5 Conclusion

C. maltaromaticum strains, individually or as a pool, showed strong inhibitory effect towards *Brochothrix thermosphacta* and *Listeria monocytogenes*. The strains were effective against *Pseudomonas fluorescens* to a lesser extent. Among *C. maltaromaticum* strains, CM_B824 showed the greatest inhibition of spoilage bacteria and antilisterial effect.

These results are encouraging to future studies on the application of the *C. maltaromaticum* strains in different meat matrices to assess their potential as bioprotective cultures to extend the shelf life and the safety of products.

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CAPÍTULO 3

Carnobacterium maltaromaticum as bioprotective culture against spoilage bacteria in meat products

Abstract

This study aimed to evaluate the bioprotective effect of *Carnobacterium maltaromaticum* (CM) against a pool containing three strains of *Brochothrix thermosphacta* (BT - ATCC®11509™, s109 and s153 lab. ref.) and *Pseudomonas fluorescens* (PF - ATCC®1355™) inoculated in ground beef and sliced cooked ham stored in modified atmosphere (MAP). Ground beef was stored in MAP containing 66% O_2, 4% N_2 and 30% CO_2 for 7 days (d), initially at 4 °C for 3 d and after at 8°C for 4 d. Sliced cooked ham was stored in MAP containing 70% N_2/30% CO_2 for 10 days at 4 °C, followed by 18 days at 8 °C. Microbiological and physicochemical analyses were carried out at 0, 3 and 7 d (ground beef) and 0, 5, 10, 19 and 28 (ham) days of storage. CM counts remained stable in the presence of the inoculated and autochthonous spoilage bacteria until the end of shelf life in all treatments. The *C. maltaromaticum* strains (CM_824, CM_827 and CM_289) reduced the population of both inoculated and autochthonous spoilage bacteria (*Brochothrix spp.*, *B. thermosphacta*, *Pseudomonas spp.*, *P. fluorescens* and Enterobacteria). The inhibitory effect was observed in both food matrices (sliced cooked ham and ground beef) and atmospheres tested. *C. maltaromaticum* strains showed potential to be used as natural preservatives in meat products, extending the shelf life and maintaining the physical chemical parameters of the products.

Keywords: Meat preservation; bioprotective culture; shelf life; Carnobacteria; lactic acid bacteria.

1 Introduction

Meat production has increased from 30 million tons in 1970 to 335.2 million tons (carcass weight equivalent) in 2019. Beef and pork contributed with 72.2 and 110.5 million tons of the total meat production in 2019, respectively (FAO, 1995; 2019). Spoilage accounts for 40% of losses of food in industries and market (SPERBER, 2009). These losses have a huge negative economic and credibility impact to meat and other industries (NYCHAS et al., 2008; MOHAREB et al., 2015; STANBOROUGH et al., 2016; LORENZO et al., 2018).

Meat can be contaminated during slaughter, processing or packaging stages, which can lead to an initial microbial count of 2 to 3 log CFU/g (NYCHAS et al., 2008). Due its rich nutrient content, the microbial count can grow quickly in meat during storage (IULIETTO et al., 2015) and decrease its shelf life. *Pseudomonas spp.* and *Brochothrix thermosphacta*, are the bacterial groups most associated with spoilage in meat stored at low temperatures (RIEDER et al., 2012; STELLATO et al., 2016). *Pseudomonas* and *Brochothrix* take advantage of the selective combination of low temperature and long storage period over other microorganisms, including pathogens. Thus, both genera of bacteria represent an obstacle to the shelf life extension of refrigerated meat products. They contribute to spoilage by producing undesirable molecules that cause off-flavors, off-odors and changes the texture, leading to consumer's rejection of the product (ZAGOREC; CHAMPOMIER-VERGÈS, 2017).

High contamination rates of meat products by *Pseudomonas* spp. (7 log CFU/g) negatively affect the sensory quality of meat due to odor and color alterations caused by the secretion of greenish pigments and volatile compounds (GONÇALVES et al., 2017). *Pseudomonas fluorescens* is a strong proteolytic and lipolytic bacteria in chilled temperatures. This microorganism is also able to grow fast under stressful environments and can overcome other spoilage competitors by producing bacteriocins and resisting to bacteriocins. Moreover, *P. fluorescens* is an important spoilage bacterium, mainly in aerobically stored meat. It produces a yellow-greenish water-soluble fluorescent pigment called pyoverdine, which has high affinity with Fe^{3+} present in the meat, utilizing it for its growth. *P. fluorescens* develop biofilms rapidly, and cause meat discoloration and sliminess (ROBACH, COSTILOW, 1961; MEYER; ABDALLAH, 1978; GONÇALVES et al., 2017; WICKRAMASINGHE et al., 2019). Thus, in aerobically stored beef, *P. fluorescens* quickly consumes the O_2 and accelerates the

oxidation process of oxymyoglobin (CHAN et al., 1998), leading consumers to reject the product based on its appearance (TSHABALALA; KOCK; BUYS, 2012; GONÇALVES et al., 2017). Although, it can adapt well to stressful environments (LIU et al., 2018) and be resistant to high concentrations of CO_2 showing slight sings of growth (TAN; GILL, 1982; HENDRICKS; HOTCHKISS, 1997; STOOPS et al., 2012). This microorganism is present in meat stored in modified atmosphere packaging (MAP) (ERCOLINI et al., 2006; CHAILLOU et al., 2014).

B. thermosphacta, a facultative anaerobic bacterium, is one of the most neglected microorganisms in the study of meat deterioration (PIN; FERNANDO; ORDÓÑEZ, 2002). *B. thermosphacta* is a problem for hamburgers and fresh sausages, stored under vacuum or MAP, due to the production of metabolites such as acetoin, diacetyl and 3-methylbutanol, which cause undesirable odors in the product (PENNACCHIA; ERCOLINI; VILLANI, 2011; CASABURI et al., 2015; STANBOROUGH et al., 2016; MANSUR et al., 2019). Moreover, *B. thermosphacta* and *Pseudomonas spp.*, along with other spoilage bacteria such as Enterobacteria, can produce volatile compounds that leads to the sulfuric and cheesy odors associated with spoilage in beef and sliced cooked ham (GWIDA et al., 2014; CASABURI et al., 2015; GEERAERTS et al., 2019; MANSUR et al., 2019; WEINROTH et al., 2019).

There are several techniques that try to control the microbial spoilage on meat. The microbial growth is affected by many factors such as storage temperature, packaging condition, food composition (pH, water activity, fat content, NaCl and nitrite concentrations) and contamination levels. The spoilage community is also influenced by interactions among bacterial niches that promotes or inhibit the growth of other bacteria (ZHANG; BARANYI; TAMPLIN, 2015). Biopreservation shows as a natural alternative to extend the shelf life of food products by adding a certain amount of microorganism to control spoilage and pathogenic strains. The term “biopreservation” or “biocontrol” means to use microorganisms or their metabolites naturally present on the food or added to it (SALAS et al., 2017). With this technique, it is possible to substitute the use of synthetic additives to control the meat microbiota (SINGH, 2018). This turns out to be a crucial strategy for the preservation of green label products and the attraction of consumers (ZHOU; XU; LIU, 2010). The biological agents used must be safe, non-pathogenic and non-toxin producers. In the literature, LAB (lactic acid bacteria) are the most used group of bacteria as biopreservatives. They have

characteristics of protective cultures and are recognized as safe (SINGH, 2018; MOKOENA, 2017).

C. maltaromaticum is one of the most predominant lactic acid bacteria (LAB) in aerobic, modified atmosphere or vacuum packaged meat (BARAKAT; GRIFFITHS; HARRIS, 2000; LAURSEN et al., 2005). Characteristics of this genera favor its survival in diverse conditions: growth in a moderate range of pH (5.00-9.50) and temperature (-1.5 to 37 °C) and tolerance to NaCl concentrations as high as 5% (w/v) (LEISNER et al., 2007; EDIMA et al., 2008; WAYNE et al., 2012; ZHU et al., 2018). *C. divergens* and *C. maltaromaticum* are found in fresh short and long-term stored meat (LAURSEN et al., 2005). Some strains are related to spoilage (JORGENSEN; HUSS; DALGAARD, 2000; SAKALA, 2002), but others are associated to preservation of dairy products (AFZAL et al., 2010; SPANU et al., 2018), seafood (ALVES et al., 2005) and meat (SCHÖBITZ et al., 1999; ROLLER et al., 2002; DANIELSKI et al., 2020).

Studies reporting the biopreservative effect of *C. maltaromaticum* in meat and meat products are scarce. In a previous study, the antilisterial effect of *C. maltaromaticum* in sliced cooked ham was proven (Danielski et al., 2020). In this way, it is also relevant to verify the potential of the strains of *C. maltaromaticum* in inhibiting relevant spoilage bacteria in meat products. The aim of this study was to evaluate the effect of use of *C. maltaromaticum* on the inhibition of *B. thermosphacta* and *P. fluorescens* and on the physical chemical quality in ground beef and cooked ham.

2 Material and methods

2.1 Bacterial strains

A pool of three strains of *Carnobacterium maltaromaticum* (CM) was used in this study: CM_B824, CM_B827 and CM_B289. These strains were isolated by Imazaki et al. (2018) from Australian beef (*longissimus dorsi*) with an extremely long shelf life (140 d) stored in vacuum packaging at -1 °C. The strains were selected among other *C. maltaromaticum* strains, representing three different phylogenetic groups with carnobacteriocin BM1 and B2 (only CM_B824) genes.

Spoilage bacteria used in this study were *Pseudomonas fluorescens* ATCC®1355™ (PF) and a *pool* of *Brochothrix thermosphacta* ATCC®11509™, s109 and s153 (lab reference) (BT). The *B. thermosphacta* strain s109 was isolated from beef and strain s153 from bone-in ham (*jambon a l'os*).

2.2 Experimental design

2.2.1 Inoculum preparation

The strains of CM (CM_B824, CM_B827 and CM_B829), PF (ATCC®1355™) and BT (s153, s109 and ATCC®11509™) were kept stored at - 80 °C until use. Strains were grown in tubes containing 10 mL brain heart infusion broth (BHI - OXOID) at 25 °C for 24 h. The inoculum count was determined in Plate Counting Agar – PCA (OXOID) plates incubated at 25 °C for 48 h for CM, in Streptomycin-thallous acetate-actidione agar base with STAA supplement (Streptomycin sulphate 500 mg/L; Thallous acetate 50 mg/L and Cycloheximide 50 m/L) (OXOID) for BT and in *Pseudomonas* Agar Plate supplemented with CFC (Cetrimide 10 mg/L; Fucidin 10 mg/L and Cephalosporin 50 mg/L) (OXOID) for PF. Both BT and PF plates were incubated at 25 °C for 24 h. At these conditions, the count of CM inoculum in BHI reached ~9 log CFU/mL and count of BT and PF, ~ 8 log CFU/mL.

