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

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ABSTRACT

The bioprotective effects of *Carnobacterium maltaromaticum* (CM) strains were assessed *in vitro* and in sliced cooked ham. CM strains were tested *in vitro* against *Listeria monocytogenes* (LM), *Escherichia coli* O157:H7 (EC) and *Salmonella* Typhimurium (ST). *In vitro* effect was evaluated using co-culture (with and without EDTA) and cell-free supernatant (CFS). CFS was tested by agar well diffusion and minimum inhibitory concentration. In cooked ham, the inhibitory effect of CM on L. *innocua* (LI) and on the physicochemical parameters were evaluated for 7 days at 4 °C. In co-cultures at −1 °C and 4 °C, all CM isolates inhibited LM. A slight inhibition was observed against the Gram-negative bacteria with the addition of EDTA. CFS did not show inhibitory effect under the studied conditions. In cooked ham, CM inhibited LI growth and did not affect the physicochemical parameters of the product during storage. CM strains show potential to be used as bioprotective cultures in cold-stored cooked ham and improve its safety.

1. Introduction

Foodborne disease outbreaks are caused by the ingestion of contaminated food with pathogenic microorganisms. The World Health Organization (WHO) considers foodborne outbreaks as critical threats to global health (WHO, 2015). In the Unites States, an estimated 9 million people get sick, 56,000 are hospitalized, and 1300 die of foodborne disease each year (IFSAC, 2019). *Salmonella* spp. is reported as the cause of 14% of the foodborne diseases in the European Union (EFSA & ECDC, 2018). Other bacteria play an essential role in these outbreaks including *Escherichia coli* O157:H7 and *Listeria monocytogenes*. These three pathogens are associated to the highest level of frequency, severity of ilnnes, hospitalizations and deaths caused by foodborne illness, linked mainly with chicken, pork and beef (EFSA & ECDC, 2018; IFSAC, 2019).

Meat products characteristics such as high protein content, low acidity, and high-water activity make them susceptible to microbial growth (Sánchez-Ortega et al., 2014). According to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (2017) in 2015, meat products were linked to the most critical cases of foodborne outbreaks in the European Union. Moreover, in Brazil, meat and meat products s were responsible for 12.8% of the outbreaks from 2009 to 2018 (Brazil, 2019). However, the frequency is probably higher than that reported due to the lack of notification of foodborne illness when the symptoms are mild, and patients do not seek medical assistance (de Oliveira, de Paula, Cardoso, & Tondo, 2010).

The food industry has largely investigated the potential use of LAB as biopreservatives. Their inhibitory effect against pathogenic and spoilage bacteria (Alves, Martinis, Destro, Vogel, & Gram, 2005; dos Reis et al., 2011; Hammi et al., 2016; Ho, Lo, Bansal, & Turner, 2018; Huang, Ye, Yu, Wang, & Zhou, 2016; Rivas, Castro, Vallejo, Marguet, & Campos, 2014) is an important indicator of their possible use as protective cultures in food matrices, where they could replace some synthetic preservatives (Engelhardt, Albano, Kiskó, Mohácsi-Farkas, & Teixeira, 2015; Huang et al., 2016).

Carnobacteria are ubiquitous lactic acid bacteria (LAB) isolated from cold and temperate environments and can be found as natural

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

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

2. Material and methods

2.1. Bacterial strains, media, and growth conditions

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

Foodborne pathogenic bacteria, *L. monocytogenes* ATCC®19117™ (LM), *E. coli* O157:H7 ATCC®35150™ (EC) and *Salmonella* Typhimurium ATCC®14028™ (ST) were used for the *in vitro* essay, and a pool of *Listeria innocua* (LI_33314, LI_33016 and LI_HPB586), isolated from meat products, belonging to the culture collection of LAPIAgro, was used as surrogate for LM in the cooked ham essay.

CM strains were grown in brain heart infusion (BHI) broth (Kasvi, São José dos Pinhais, Brazil) at 25 °C for 48 h, and pathogenic and LI strains at 37 °C for 24 h in the same medium. Growth was verified by optical density at 540 nm for the pathogenic bacteria and LI and 620 nm for CM (Gutiérrez, Martínez-Blanco, Rodríguez-Aparicio, & Ferrero, 2016).

2.2. In vitro assay

2.2.1. Antimicrobial effect of CM in co-culture

The antimicrobial effect of CM in co-culture, where both CM strains and pathogens were grown together (cell-to-cell contact), was determined inoculating Falcon® flasks with 30 mL of BHI broth with each strain of CM at 6.0 log CFU/mL in order to account for natural