2.2.2 Ground beef

Beef knuckle, purchased from a local butcher's shop in Belgium, was grounded through an 8-mm grinding plate. Ground beef was aseptically mixed, using a stand mixer (Kitchen Grand Chef, Kenwood) with CM, BT and PF, grown in BHI at the concentrations of 6.76, 3.49 and 3.48 log CFU/mL, respectively. Six batches of beef burgers were assigned to six different treatments, in triplicate: 1) NC – negative control

inoculated only with sterilize deionized water; 2) CM – *C. maltaromaticum* pool; 3) BT – *B. thermosphacta* pool; 4) PF – *P. fluorescens*; 5) CM+BT; 6) CM+PF. In the CM treatments, the meat was inoculated with CM and mixed for 10 min. In CM + spoilage bacteria treatments (CM+BT and CM+PF), after CM inoculation and mixing, meat was inoculated with each spoilage bacteria, followed by a period of 10 min of mixing. In the other treatments (BT and PF), the meat was inoculated with each spoilage bacteria and then mixed for 10 min.

Beef patties (80 g) (n = 54) were formed using a meat former (12-cm diameter) and packaged in a PP/EVOH/PP tray (dimensions: 187 × 137 × 50 mm, oxygen permeability: 4 cm^3/m^2 x day x bar, 23 °C and 0% RH) in modified atmosphere (MAP) containing 66% O_2, 4% N_2 and 30% CO_2, and sealed with PET/PP film (Wipak, Helsinki, Finland) (oxygen permeability: 8.4 cm^3/m^2 x day x bar, 23 °C and 0% RH). Ground beef patties were stored for 7 d, initially at 4 °C for 3 d and after at 8°C for 4 d. At 0, 3 and 7 days of storage, samples were withdrawn for analyses. The storage conditions were according to the *Norme Française* NF V01-003 (2010) used to simulate the real conditions of commercial chilled meat at retail (1/3 of the shelf life at initially at 4 °C followed by 2/3 at 8 °C).

2.2.3 Cooked ham

Cooked ham from the same batch stored at 4 °C was purchased from a medium scale producer in Belgium. Ham was processed with pork muscles (*M. semimembranosus, M. semitendinosus* and *M. biceps femoris)*, injected with a brine solution (15 g brine/100 g ham meat), containing the following ingredients (g/ Kg): water, 100; salt, 20; dextrose 3.6; $NaNO_2$, 0.130; sodium isoascorbate, 0.5 and condiments (onion, carrot, parsley, clove, pepper, laurel), 2.

Ham slices (n = 180 slices) were assigned to the following treatments, in triplicate: (1) negative control (NC), inoculated with saline solution (2%) to avoid dehydration; (2) CM as positive control - *C. maltaromaticum* pool; (3) BT – *B. thermosphacta* pool; (4) PF – *P. fluorescens*; (5) CM + BT; (6) CM + PF. For the inoculation, slices were placed in a steel mesh basket strainer and immersed in 1000 mL of saline solution (0.9%), containing the bacterial inoculum: CM at 6.37, BT at 3.99 and PF at 3.79 log CFU/mL. In the CM treatments, slices were maintained in contact with the solution containing the inoculum for 10 min, to facilitate cells attachment to the

product surface. In the CM + spoilage bacteria treatments, after the CM contact period, the solution was inoculated with each spoilage bacteria and slices were kept in the solution for 10 min. In the other treatments, the saline solution was inoculated with each spoilage bacteria and ham slices were kept in the solution for 10 min. After inoculation, the slices were suspended out of the solution for 10 min to allow the liquid to dry.

The slices were packaged in PP/EVOH/PP trays (dimensions: 187 × 137 × 50 mm, oxygen permeability: 4 cm^3/m^2 x day x bar, 23 °C and 0% RH) in modified atmosphere (MAP), containing 70% N_2/30% CO_2, and sealed with PET/PP film (Wipak, Helsinki, Finland) (oxygen permeability: 8.4 cm^3/m^2 x day x bar, 23 °C and 0% RH). The samples were stored for 10 days at 4 °C, followed by 18 days at 8 °C, accordingly to the *Norme Française* NF V01-003 (2010) used to simulate the real conditions of commercial chilled meat at retail. Analyses were performed at 0, 5, 10, 19 and 28 days of storage.

2.4 Microbiological analyses

Ground beef patties and sliced cooked ham samples (25 g) were diluted in sterile stomacher bags containing 225 mL of saline solution (0.9% NaCl). The samples were homogenized using a stomacher (Masticator Basic 2000, IUL, Barcelona, Spain) for 120 s and serially diluted and plated using a spiral plater (EasySpital® Pro, Interscience, St Nom la Bretèche, France). The microbiological counts were carried out using the following culture media and incubation conditions: total bacterial count - TBC, plates incubated at 25 °C for 24 h (Plate Counting Agar – PCA, OXOID); lactic acid bacteria – LAB (plates incubated at 25 °C for 48-72 h, MRS – OXOID); Enterobacteriaceae (plates incubated at 37 °C for 24 h, Violet Red Bile Glucose, VRBG – BIORAD); *Brochothrix* sp. (plates incubated at 25 °C for 24 h, *Streptomycin-thallous acetate-actidione* agar (STAA) plus STAA supplement (Streptomycin sulphate 500 mg/L; Thallous acetate 50 mg/L and Cycloheximide 50 m/L) (OXOID); *Pseudomonas* sp. (plates incubated at 25 °C for 24 h, *Pseudomonas* Agar Plate - OXOID; supplemented with CFC - Cetrimide 10 mg/L; Fucidin 10 mg/L and Cephalosporin 50 mg/L - OXOID). CM population was calculated as the difference between counts on PCA and the other culture media.

2.5 Physicochemical analyses

2.5.1. pH

pH was measured in triplicate in 5 g of sample, homogenized with 45 mL of deionized water for 1 min using a blender. The pH value was determined using a pH-meter (Model 104063123, Ingold, Houston, US) calibrated with buffer solutions at pH 4.0 and 7.0.

2.5.2 Instrumental color

The instrumental color was measured using a spectrophotometer (Labscan II, Hunterlab, Houston, US), light source D65, opening diameter of 8 mm, observation angle of 10° and color coordinates CIEL L*, a*, b*. The data was collected from five different spots on the surface of the meat product. The color disparities (ΔE) were calculated with the formula $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (HUNT et al., 2012).

2.5.3 Headspace gas composition

Changes in the gas composition inside the packages (CO_2, O_2, N_2 %) during storage were evaluated with a gas analyzer (Checkmate 3, Dansensor, Ringsted, Denmark) using a sterile needle for collection through a 15 mm diameter *septum* attached to the package (SPANU et al., 2018).

2.6 Statistical analysis

Each meat matrix assay was performed two times independently. The data was expressed in mean ± SE (standard error) and 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´s test ($P < 0.05$). Differences between assay replicates were not significant. When the interaction between treatment and storage time was not significant it was withdrawn from the model and the main effects were evaluated.

The microbiological analyses were conducted in duplicate ($n = 4$) and physicochemical analyses in triplicate ($n = 6$). The analysis was performed using the

software Statgraphics® Centurion XVI version 16.1.11 (Statpoint Technologies, Warrenton, Virginia, USA).

To predict the shelf life of the meat products, the experimental data was analyzed with a dose-response curve model using the software Table Curve 2D (version 3; Systat Software Inc., Richmond California, USA. A logistic regression model was used to predict the product storage time (days) considering the spoilage threshold based on the observed microbial growth using the following equation:

$$Y= a+b/(1+(x/c)^d)$$

Where *y* is the microbial population count ($Y=F(x)_\infty$); *x* is the storage time (days) and *a*, *b*, *c*, and *d* are independent variables.

The spoilage threshold used for *Brochothrix spp.* was 6 log CFU/g and 7 log CFU/g for *Pseudomonas spp.* (MILLS; DONNISON; BRIGHTWELL, 2014; PELLISSERY et al., 2020).

3 Results

3.1 Meat matrix assay

3.1.1 Ground beef

For all microbiological counts, there was a significant interaction between storage time and treatments ($P < 0.05$). A significant increase in the count of all groups of bacteria was observed with the temperature abuse, 8 °C, after 3 d of storage at 4 °C.

CM showed inhibitory effect towards *Pseudomonas*, both on *P. fluorescens* and on the naturally present *Pseudomonas* sp. in meat throughout the storage time ($P < 0.05$). *P. fluorescens* count in CM+PF slightly increased Δ 0.73 log CFU/g after 7 days of storage, whereas PF count increased 1.39 log CFU/g in the same period. During storage, the count of naturally present *Pseudomonas sp.* increased Δ 3.56 log CFU/g in NC and moderately increased Δ 0.96 log CFU/g in CM ($P < 0.05$) (BURT, 2004). Similar inhibitory effect was observed towards *Brochothrix sp.* populations. During storage, the count of *Brochothrix sp.* increased Δ 4.68 log CFU/g in NC and slightly increased Δ 3.49 log CFU/g in CM. The ground beef inoculated with *B. thermosphacta,* the presence of CM (CM + BT) moderately inhibited the growth of BT, showing a difference of Δ 2.41 log CFU/g in the population increase compared to ground beef inoculated only with *B. thermosphacta* (BT) during storage ($P < 0.05$) (Table 1) (BURT, 2004).

The addition of CM in ground beef was slightly effective in inhibiting the Enterobacteriaceae (EB) growth and the artificial inoculation of *P. fluorescens* and *B. thermosphacta* in the meat did not influence the antimicrobial effect of CM towards EB (BURT, 2004). In general, counts of EB in the CM treated ground beef (CM, CM + PF and CM + BT) were lower than in the other treatments ($P < 0.05$). When compared to NC, EB growth was reduced in Δ 0.99 (CM), Δ 0.96 (CM+BT) and Δ 0.12 log CFU/mL (CM+PF) in the presence of Carnobacteria (Table 1).

Lactic acid bacteria (LAB) counts were affected by treatments ($P < 0.05$). At 3 d of storage, LAB growth (other than Carnobacteria) was lower in the treatments added with CM and at 7 d, LAB count was similar in all treatments, except for PF, which showed a slightly higher count than other treatments ($P < 0.05$) (Table 1). Total aerobic

bacteria count varied between treatments during storage ($P < 0.05$) and there was no significant effect of CM on the growth of total aerobics.

Table 1. Effect of *C. maltaromaticum* on the microbiological count in ground beef stored in MAP (66/30/4% O2/ CO2/N2) for 7 days (3 days at 4 °C and 4 days at 8 °C).

Treatments	Storage time (days)				
	0	3	7	Mean	
	Pseudomonas spp. (Log CFU/ g)				*P*
NC	2.56 ± 0.04^{eC}	2.98 ± 0.04^{bB}	6.12 ± 0.03^{aA}	3.89^{c}	0.0000
CM	2.95 ± 0.03^{cB}	2.95 ± 0.09^{bB}	3.91 ± 0.01^{dA}	3.27^{d}	0.0001
BT	2.78 ± 0.04^{dC}	3.83 ± 0.12^{aB}	4.84 ± 0.03^{cA}	3.82^{c}	0.0000
CM+BT	3.18 ± 0.04^{bB}	3.22 ± 0.02^{bB}	3.89 ± 0.04^{dA}	4.43^{d}	0.0000
PF	4.13 ± 0.03^{aB}	3.89 ± 0.03^{aB}	5.52 ± 0.27^{bA}	4.51^{a}	0.0006
CM+PF	4.09 ± 0.03^{aC}	3.72 ± 0.03^{aB}	4.82 ± 0.02^{cA}	4.21^{b}	0.0000
Mean	3.28^{C}	3.43^{B}	4.85^{A}		
P	0.0000	0.0000	0.0000	0.0000	
	Brochothrix spp. (Log CFU/ g)			Mean	*P*
NC	2.00 ± 0.00^{bC}	3.38 ± 0.08^{cB}	6.68 ± 0.01^{bA}	4.02^{b}	0.0000
CM	2.00 ± 0.00^{bC}	3.06 ± 0.08^{dB}	5.49 ± 0.06^{cA}	3.52^{c}	0.0000
BT	3.67 ± 0.01^{aC}	3.89 ± 0.03^{bB}	7.21 ± 0.02^{aA}	4.92^{a}	0.0000
CM+BT	3.76 ± 0.04^{aB}	3.90 ± 0.04^{bB}	4.89 ± 0.12^{dA}	4.18^{b}	0.0001
PF	2.47 ± 0.23^{bC}	3.32 ± 0.04^{cdB}	6.69 ± 0.01^{bA}	4.16^{b}	0.0000
CM+PF	2.30 ± 0.17^{bC}	4.26 ± 0.08^{aB}	5.50 ± 0.07^{cA}	4.02^{b}	0.0000
Mean	2.70^{C}	3.64^{B}	6.08^{A}		0.0000
P	0.0000	0.0000	0.0000	0.0000	
	Enterobacteriacea (Log CFU/ g)			Mean	*P*
NC	2.36 ± 0.06^{aB}	2.46 ± 0.09^{bcB}	4.76 ± 0.05^{abA}	3.19^{bc}	0.0000
CM	2.10 ± 0.01^{aB}	2.00 ± 0.00^{cB}	3.77 ± 0.04^{cA}	2.62^{d}	0.0000
BT	2.40 ± 0.20^{aB}	2.32 ± 0.16^{bcB}	4.71 ± 0.04^{abA}	3.14^{c}	0.0000
CM+BT	2.40 ± 0.20^{aB}	2.59 ± 0.15^{bB}	3.80 ± 0.16^{cA}	2.93^{c}	0.0023
PF	2.56 ± 0.14^{aB}	2.73 ± 0.09^{bB}	5.03 ± 0.06^{aA}	3.44^{ab}	0.0000
CM+PF	2.67 ± 0.03^{aC}	3.48 ± 0.00^{aB}	4.64 ± 0.04^{bA}	3.60^{a}	0.0000
Mean	4.41^{C}	2.60^{B}	4.45^{A}		0.0000
P	0.1569	0.0000	0.0000	0.0000	
	Carnobacterium spp. (Log CFU/ g)			Mean	*P*
NC	3.55 ± 0.04^{cC}	4.52 ± 0.02^{bB}	6.65 ± 0.09^{abA}	4.91^{c}	0.0000
CM	6.24 ± 0.03^{aB}	5.56 ± 0.09^{aC}	7.29 ± 0.06^{aA}	4.36^{a}	0.0000
BT	3.61 ± 0.04^{cC}	4.33 ± 0.12^{bB}	5.56 ± 0.26^{cA}	4.76^{d}	0.0005
CM+BT	5.69 ± 0.00^{bB}	5.37 ± 0.04^{aC}	7.32 ± 0.07^{aA}	6.13^{a}	0.0000
PF	3.61 ± 0.14^{cB}	3.31 ± 0.18^{cB}	7.43 ± 0.06^{aA}	4.78^{c}	0.0000
CM+PF	5.69 ± 0.00^{bA}	5.51 ± 0.05^{aA}	6.13 ± 0.35^{bcA}	4.77^{b}	0.1743
Mean	4.73A^{B}	4.77^{A}	4.60^{B}		0.041

P	0.0000	0.0000	0.0000	0.0000	
	Lactic acid bacteria (Log CFU/ g)			Mean	*P*
NC	2.20 ± 0.10^{aC}	4.19 ± 0.20^{abB}	6.08 ± 0.18^{bA}	4.16^{ab}	0.0000
CM	2.16 ± 0.16^{aC}	3.47 ± 0.37^{bB}	5.91 ± 0.02^{bA}	3.84^{d}	0.0001
BT	2.10 ± 0.10^{aC}	4.67 ± 0.06^{abB}	5.95 ± 0.09^{bA}	3.99^{cd}	0.0000
CM+BT	2.43 ± 0.13^{aC}	4.23 ± 0.09^{bB}	5.90 ± 0.03^{bA}	4.19^{ab}	0.0000
PF	2.20 ± 0.10^{aC}	4.50 ± 0.04^{aB}	6.29 ± 0.06^{aA}	4.33^{a}	0.0000
CM+PF	2.20 ± 0.10^{aC}	4.09 ± 0.06^{bB}	5.76 ± 0.08^{cA}	4.02^{abc}	0.0000
Mean	2.22^{C}	4.07^{B}	5.98^{A}		0.0000
P	0.4954	0.0136	0.0278	0.0009	
	Total aerobic bacteria (Log CFU/ g)			Mean	*P*
NC	3.65 ± 0.04^{eC}	4.18 ± 0.02^{cB}	7.09 ± 0.03^{bA}	4.98^{e}	0.0000
CM	6.24 ± 0.03^{aB}	5.57 ± 0.09^{aC}	7.31 ± 0.06^{abA}	4.97^{a}	0.0000
BT	3.99 ± 0.01^{dC}	4.67 ± 0.06^{bB}	6.15 ± 0.12^{cA}	4.94^{e}	0.0000
CM+BT	5.70 ± 0.00^{bB}	5.40 ± 0.04^{aC}	7.33 ± 0.06^{abA}	6.15^{b}	0.0000
PF	4.19 ± 0.05^{cC}	4.62 ± 0.02^{bB}	7.54 ± 0.04^{aA}	5.45^{d}	0.0000
CM+PF	5.70 ± 0.00^{bB}	5.56 ± 0.04^{aB}	6.45 ± 0.15^{cA}	5.90^{c}	0.0008
Mean	4.91^{A}	5.00^{B}	6.98^{C}		0.0000
P	0.0000	0.0000	0.0000	0.0000	

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. 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 ($P < 0.05$).

Regarding meat quality parameters, there was a slight decrease in the pH of ground beef inoculated with *C. maltaromaticum* (CM; CM+BT; CM+PF) during storage. CM treated ground beef showed lower pH values than other treatments ($P < 0.05$) after 7 d of storage. However, differences in pH values between treatments were lower than Δ 0.30 (Table 2).

Considering changes on the aerobic gaseous composition of the ground beef, the O_2 concentration increased from 0 to 3 d and decreased at 7 d of storage in all treatments. Treatments inoculated with *C. maltaromaticum* showed higher O_2 consumption (CM (Δ -0.86), CM+BT (Δ -0.90) and CM+PF (Δ -1.13)) than other treatments during storage ($P < 0.05$). On the other hand, CO_2 levels decreased from 0 to 3 d in all treatments and increased only in CM treatments (CM (Δ 1.13), CM+BT (Δ 1.47) and CM+PF (Δ 1.10) from the 3th to 7th day of storage (Table 2).

Table 2. pH and dynamic behavior of headspace gases of ground beef stored in MAP (66/30/4% O_2/ CO_2/N_2) for 3 days at 4°C and 4 days at 8 °C.

Treatment	Storage days 0	3	7	Mean	*P*
	Physicochemical parameter				
	pH				
NC	5.69 ± 0.00^{bB}	5.71 ± 0.01^{bB}	5.85 ± 0.01^{aA}	5.75^{a}	0.0000
CM	5.70 ± 0.01a^{bC}	5.70 ± 0.00^{bBC}	5.68 ± 0.01^{bcA}	5.69^{cd}	0.0260
BT	5.70 ± 0.01a^{bB}	5.72 ± 0.01^{abB}	5.81 ± 0.00^{aA}	5.74^{ab}	0.0000
CM+BT	5.72 ± 0.00^{aB}	5.74 ± 0.00^{aA}	5.67 ± 0.01^{cA}	5.71^{bc}	0.0050
PF	5.72 ± 0.00^{aB}	5.74 ± 0.00^{aB}	5.79 ± 0.01^{abA}	5.75^{a}	0.0035
CM+PF	5.72 ± 0.00^{aB}	5.69 ± 0.00^{bA}	5.55 ± 0.05^{dA}	5.66^{d}	0.0160
Mean	5.71^{A}	5.72^{A}	5.73^{A}		0.1557
P	0.0008	0.0004	0.0000	0.0000	
		O_2 (%)		Mean	*P*
NC	65.83 ± 0.03^{cC}	69.53 ± 0.03^{aA}	68.53 ± 0.03^{aB}	68.26^{a}	0.0000
CM	66.03 ± 0.03^{cdB}	69.33 ± 0.09^{aA}	65.17 ± 0.09^{bC}	66.84^{b}	0.0000
BT	66.30 ± 0.00^{aC}	70.00 ± 0.40^{aA}	68.47 ± 0.03^{aB}	67.97^{a}	0.0001
CM+BT	66.07 ± 0.03^{bB}	69.40 ± 0.10^{aA}	65.17 ± 0.33^{bC}	66.88^{b}	0.0000
PF	66.23 ± 0.07^{abC}	69.73 ± 0.07^{aA}	68.37 ± 0.09^{aB}	68.11^{a}	0.0000
CM+PF	66.2o ± 0.06^{abA}	69.40 ± 0.06^{aB}	65.07 ± 0.23^{bC}	66.89^{b}	0.0000
Mean	66.11^{C}	69.57^{A}	66.79^{B}		0.0000
P	0.0001	0.1411	0.0000	0.0000	
		CO_2 (%)		Mean	*P*
NC	29.67 ± 0.44^{aA}	26.57 ± 0.19^{aB}	27.33 ± 0.17^{bB}	27.86^{b}	0.0007
CM	30.07 ± 0.03^{aB}	26.63 ± 0.03^{aC}	31.20 ± 0.06^{aA}	29.30^{a}	0.0000
BT	29.97 ± 0.13^{aB}	26.53 ± 0.12^{aC}	27.67 ± 0.09^{bA}	28.05^{b}	0.0000
CM+BT	29.93 ± 0.03^{aB}	26.70 ± 0.06^{aC}	31.40 ± 0.40^{aA}	29.34^{a}	0.0000
PF	29.67 ± 0.03^{aA}	26.57 ± 0.09^{aC}	27.90 ± 0.10^{bB}	28.04^{b}	0.0000
CM+PF	29.97 ± 0.09^{aB}	26.70 ± 0.00^{aC}	31.07 ± 0.13^{aA}	29.24^{a}	0.0000
Mean	29.88^{A}	26.62^{C}	29.43^{B}		0.0000
P	0.5805	0.7619	0.0000	0.0000	
		N_2 (%)		Mean	*P*
NC	4.50 ± 0.40^{aA}	3.90 ± 0.15^{aA}	4.13 ± 0.19^{aA}	4.18^{a}	0.3539
CM	3.90 ± 0.00^{aA}	4.03 ± 0.09^{aA}	3.63 ± 0.13^{abA}	3.86^{ab}	0.0554
BT	3.73 ± 0.13^{aA}	3.47 ± 0.28^{aA}	3.87 ± 0.10^{abA}	3.69^{b}	0.3923
CM+BT	4.00 ± 0.06^{aA}	3.90 ± 0.06^{aA}	3.43 ± 0.07^{bB}	3.78^{b}	0.0066
PF	4.10 ± 0.10^{aA}	3.70 ± 0.15^{aA}	3.73 ± 0.12^{abA}	3.84^{ab}	0.1194
CM+PF	3.83 ± 0.03^{aA}	3.90 ± 0.06^{aA}	3.87 ± 0.12^{abA}	3.87^{ab}	0.8424
Mean	4.01^{A}	3.82^{AB}	3.78^{B}		0.0148
P	0.1149	0.2317	0.0381	0.0326	

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. 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 ($P < 0.05$).

For the color parameter, there was significant interaction between treatment and storage time for L* and b* ($P < 0.05$). In general, ground beef inoculated with PF showed higher luminosity and lower redness and yellowness over time than that inoculated with CM and BT. Time influenced color coordinates in all treatments, which showed increased luminosity and decreased redness and yellowness over storage. Total color differences (ΔE*) in all treatments over storage (CM = 17.09; CM+PF = 16.84; CM+BT = 17.42; BT = 16.86; PF = 16.15; NC = 18.18) were higher than the theoretical customer decline value (ΔE* > 3) (Table 3).

Table 3. Effect of the addition of a pool of *C. maltaromaticum* and spoilage bacteria on the Instrumental color (L*, a*, b* and ΔE*) of ground beef during storage in MAP (66/30/4% O_2/ CO_2/N_2) for 3 days at 4°C and 4 days at 8 °C.

	Storage days				
Treatment	0	3	7		
		L*		Mean	*P*
NC	37.22 ± 0.38^{bcB}	40.52 ± 0.53^{aA}	42.26 ± 0.60^{abA}	40.00^{b}	0.0000
CM	37.14 ± 0.47^{bcB}	40.40 ± 0.61^{aA}	41.26 ± 0.43^{abA}	39.60^{bc}	0.0000
BT	37.97± 0.49^{abB}	41.00 ± 0.62^{aA}	40.53 ± 0.51^{bA}	39.84^{b}	0.0006
CM+BT	36.09 ± 0.36^{cdC}	39.84 ± 0.36^{aB}	41.66 ± 0.62^{abA}	39.19^{bc}	0.0000
PF	39.33 ± 0.48^{aC}	41.51 ± 0.26^{aB}	43.39 ± 0.44^{aA}	41.41^{a}	0.0000
CM+PF	34.89 ± 0.36^{dB}	39.67 ± 0.31^{aA}	40.99 ± 0.60^{bA}	38.52^{c}	0.0000
Mean	37.11^{C}	40.49^{B}	41.68^{A}		0.0000
P	0.0000	0.0639	0.0050	0.0000	
		a*		Mean	*P*
NC	20.31 ± 0.56^{aA}	19.95 ± 0.39^{aA}	14.09 ± 0.51^{aB}	18.12^{a}	0.0000
CM	18.98 ± 0.54^{abA}	18.57 ± 0.49^{aA}	14.69 ± 0.48^{aB}	17.42^{ab}	0.0000
BT	19.33 ± 0.81^{abA}	18.82 ± 0.49^{aA}	14.89 ± 0.63^{aB}	17.68^{ab}	0.0000
CM+BT	18.59 ± 0.46^{abA}	18.96 ± 0.34^{aA}	13.69 ± 0.33^{aB}	17.08^{ab}	0.0000
PF	17.89 ± 0.44^{bB}	19.45 ± 0.26^{aA}	13.42 ± 0.36^{aC}	16.92^{b}	0.0000
CM+PF	19.12 ± 0.53^{abA}	18.70 ± 0.32^{aA}	13.43 ± 0.47^{aB}	17.09^{ab}	0.0000
Mean	19.04^{A}	19.08^{A}	14.04^{B}		0.0000
P	0.0890	0.1300	0.1186	0.0258	
		b*		Mean	*P*
NC	27.76 ± 0.36^{aA}	20.59 ± 0.25^{aB}	11.44 ± 0.15^{abcC}	19.93^{a}	0.0000
CM	26.71 ± 0.36^{aA}	18.92 ± 0.42^{bB}	10.68 ± 0.35^{bcC}	18.77^{bc}	0.0000
BT	26.70 ± 0.63^{aA}	19.60 ± 0.28^{abB}	10.64 ± 0.37^{bcC}	18.98^{bc}	0.005

CM+BT	26.05 ± 0.49^{aA}	19.45 ± 0.21^{abB}	10.29 ± 0.29^{cC}	18.59^{c}	0.0000
PF	26.70 ± 0.36^{aA}	19.92 ± 0.24^{abB}	11.72 ± 0.25^{abC}	19.45^{ab}	0.0000
CM+PF	26.59 ± 0.41^{aA}	18.74 ± 0.31^{bB}	11.96 ± 0.37^{aC}	19.09^{abc}	0.0000
Mean	26.75^{A}	19.54^{B}	11.12^{C}		0.0000
P	0.1832	0.0003	0.0007	0.0001	

	ΔE
NC	18.18
CM	17.10
BT	16.86
CM+BT	17.42
PF	16.15
CM+PF	16.84

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. 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 ($P < 0.05$).

C. maltaromaticum extended the shelf life for all treatments when compared with the controls. Moreover, the microbial population did not reach the spoilage threshold (7 log CFU/g for *Pseudomonas spp.* and 6 log CFU/g for *Brochothrix spp*.). Overall, Carnobacteria showed a bioprotective effect that doubled the amount of days needed for the spoilage bacteria to growth to certain concentration (log). The commercial shelf life of ground beef (7 d) was increased 29.57% in CM and 40.43 % in CM+BT, regarding *Brochothrix spp*. population and 36.28% in CM and 14.29 in CM+PF, regarding *Pseudomonas spp*. In addition, the inoculated and the autochthonous spoilage population growth stabilized at lower concentrations in the presence of Carnobacteria: 5.6 (CM) and 5 log CFU/g (CM+BT) for *Brochothrix spp*. and at 4.2 (CM) and 4.9 log CFU/g (CM+PF) for *Pseudomonas spp.* (Table 4).

Table 4. Effect of *C. maltaromaticum* on the prediction of shelf life based on the spoilage threshold in ground beef during storage in MAP (66/30/4% O_2/ CO_2/N_2) for 3 days at 4°C and 4 days at 8 °C.

Growth (log)	*Brochothrix spp.*				*Pseudomonas spp.*			
	Treatments							
	NC	CM	BT	CM+BT	NC	CM	PF	CM+PF
	Predicted shelf life (days)							
3	3	3	NR	NR	3	5	NR	NR

3.1	3	3	NR	NR	3	5	NR	NR
3.2	3	3	NR	NR	3	6	NR	NR
3.3	3	3	NR	NR	3	6	NR	NR
3.4	3	3	NR	NR	4	6	NR	NR
3.5	3	3	NR	NR	4	6	NR	NR
3.6	3	3	NR	NR	4	6	NR	NR
3.7	3	4	2	NR	4	7	NR	NR
3.8	3	4	3	2	4	7	NR	NR
3.9	3	4	3	3	4	7	NR	NR
4	3	4	3	3	4	7	NR	6
4.1	3	4	3	5	4	8	5	6
4.2	3	4	4	4	4	10	5	6
4.3	4	4	4	5	4	NR	6	6
4.4	4	4	4	5	4	NR	6	6
4.5	4	4	4	5	5	NR	6	7
4.6	4	4	4	6	5	NR	6	7
4.7	4	5	4	6	5	NR	6	7
4.8	4	5	4	6	5	NR	6	7
4.9	4	5	4	7	5	NR	6	7
5	4	5	4	8	5	NR	6	8
5.1	4	5	4	10	5	NR	6	NR
5.2	5	5	4	NR	5	NR	7	NR
5.3	4	6	5	NR	5	NR	7	NR
5.4	4	5	5	NR	6	NR	7	NR
5.5	4	7	5	NR	6	NR	7	NR
5.6	4	9	5	NR	6	NR	7	NR
5.7	5	NR	5	NR	6	NR	8	NR
5.8	5	NR	5	NR	6	NR	9	NR
5.9	5	NR	5	NR	6	NR	NR	NR
6	5	NR	5	NR	7	NR	NR	NR
6.1	5	NR	5	NR	7	NR	NR	NR
6.2	5	NR	5	NR	7	NR	NR	NR
6.3	5	NR	5	NR	8	NR	NR	NR
6.4	6	NR	6	NR	9	NR	NR	NR
6.5	6	NR	6	NR	10	NR	NR	NR
6.6	6	NR	6	NR	NR	NR	NR	NR
6.7	7	NR	6	NR	NR	NR	NR	NR
6.8	10	NR	6	NR	NR	NR	NR	NR
6.9	NR	NR	6	NR	NR	NR	NR	NR
7	NR	NR	6	NR	NR	NR	NR	NR
R^2	0.998823	0.996798	0.999527	0.963082	0.998891	0.968915	0.897622	0.88804

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM+BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM+PF = pool of *C. maltaromaticum* + *P. fluorescens*.+

*NR (not reached): microbial population cannot reach the determined log in the study's conditions. Spoilage threshold: 7 log CFU/g for *Pseudomonas spp.* and 6 log CFU/g for *Brochothrix spp*.

3.1.2 Sliced cooked ham

Storage time and treatment showed interaction for the microbiological counts ($P < 0.05$), except for LAB count, which was not affected by treatment. Bacterial growth increased with storage time in all treatments. The temperature abuse (from the 10th day) showed significant influence on the count of *P. fluorescens*, *B. thermosphacta*, Enterobacteria, LAB and total aerobics. The highest growth rate was from the 10th day to the 19th day. However, the count of *Carnobacterium* sp. was not affected by temperature abuse in the CM treated cooked ham, showing that the population of *C. maltaromaticum* used in inoculum remained more stable during temperature fluctuations than the P. *fluorescens* and *B. thermosphacta* populations.

Comparing the treatment CM+PF with the spoilage control PF, there was a slightly significant inhibitory effect of CM on the P. *fluorescens* growth (BURT, 2004). The count of P. *fluorescens* in PF increased Δ 3.52 log CFU/g during 28 days of storage, whereas, when in the presence of CM the count increased Δ 1.78 log CFU/g, in the same storage period. The naturally present *Pseudomonas sp.* was also inhibited by *C. maltaromaticum*. Although there was no difference in the count of *Pseudomonas* sp. in CM and NC at the 28th day of storage, the population of *Pseudomonas* sp. in NC increased from 2.73 to 6.87 log CFU/g (Δ 4.14 log CFU/g), whereas in CM, the population slightly increased from 2.36 to 6.09 log CFU/g (Δ 3.73 log CFU/g) (BURT, 2004).

C. maltaromaticum showed significant inhibitory effect on either *B. thermosphacta* or the naturally present *Brochothrix* sp. in cooked ham. In the presence of *C. maltaromaticum*, *Brochothrix* sp. growth in cooked ham during 28 days of storage was ~1 log CFU/g (CM Δ 1.75 log CFU/g and CM + BT Δ 2.11 log CFU/g) lower than without *C. maltaromaticum* inoculation (NC Δ 2.71 log CFU/g and BT Δ 3.56 log CFU/g) (Table 3).

On the other hand, *C. maltaromaticum* count (CM) during storage was not influenced by the presence of *B. thermosphacta* (CM+BT) or *P. fluorescens* (CM+PF) ($P > 0.05$) (Table 3).

Enterobacteriaceae (EB) counts were slightly reduced in the presence of *C. maltaromaticum* (CM Δ 1.34 log CFU/g and CM+PF Δ 1.49 log CFU/g) as compared to NC, during storage (Table 4) (BURT, 2004).

Table 5. Effect of *C. maltaromaticum* on the microbiological count in sliced cooked ham stored in MAP (70/30% N_2/CO_2) for 28 days with temperature abuse (10 days at 4 °C, followed by 18 days at 8 °C).

Treatments	Storage time (days)					Mean	
	0	5	10	19	28		
	Pseudomonas spp. (Log CFU/ g)						*P*
NC	2.73 ± 0.38^{cC}	1.84 ± 0.16^{bC}	2.26 ± 0.38^{bC}	5.21 ± 0.30^{cB}	6.87 ± 0.13^{abA}	3.78^{c}	0.0000
CM	2.36 ± 0.06^{cdB}	1.52 ± 0.00^{bB}	2.26 ± 0.38^{bB}	6.62 ± 0.04^{bA}	6.09 ± 0.27^{bA}	3.77^{c}	0.0000
BT	1.52 ± 0.00^{dC}	1.52 ± 0.00^{bC}	2.94 ± 0.24^{bB}	6.66 ± 0.01^{bA}	6.57 ± 0.25^{abA}	3.84^{c}	0.0000
CM+BT	2.46 ± 0.09^{cC}	1.98 ± 0.46^{bC}	1.84 ± 0.24^{bC}	4.43 ± 0.02^{dB}	5.98 ± 0.25^{bA}	3.34^{d}	0.0000
PF	3.81 ± 0.19^{bC}	5.14 ± 0.06^{aB}	5.42 ± 0.04^{aB}	7.33 ± 0.06^{aA}	7.64 ± 0.12^{aA}	5.87^{a}	0.0000
CM+PF	4.81 ± 0.19^{aB}	5.02 ± 0.36^{aB}	5.49 ± 0.03^{aAB}	5.24 ± 0.12^{cB}	6.59 ± 0.37^{abA}	5.43^{b}	0.0048
Mean	2.95^{d}	2.84^{d}	3.37^{c}	5.92^{b}	6.62^{a}		
P	0.0000	0.0000	0.0000	0.0000	0.0059		
	Brochothrix spp. (Log CFU/ g)					Mean	*P*
NC	2.70 ± 0.23^{bBC}	1.52 ± 0.00^{bC}	1.52 ± 0.00^{cC}	3.88 ± 0.93^{bcAB}	5.41 ± 0.37^{bA}	3.01^{b}	0.0006
CM	1.52 ± 0.00^{cB}	1.52 ± 0.00^{bB}	1.52 ± 0.00^{cB}	2.07 ± 0.34^{cdB}	3.27 ± 0.20^{cA}	1.98^{c}	0.0001
BT	4.37 ± 0.01^{aC}	5.05 ± 0.15^{aB}	5.61 ± 0.04^{bBC}	6.72 ± 0.49^{aAB}	7.93 ± 0.31^{aA}	5.94^{a}	0.0000
CM+BT	4.46 ± 0.07^{aA}	4.16 ± 1.32^{bA}	6.12 ± 0.07^{aA}	6.22 ± 0.24^{aA}	6.57 ± 0.04^{bA}	5.51^{a}	0.0543
PF	1.52 ± 0.00^{cB}	1.52 ± 0.00^{bdB}	1.52 ± 0.00^{cB}	6.12 ± 0.24^{abA}	5.66 ± 0.17^{bA}	3.27^{b}	0.0000
CM+PF	1.52 ± 0.00^{cB}	1.52 ± 0.00^{bB}	1.52 ± 0.00^{cB}	1.52 ± 0.00^{dB}	2.49 ± 0.28^{cA}	1.72^{c}	0.001
Mean	2.69^{c}	2.55^{c}	2.97^{c}	4.42^{b}	5.22^{a}		
P	0.0000	0.001	0.0000	0.0000	0.0000		
	Enterobacteriacea (Log CFU/ g)					Mean	*P*
NC	1.52 ± 0.00^{bC}	1.52 ± 0.00^{bC}	1.52 ± 0.00^{bC}	6.77 ± 0.18^{aB}	6.38 ± 0.11^{abA}	3.54^{b}	0.0000
CM	1.52 ± 0.00^{bB}	1.78 ± 0.26^{bB}	1.52 ± 0.00^{bB}	4.41 ± 0.12^{bcB}	5.04 ± 0.59^{bcA}	2.86^{cd}	0.0000
BT	4.47 ± 0.02^{aB}	4.93 ± 0.06^{aB}	4.95 ± 0.19^{aB}	6.20 ± 0.18^{aA}	6.62 ± 0.03^{aA}	5.44^{a}	0.0000
CM+BT	4.47 ± 0.00^{aD}	4.89 ± 0.07^{aC}	5.23 ± 0.05^{aC}	6.41 ± 0.05^{aB}	7.00 ± 0.00^{aA}	5.60^{a}	0.0000
PF	1.52 ± 0.00^{bC}	1.52 ± 0.00^{bC}	1.52 ± 0.00^{bC}	5.04 ± 0.22^{bB}	6.02 ± 0.39^{abcA}	3.13^{c}	0.0000
CM+PF	1.52 ± 0.00^{bC}	1.68 ± 0.15^{bC}	1.52 ± 0.00^{bC}	3.99 ± 0.33^{cB}	4.89 ± 0.11^{cA}	2.72^{d}	0.0000
Mean	2.51^{c}	2.72^{c}	2.71^{c}	5.47^{b}	5.99^{a}		

P	-	0.0000	0.0000	0.0000	0.0013		
			Carnobacterium spp. (Log CFU/ g)			Mean	*P*
NC	3.47 ± 0.04^{dB}	1.78 ± 0.13^{cC}	4.21 ± 0.14^{cbB}	6.43 ± 0.24^{bA}	6.43 ±0.29^{aA}	4.46^{d}	0.0000
CM	6.50 ± 0.02^{aA}	6.38 ± 0.14^{aAB}	6.66 ± 0.01^{aA}	6.08 ± 0.09^{bcB}	6.58 ± 0.04^{aA}	6.44^{a}	0.0032
BT	5.18 ± 0.10^{bcB}	4.54 ± 0.08^{bB}	4.55 ± 0.22^{bcB}	7.53 ± 0.01^{aA}	7.25 ± 0.21^{aA}	5.81^{b}	0.0000
CM+BT	5.83 ± 0.22^{abAB}	6.42 ± 0.09^{aA}	6.35 ± 0.18^{aAB}	5.51 ± 0.28^{cB}	6.56 ± 0.12^{aA}	6.14^{ab}	0.012
PF	4.68 ± 0.16^{cB}	4.65 ± 0.1^{bcB}	5.18 ± 0.09^{bB}	5.67 ± 0.18^{bcAB}	6.42 ± 0.38^{aA}	5.31^{c}	0.001
CM+PF	6.34 ± 0.19^{aAB}	6.22 ± 0.27^{aA}	6.62 ± 0.02^{aAB}	5.58 ± 0.12^{bcB}	6.09 ± 0.35^{aA}	6.17^{a}	0.012
Mean	5.33^{c}	4.99^{d}	5.59^{c}	6.13^{b}	6.56^{a}		
P	0.0000	0.0000	0.0000	0.0000	0.129		
			Lactic acid bacteria (Log CFU/ g)			Mean	*P*
NC	1.52 ± 0.00^{aC}	1.84 ± 0.32^{aC}	4.42 ± 0.09^{aB}	4.92 ± 0.22^{bAB}	6.58 ± 0.79^{aA}	3.86^{a}	0.0000
CM	1.52 ± 0.00^{aC}	2.99 ± 0.12^{aB}	4.18 ± 0.53^{aB}	6.43 ± 0.39^{aA}	6.40 ± 0.29^{aA}	4.31^{a}	0.0000
BT	1.52 ± 0.00^{aC}	1.68 ± 0.16^{aC}	4.57 ± 0.02^{aB}	6.50 ± 0.24^{aA}	7.00 ± 0.00^{aA}	4.26^{a}	0.0000
CM+BT	1.52 ± 0.00^{aC}	2.35 ± 0.42^{aC}	4.39 ± 0.09^{aB}	5.91 ± 0.41^{abA}	5.88 ± 0.18^{aA}	4.01^{a}	0.0000
PF	1.52 ± 0.00^{aC}	1.68 ± 1.16^{aC}	4.47 ± 0.02^{aB}	6.48 ± 0.04^{aA}	6.78 ± 0.04^{aA}	4.19^{a}	0.0000
CM+PF	1.52 ± 0.00^{aC}	2.29 ± 0.44^{aC}	4.33 ± 0.19^{aB}	6.24 ± 0.41^{abA}	6.72 ± 0.11^{aA}	4.22^{a}	0.0000
Mean	1.52^{e}	2.14^{d}	4.39^{c}	6.08^{b}	6.56^{a}		
P	-	0.0587	0.4824	0.0199	0.3767		
			Total aerobic bacteria (Log CFU/ g)			Mean	*P*
NC	1.52 ± 0.00^{eC}	2.10 ± 0.29^{cC}	4.64 ± 0.08^{cB}	6.51 ± 0.18^{cA}	7.17 ± 0.13^{aA}	4.39^{c}	0.0000
CM	6.50 ± 0.02^{aABC}	6.38 ± 0.14^{aBC}	6.66 ± 0.01^{aAB}	6.23 ± 0.07^{bC}	6.79 ± 0.10^{aA}	6.52^{a}	0.0048
BT	5.32 ± 0.07^{cdB}	5.41 ± 0.03^{bB}	5.24 ± 0.13^{bB}	7.77 ± 0.09^{aA}	7.67 ± 0.16^{aA}	6.28^{a}	0.0000
CM+BT	5.88 ± 0.19^{bcD}	6.47 ± 0.07^{aBC}	6.61 ± 0.08^{aAB}	6.05 ± 0.05^{cCD}	7.00 ± 0.00^{aA}	6.40^{a}	0.0001
PF	4.80 ± 0.09^{dC}	5.13 ± 0.09^{bC}	5.57 ± 0.06^{bBC}	6.62 ± 0.04^{bAB}	6.91 ± 0.49^{aA}	5.80^{b}	0.0002
CM+PF	6.35 ± 0.19^{abA}	6.29 ± 0.20^{aA}	6.65 ± 0.01^{aA}	6.01 ± 0.04^{cA}	6.73 ± 0.27^{aA}	6.41^{a}	0.0893
Mean	5.06^{d}	5.29^{d}	5.89^{c}	6.53^{b}	7.05^{a}		
P	0.0000	0.0000	0.0000	0.0000	0.0000		

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM + BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM + PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. Different lowercase letters in the same column show significant differences among treatments ($P < .05$). Different uppercase letters in the same row show significant differences among storage time ($P < .05$).

*Values of bacterial count below the detection limit were considered as 1.52 log CFU/ g for the statistical analysis.

Considering physicochemical characteristics of the product, the pH decreased with the progression of the storage time in all treatments. Similarly, to the pH behavior in ground beef, cooked ham inoculated with *C. maltaromaticum* (CM, CM + BT, CM + PF) showed lower pH values during storage than non-inoculated ham (NC) ($P < 0.05$) (Table 5). Although, differences in pH values were low (< 0.28).

Regarding atmosphere composition (70/30% N_2/CO_2), initial concentrations of CO_2 started with slight variations in all treatments. In general, treatments added with CM (CM+PF, CM+BT and CM) showed greater decrease in CO_2 and increase in N_2 concentration throughout storage. At 28 d of storage, NC and BT showed the lowest decrease in CO_2 (Δ -0.80 and -1.30 respectively) and increase in N_2 (Δ 1.48 and 1.98) concentrations. The residual oxygen was nearly completely consumed in all treatments at the end of storage (Table 5).

Concerning the instrumental colors parameters, conversely to the observed for ground beef, storage time showed no effect on the luminosity and redness of cooked ham. These coordinates were also not affected by treatments at the end of storage. There were differences between treatments and time for yellowness. However, differences in b* values were low and would not interfere the visual quality of the product, since ΔE* values were below 3.00 in all treatments. As color disparity (ΔE* > 3) represents consumers visual rejection of the product, treatments inoculated with *C. maltaromaticum* maintained the visual appearance throughout storage and showed lower color disparity (CM = 0.76, CM+PF = 1.34, CM+BT = 1.86) than other treatments (NC = 1.12, PF = 1.85, BT = 2.09).

Table 6. pH and dynamic behavior of headspace gases of sliced cooked ham stored in MAP (70/30% N_2/CO_2) for 28 days with temperature abuse (10 days at 4 °C, followed by 18 days at 8 °C).

Treatment	Storage days 0	5	10	19	28	Mean	*P*
			Physicochemical parameter				
			pH				
NC	6.07 ± 0.01^{bAB}	6.09 ± 0.03^{aA}	6.07 ± 0.01^{bAB}	6.10 ± 0.04^{aAB}	5.94 ± 0.06^{aB}	6.05^{a}	0.0284
CM	5.93 ± 0.01^{cB}	6.13 ± 0.01^{aA}	5.93 ± 0.01^{cA}	5.88 ± 0.03^{bA}	5.66 ± 0.06^{cC}	6.90^{ab}	0.0000
BT	6.00 ± 0.01^{bA}	6.02 ± 0.05^{aA}	6.01 ± 0.01^{bA}	5.86 ± 0.01^{bAB}	5.77 ± 0.01^{abcB}	6.93^{b}	0.0012
CM+BT	6.18 ± 0.01^{aA}	6.05 ± 0.01^{aB}	6.18 ± 0.01^{aA}	5.81 ± 0.04^{bcC}	5.91 ± 0.01^{abC}	5.87^{c}	0.0000
PF	6.05 ± 0.03^{bA}	5.87 ± 0.02^{bB}	6.07 ± 0.03^{bA}	5.70 ± 0.02^{cdC}	5.66 ± 0.00^{cC}	6.03^{a}	0.0000
CM+PF	6.05 ± 0.00^{bA}	5.87 ± 0.00^{bB}	6.05 ± 0.00^{bA}	5.64 ± 0.03^{dD}	5.74 ± 0.04b^{cC}	5.87^{c}	0.0000
Mean	6.05^{A}	6.00^{B}	6.05^{A}	5.83^{C}	5.78^{C}		0.0000
P	0.0000	0.0000	0.0000	0.0000	0.0006	0.000	
			O_2 residue (%)			Mean	*P*
NC	0.68 ± 0.00^{aC}	0.86 ± 0.00^{aB}	0.94 ± 0.01^{aA}	0.29 ± 0.11^{aD}	0.00 ± 0.00^{aE}	0.55^{a}	0.0000
CM	0.66 ± 0.00^{abB}	0.62 ± 0.02^{cAB}	0.95 ± 0.00^{bA}	0.29 ± 0.01^{abC}	0.00 ± 0.00^{aD}	0.32^{c}	0.0000
BT	0.68 ± 0.01^{aC}	0.84 ± 0.00^{aB}	0.96 ± 0.01^{aA}	0.21 ± 0.03^{abD}	0.00 ± 0.00^{aE}	0.52^{ab}	0.0000
CM+BT	0.66 ± 0.01^{cA}	0.67 ± 0.01^{aA}	0.10 ± 0.00^{aC}	0.16 ± 0.01^{bB}	0.10 ± 0.10^{aC}	0.34^{c}	0.0000
PF	0.63 ± 0.00^{aA}	0.84 ± 0.00^{bA}	0.93 ± 0.03^{bB}	0.02 ± 0.02^{abB}	0.00 ± 0.00^{aB}	0.48^{b}	0.0000
CM+PF	0.64 ± 0.00^{bcA}	0.56 ± 0.01^{dB}	0.09 ± 0.00^{bD}	0.14 ± 0.00^{abC}	0.00 ± 0.00^{aE}	0.29^{c}	0.0000
Mean	0.66^{B}	0.73^{A}	0.52^{C}	0.15^{D}	0.02^{E}		0.0000
P	0.0000	0.0000	0.0000	0.0269	0.4175	0.0000	
			CO_2 (%)			Mean	*P*
NC	28.40 ± 0.07^{bA}	26.00 ± 0.03^{aB}	26.00 ± 0.20^{bB}	25.30 ± 0.38^{cB}	27.60 ± 0.42^{aA}	26.74^{ab}	0.0000
CM	28.60 ± 0.28^{abA}	26.40 ± 0.17^{aC}	27.30 ± 0.09^{aB}	25.90 ± 0.09^{bcCD}	25.10 ± 0.25^{bD}	26.73^{ab}	0.0000
BT	28.30 ± 0.09^{bA}	26.30 ± 0.06^{aBC}	25.70 ± 0.24^{bC}	26.50 ± 0.12^{abB}	27.00 ± 0.20^{aB}	26.72^{ab}	0.0000
CM+BT	29.10 ± 0.12^{bA}	26.60 ± 0.26^{aB}	27.30 ± 0.10^{bB}	26.90 ± 0.06^{abcB}	25.60 ± 0.23^{bB}	27.05^{a}	0.0000

PF	29.60 ± 0.35^{abA}	26.00 ± 0.09^{aB}	25.30 ± 0.18^{aB}	26.00 ± 0.30^{aB}	25.60 ± 0.23^{bB}	26.51^{b}	0.0000
CM+PF	29.50 ± 0.03^{abA}	26.30 ± 0.06^{aC}	27.40 ± 0.15^{aB}	26.20 ± 0.15^{abC}	25.30 ± 0.22^{bD}	26.89^{a}	0.0000
Mean	28.94^{A}	26.26^{BC}	26.46^{B}	26.13^{BC}	26.07^{C}		0.0000
P	0.0039	0.0742	0.0000	0.0027	0.0000	0.0000	
			N_2 (%)			Mean	*P*
NC	70.92 ± 0.07^{aC}	73.14 ± 0.03^{aB}	73.07 ± 0.21^{abcB}	74.44 ± 0.27^{aA}	72.40 ± 0.27^{cB}	72.71^{ab}	0.0000
CM	70.74 ± 0.28^{abC}	72.98 ± 0.19^{aB}	72.61 ± 0.09^{cB}	73.89 ± 0.08^{abA}	74.90 ± 0.25^{bA}	72.95^{ab}	0.0000
BT	71.02 ± 0.08^{aB}	72.86 ± 0.06^{aA}	73.35 ± 0.23^{abA}	73.44 ± 0.11^{bcA}	73.00 ± 0.20^{aA}	72.76^{ab}	0.0000
CM+BT	70.25 ± 0.13^{bC}	72.73 ± 0.26^{aB}	72.60 ± 0.10^{cAB}	72.93 ± 0.05^{abAB}	74.40 ± 0.15^{aA}	72.61^{b}	0.0000
PF	69.77 ± 0.35^{abC}	73.16 ± 0.09^{aB}	73.79 ± 0.20^{bcB}	74.00 ± 0.28^{cB}	74.40 ± 0.23^{aA}	73.01^{a}	0.0000
CM+PF	69.86 ± 0.03^{bD}	73.18 ± 0.06^{aBC}	72.51 ± 0.15^{cC}	73.66 ± 0.15^{bcB}	74.70 ± 0.22^{aA}	73.83^{ab}	0.0000
Mean	70.40^{C}	73.01^{B}	73.02^{B}	73.72^{A}	73.91^{A}		0.0000
P	0.0051	0.2642	0.0025	0.0013	0.0000	0.0184	

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM+BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM+PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. Different lowercase letters in the same column show significant differences among treatments ($P < .05$). Different uppercase letters in the same row show significant differences among storage time ($P < 0.05$).

Table 7. Effect of the addition of a pool of *C. maltaromaticum* and spoilage bacteria on the Instrumental color (L*, a*, b* and ΔE*) of sliced cooked ham during storage in MAP (70/30% N_2/CO_2) for 28 days (10 days at 4 °C, followed by 18 days at 8 °C).

Treatment	Storage days 0	5	10	19	28	Mean	*P*
			L*				
NC	65.3 ± 0.67^{aA}	66.26 ± 0.54^{aA}	65.66 ± 0.89^{aA}	64.82 ± 1.33^{aA}	66.37 ± 0.96^{aA}	65.59^{a}	0.8623
CM	65.58 ± 0.74^{aA}	63.59 ± 1.14^{aA}	66.67 ± 0.82^{aA}	65.39 ± 0.78^{aA}	65.45 ± 0.80^{aA}	65.11^{ab}	0.3346
BT	66.65 ± 0.95^{aA}	66.33 ± 0.86^{aA}	66.62 ± .66^{aA}	63.54 ± 1.13^{aA}	64.69 ± 0.87^{aA}	65.51^{ab}	0.0553
CM+BT	62.90 ± 1.18^{aA}	65.62 ± 1.05^{aA}	63.55 ± 1.39^{aA}	65.55 ± 1.00^{aA}	64.71 ± 0.88^{aA}	64.39^{ab}	0.9958

PF	64.38 ± 1.49^{aA}	63.38 ± 1.12^{aA}	63.84 ± 0.80^{aA}	66.14 ± 1.15^{aA}	63.24 ± 1.37^{aA}	64.53^{ab}	0.6355
CM+PF	64.89 ± 1.16^{aA}	64.78 ± 1.26^{aA}	64.16 ± 0.91^{aA}	62.76 ± 1.03^{aA}	63.79 ± 1.06^{aA}	63.69^{b}	0.3118
Mean	65.04^{A}	64.90^{A}	65.41^{A}	64.34^{A}	64.31^{A}		0.2839
P	0.9690	0.2154	0.2847	0.5677	0.0490	0.0259	
			a*				*P*
NC	7.65 ± 0.30^{aA}	7.88 ± 0.30^{aA}	8.91 ± 0.61^{aA}	8.51 ± 0.72^{aA}	7.82 ± 0.47^{aA}	7.87^{a}	0.6903
CM	7.99 ± 0.35^{aA}	8.61 ± 0.65^{aA}	7.94 ± 0.39^{aA}	7.34 ± 0.50^{aA}	7.89 ± 0.30^{aA}	8.04^{a}	0.2225
BT	7.88 ± 0.54^{aA}	6.87 ± 0.43^{aA}	7.56 ± 0.42^{aA}	9.00 ± 0.67^{aA}	7.67 ± 0.35^{aA}	7.98^{a}	0.2902
CM+BT	8.41 ± 0.55^{aA}	8.39 ± 0.47^{aA}	8.33 ± 0.57^{aA}	7.88 ± 0.47^{aA}	8.39 ± 0.36^{aA}	8.19^{a}	0.9024
PF	7.13 ± 0.80^{aA}	8.75 ± 0.59^{aA}	8.07 ± 0.42^{aA}	7.63 ± 0.60^{aA}	8.33 ± 0.63^{aA}	7.92^{a}	0.5653
CM+PF	7.74 ± 0.56^{aA}	8.29 ± 0.55^{aA}	7.77 ± 0.41^{aA}	8.21 ± 0.57^{aA}	8.38 ± 0.51^{aA}	8.41^{a}	0.6178
Mean	7.71^{A}	8.31^{A}	7.99^{A}	8.12^{A}	8.22^{A}		0.2954
P	0.9329	0.4442	0.7211	0.5897	0.3112	0.5826	
			b*				*P*
NC	8.57 ± 0.14^{cBC}	8.17 ± 0.11^{aC}	8.82 ± 0.17^{aB}	10.07 ± 0.13^{aA}	8.84 ± 0.13^{aB}	8.85^{ab}	0.0000
CM	9.32 ± 0.15^{aB}	8.00 ± 0.21^{aC}	8.69 ± 0.10^{aC}	10.06 ± 0.15^{aA}	8.58 ± 0.16^{abBC}	8.97^{ab}	0.0000
BT	8.92 ± 0.18^{abcB}	8.33 ± 0.08^{aC}	8.52 ± 0.13^{aBC}	10.09 ± 0.18^{aA}	8.21 ± 0.12^{bC}	8.81^{ab}	0.0000
CM+BT	8.73 ± 0.22^{abcBC}	8.62 ± 0.18^{aBC}	8.15 ± 0.26^{aC}	10.49 ± 0.15^{aA}	9.15 ± 0.17^{aB}	9.06^{a}	0.0000
PF	8.45 ± 0.18^{bcB}	8.20 ± 0.23^{aB}	8.00 ± 0.15^{aB}	9.88 ± 0.19^{aA}	8.89 ± 0.21^{abB}	8.68^{b}	0.0000
CM+PF	9.18 ± 0.22^{bcB}	8.57 ± 0.18^{aC}	8.29 ± 0.19^{aBC}	10.13 ± 0.18^{aA}	8.75 ± 0.16^{abBC}	8.98^{ab}	0.0000
Mean	8.86^{B}	8.28^{D}	8.47^{CD}	10.1^{2A}	8.74^{BC}		0.0000
P	0.0023	0.5488	0.0980	0.2204	0.0038	0.0069	
			ΔE				
NC			1.12				
CM			0.76				
BT			2.09				
CM+BT			1.86				
PF			1.71				

CM+PF		1.34

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM+BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM+PF = pool of *C. maltaromaticum* + *P. fluorescens*.

Values are expressed as Mean ± SE. Different lowercase letters in the same column show significant differences among treatments ($P < .05$). Different uppercase letters in the same row show significant differences among storage time ($P < 0.05$).

In terms of shelf life gain, the autochthonous population of *Brochothrix spp.* and *Pseudomonas spp*. remained under the spoilage threshold count in both NC and CM treatments. Considering *Brochothrix spp* population, Carnobacteria showed no evident effect on the extension of the shelf life of cooked ham according to the logistic model used. Even though, *Brochothrix spp.* growth was stabilized at 6.6 log CFU/g in CM+BT, whereas in BT the growth stopped at 7.0 log.

Carnobacteria (CM) stabilized the autochthonous *Pseudomonas spp*. population at lower concentration (6.4 log CFU/g) when compared to control (NC – 6.8 log CFU/g). The presence of Carnobacteria population increased 4.68% shelf life in CM+PF, which represented 10 d shelf life increase (PF) (Table 8).

Table 8. Effect of *C. maltaromaticum* on the prediction of shelf life based on the spoilage threshold in in sliced cooked ham during storage in MAP (70/30% N_2/CO_2) for 28 days (10 days at 4 °C, followed by 18 days at 8 °C).

Growth (log)	*Brochothrix spp.*				*Pseudomonas spp.*			
	Treatments							
	NC	CM	BT	CM+BT	NC	CM	PF	CM+PF
	Predicted shelf life (days)							
3	14	24	NR	NR	17	10	NR	NR
3.1	15	25	NR	NR	17	10	NR	NR
3.2	15	26	NR	NR	17	10	NR	NR
3.3	15	29	NR	NR	18	10	NR	NR
3.4	15	NR	NR	NR	18	10	NR	NR
3.5	15	NR	NR	NR	18	11	NR	NR
3.6	16	NR	NR	NR	18	11	NR	NR
3.7	16	NR	NR	NR	18	11	NR	NR
3.8	16	NR	NR	NR	18	11	NR	NR
3.9	17	NR	NR	NR	18	11	0	NR
4	17	NR	NR	NR	18	11	1	NR
4.1	17	NR	NR	NR	18	11	1	NR
4.2	17	NR	NR	NR	18	11	2	NR
4.3	18	NR	NR	NR	18	11	2	NR
4.4	18	NR	0	NR	18	11	3	NR
4.5	18	NR	1	NR	18	11	3	NR
4.6	19	NR	2	1	19	11	3	NR
4.7	20	NR	3	1	19	11	4	NR
4.8	21	NR	3	2	19	11	4	NR
4.9	NR	NR	4	2	19	11	5	NR
5	NR	NR	5	3	19	11	5	NR
5.1	NR	NR	6	3	19	11	6	9

5.2	NR	NR	7	4	19	11	6	17
5.3	NR	NR	7	4	19	11	7	19
5.4	NR	NR	8	5	19	11	8	21
5.5	NR	NR	9	5	19	11	8	22
5.6	NR	NR	10	6	19	11	9	23
5.7	NR	NR	11	6	19	11	9	24
5.8	NR	NR	11	7	20	11	10	24
5.9	NR	NR	12	8	20	11	11	25
6	NR	NR	13	9	20	11	11	26
6.1	NR	NR	14	10	20	11	12	26
6.2	NR	NR	15	11	20	12	13	27
6.3	NR	NR	15	13	20	12	13	27
6.4	NR	NR	16	16	20	NR	14	27
6.5	NR	NR	17	20	21	NR	15	28
6.6	NR	NR	18	32	21	NR	16	28
6.7	NR	NR	19	NR	21	NR	16	28
6.8	NR	NR	19	NR	22	NR	17	29
6.9	NR	NR	20	NR	NR	NR	18	29
7	NR	NR	21	NR	NR	NR	19	29
R^2	0.834974	0.879554	0.879554	0.961514	0.936228	0.959526	0.954111	0.653685

Treatments: NC = negative control; CM = pool of *C. maltaromaticum*; BT = pool of *B. thermosphacta*; PF = *P. fluorescens*; CM+BT = pool of *C. maltaromaticum* + pool of *B. thermosphacta*; CM+PF = pool of *C. maltaromaticum* + *P. fluorescens*.

*NR (not reached): microbial population cannot reach the determined log in the study's conditions.

Spoilage threshold: 7 log CFU/g for *Pseudomonas spp.* and 6 log CFU/g for *Brochothrix spp*.

4 Discussion

C. maltaromaticum reduced the growth of the target spoilage strains in both types of meat products. In the treatments added with *C. maltaromaricum*, the count of *Pseudomonas* sp. and *Brochothrix* sp. was lower than the maximal bacteriological count (7 log CFU/g for *Pseudomonas sp.* and 6 log CFU/g for *Brochothrix sp.*) considered as indicator of spoilage in meat products (MILLS; DONNISON; BRIGHTWELL, 2014; PELLISSERY et al., 2020). *C. maltaromaticum* inhibition effect towards the spoilage strains may occurs mainly due to the following mechanisms: (i) competition for nutrients which leads to nutrients depletion; (ii) displacement or exclusion: strong and long adhesion to food matrix. (iii) production of antagonist compounds as lactic acid, H_2O_2, diacetyl, CO_2 (VERMEIREN; DEVLIEGHERE; DEBEVERE, 2004; NILSSON et al., 2005; KASRA-KERMANSHAHI; MOBARAK-QAMSARI, 2015; SAID et al., 2019) (iv) faster growth in the matrix (AMÉZQUITA; BRASHEARS, 2002; BRILLET-VIEL et al., 2005) and bacteriocins production, leading to bactericide or bacteriostatic effects on spoilage bacteria (BALI et al., 2014; BRILLET-VIEL et al., 2016). Zhang, Gänzle and Yang (2019) also found that the organic acids formic and acetic play a key role as antibacterial compounds produced by *Carnobacterium* sp. in vacuum-packaged meat.

Other studies report the inhibitory effect of *C. maltaromaticum* towards spoilage bacteria *in vitro* and in other foods. Zhang, Baranyi and Tamplin (2015) verified the *in vitro* inhibitory effect towards some strains of *B. thermosphacta* and *Pseudomonas spp.* in spot-lawn (cell-to-cell contact) assay and medium broth (cell free supernatant - CFC). Spanu et al. (2018) showed the inhibition of *Pseudomonas spp.* by *Carnobacterium spp.* in *Ricotta* cheese and Laursen et al. (2006) observed the inhibition of *B. thermosphacta* in MAP (50/30/20% $CO_2/N_2/O_2$) packaged shrimp.

Regarding the Enterobacteria growth in ground beef and cooked ham, the presence of *P. fluorescens* or *B. thermosphacta* did not influence the inhibitory effect of *C. maltaromaticum* on Enterobacteria. Russo et al. (2006) explored the relation of spoilage raw beef microbiota (*B. thermosphacta,* Enterobacteria and *Pseudomonas spp.*) and LAB, with the same level of contamination. They observed that *B. thermosphacta* grew similarly in the presence of the other

bacteria, except when in co-cultured with LAB (*L. sakei*, *L. curvatus* and *Leuconostoc mesenteroides*). The bioprotective bacteria were able to reduce *B. thermosphacta* by 2 log CFU *in vitro*. Tshabalalala, Kock and Buys (2012) investigated whether *P. fluorescens* influenced the counts of *Escherichia coli* O157:H7 in fresh beef stored aerobically and under vacuum storage. They found that *E. coli* counts were only affected by a bioprotective LAB, *Lactobacillus plantarum*, and not by *P. fluorescens* regardless the inoculation level or storage time.

Our findings corroborate with the previous results obtained by Imazaki (2018), who showed an inhibitory effect of the CM strains on EB in beef stored with low-oxygen atmosphere. The heterofermentative metabolism of Carnobacteria leads to the production of molecules that can act as microbial antagonist such as CO_2, acetate and ethanol (KASRA-KERMANSHAHI; MOBARAK-QAMSARI, 2015). The inhibitory effect of CM in ground beef may be partly due the production of CO_2 that acts as antimicrobial compound against EB (MILIJASEVIC; BABIC; VESKOVIC-MORACANIN, 2015).

The same parameters that affect the microbiological growth also impact on gaseous dynamics in the package, for instance, the levels of CO_2 dissolved in the aqueous phase of the meat product (REMENANT et al., 2015). *C. maltaromaticum* can influence the gaseous composition of products stored in modified atmosphere. In ground beef, the presence of *C. maltaromaticum* (CM; CM+BT; CM+PF) increased the initial concentration of CO_2 (LAURSEN; LEISNER; DALGAARD, 2006), whereas in other treatments CO_2 concentrations were reduced during storage. The increase in CO_2 concentrations could limit EB growth (DJENANE; RONCALÉS et al., 2018), while for *B. thermosphacta* the increase of CO_2 does not affect the bacteria growth, thus the inhibitory effect results from other mechanisms of action of *C. maltaromaticum* (PIN; FERNANDO; ORDONEZ, 2002). *P. fluorescens* still shows slight signs of growth regardless high concentrations of CO_2 (TAN; GILL, 1982; HENDRICKS; HOTCHKISS, 1997; STOOPS et al., 2012). In fact, residual O_2 levels as low as 0.1% are enough to allow *Pseudomonas spp.* growth even in products stored with MAP (100% N_2; 30/70% CO_2/N_2; 100% CO_2). Moreover, package permeability allows the growth despite absence of oxygen in the packaging (CLARK; BURKI, 1972; SEYDIM et al., 2006; STOOPS et al., 2012). Conversely, in sliced cooked

ham (70/30% N_2/CO_2 MAP), there was a decrease in CO_2 concentrations during storage in all treatments. This decrease in the CO_2 concentration in ham may be related to the rate of gas absorption by the different meat matrices. In fact, sliced cooked ham showed to reach faster (60 min) (DEVLIEGHERE; DEBEVERE, 2000) absorption equilibrium of CO_2 dissolved in the matrix than ground beef (12 h) (ZHAO; WELLS; MCMLLLLN, 1995).

C. maltaromaticum in all CM treatments reached high counts at the end of the storage period in ground beef, 7.29 (CM), 7.32 (CM+BT), 6.13 log CFU/g (CM+PF) and in ham, 6.85 (CM), 6.56 (CM+BT) and 6.09 log CFU/g (CM+PF). As it was noticed *C. maltaromaticum* growth in CM treatments remained practically constant during storage and was not affected by the inoculated nor autochthonous microbiota of the products, in both MAP and meat matrices. *C. maltaromaticum* and *C. divergens* can predominate over spoilage population (LAURSEN et al., 2005) and persist in chilled meat products in diverse atmospheres until the end of the shelf life (DOULGERAKI et al., 2012; HOLCK et al., 2014). The fact that the *C. maltaromaticum* strains used were isolated from a log-term vacuum packaged Australian beef and from which 98% of the final microbial composition was prevalently *Carnobacterium* sp. corroborates the ability of these populations to remain stable over a long period of refrigerated storage (IMAZAKI, 2018). In long shelf life vacuum packaged pork and beef (60-190 days), Zhang et al. (2018) identified *Carnobacterium* strains (*C. maltaromaticum* and *C. divergens*) as the most predominant microbiota. *Carnobacterium* tends to survive and thrive contamination in abattoirs, processing, storage and distribution of the meat products (MILLS et al., 2018; LAURITSEN et al., 2019; CHEN et al., 2020). These are desirable characteristics of a bioprotective culture (SINGH, 2018; MOKOENA, 2017).

Additionally, the negligible pH decrease in the treatments added with *C. maltaromaticum* (beef at 7 d of storage CM (5.68), CM+BT (5.67) and CM+PF (5.55); ham at 28 d CM (5.66), CM+BT (5.91) and CM+PF (5.74) confirms that *Carnobacterium spp.* is not a strong acid producer (LEISNER et al. 2007) and can sustain the low pH variation in the presence of other acidifying LAB (EDIMA et al., 2008). Moreover, pH of the products remained within the normal range for beef 5.40-5.80 (MACDOUGALL; TAYLOR, 2007) and cooked ham 5.60–6.20 (ARNAU, GUERRERO, CASADEMONT; GOU, 1995).The weak acid producing

potential of *C. maltaromaticum* is an advantage over most of LAB, because of the influence of a strong pH decrease on the quality parameters of these type of meat products (AUDENAERT et al., 2010; SINGH, 2018).

Meat color is the first parameter evaluated by consumers to predict meat quality and freshness when purchasing the product. The acceptance of cooked and raw meat relies deeply on color and color uniformity (HUNT et al., 2012). In ground beef, all treatments showed perceptible color alterations at the end of storage, which is likely related to the high O_2 content (66%) in MAP. When $\Delta E > 3$, color differences are obvious for the human eye (OLIVERA et al., 2013). Jakobsen and Bertelsen (2000) showed that beef stored in MAP with O_2 content higher than 55% had issues with color stabilization throughout the shelf life. When beef is stored in aerobic atmosphere, the high concentrations of O_2 can cause myoglobin oxidation leading to meat discoloration (XIONG, 2000; ZAKRYS et al, 2009; ZHANG; XIAO; AHN, 2013). Regarding ham color, the treatments added with *C. maltaromaticum* (CM - ΔE 0.76) did not show a great impact on the color characteristics of the product as ΔE* values were close to 1. ΔE* values <1 would theoretically be imperceptible to the consumers eye (HUNT et al., 2012). Studies report the low influence of *Carnobacterium* strains on the color of beef (CASABURI et al. 2011; IMAZAKI, 2018; SAID et al., 2019), ham (DANIELSKI et al., 2020), cold-smoked salmon (BRILLET-VIEL et al. 2005) and grilled salmon (WIERNASZ et al., 2020). Moreover, Imazaki (2018) found a negligible influence of the *C. maltaromaticum* strains used in the present study on the sensory quality of raw and cooked beef patties (counts varying from 4 - 6 log CFU/g). Casaburi et al. (2011) also reported insignificant influence of high concentration (7 log CFU/g) of *C. maltaromaticum* on the sensory characteristics of beef stored in air and vacuum package. More recently, WIERNASZ et al. (2020) observed greater antilisterial effect from *C. maltaromaticum* than other LAB in salmon drill with low impact on the sensory characteristics of the product. Therefore, *C. maltaromaticum* strains are interesting alternatives as natural biopreservatives for different food products against spoilage and pathogenic bacteria.

The use of natural preservatives as a tool to extend the meat shelf life is attractive to both consumers and industry. Overall, the growth of the spoilage population was slowed down in the presence of Carnobacteria and stabilized the growth at lower concentrations (log) in comparison to controls for both products

tested. Grispoldi et al. (2020) showed that the addition of LAB (mix of several strains) along with good hygiene practice can help preserve meat quality, stabilizing the color variation and reducing spoilage population in ground meat. In ground beef, treatments inoculated with Carnobacteria showed an expressive increase of shelf life (28.57 % in CM; 36.28 % in CM+BT and 40.43 % in CM+PF). Castellano et al. (2010) showed that *Lactobacillus curvatus* CRL705 (6 log CFU/g) can increase the shelf life of vacuum-packaged raw beef (60 days at 2 °C) in 16.67 % (10 days increase) based on appearance acceptance.

The shelf life extension in sliced cooked ham (3.25 % in CM for *Brochothrix sp*. population and 4.28% in CM+PF) was lower compared to the one observed in ground beef. In general, the maximum spoilage growth was reduced in the presence of Carnobacteria. Although, the regression model showed no increase of shelf life for the treatments that the general mean of spoilage population had the same statistical value for both Carnobacteria inoculated and non-inoculated (*Pseudomonas sp*. population in NC and CM; *Brochothrix sp*. population in BT and CM+BT). Other studies support the potential of LAB to improve the shelf life of ready to eat meat products (CASTELLANO et al., 2017). Bredholt, Nesbakken and Holck (2001) reported that a non-bacteriogenic Lactobacillus sakei strain increased the shelf life of sliced cooked ham (28 days) in 17.86 % (5 days increase) at concentrations of 5 to 6 log CFU/g (acceptability score). Hu et al. (2008) *Lactobacillus sakei* increased the shelf life of vacuum-packed sliced cooked ham (without preservatives) in 133% (35 d) when compared to control without inoculum (15 d). Vermeiren, Devlieghere, and Debevere (2006) found that *Lactobacillus sakei* 10A (5 log CFU/g) slowed and reduced the growth of *B. thermosphacta* and *Leuconostoc mesenteroides* in vacuum-packaged ham without the action of a bacteriocin (postponed the shelf life in 14 days). Therefore, lactic acid bacteria are useful to extend the shelf life of meat products. *C. maltaromaticum* proved to be an effective bioprotective culture. As observed *C. maltaromaticum* showed a neglected effect on meat quality and decreased both autochthonous and inoculated spoilage and pathogenic bacteria for the products tested. In conclusion, *C. maltaromaticum* could be exploited as a natural preservative in meat to enhance safety, quality and extend the product shelf life.

5 Conclusion

The *C. maltaromaticum* strains (CM_824, CM_827 and CM_289) reduced the counts of both inoculated and autochthonous spoilage bacteria (*Brochothrix spp.*, *B. thermosphacta*, *Pseudomonas spp.*, *P. fluorescens* and Enterobacteria). The inhibitory effect was observed in both food matrices (sliced cooked ham and ground beef) and atmospheres tested. This study supports the use of *C. maltaromaticum* as a bioprotective culture, due to the following attributes: (i) inhibition of spoilage microorganisms; (ii) stable growth under storage conditions until the end of shelf life; (iii) negligible alterations of the physical chemical quality parameters of the products; (iv) extension of products shelf life.

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CAPÍTULO 4

RELEVANCE AND IMPACT

This study provides evidences of the use of *Carnobacterium maltaromaticum* as an effective bioprotective culture *in vitro* and in different chilled meat products against both spoilage and pathogenic bacteria. Thus, *C. maltaromaticum* contributes to increase the microbiological stability and safety of the product. This effect was obtained with negligible impacts on the quality characteristics (color and pH parameters) of the products tested, preventing the meat from significant discoloration and maintaining the pH under acceptable values even in the presence of the spoilage agents.

This research contributes to the use of natural alternatives to the meat industry and consumers that seek for green label and sustainable products with longer shelf life.

Further studies will include the assessment of the probiotic potential of the *C. maltaromaticum* strains aiming their use for both bioprotective and probiotic effects in meat products.

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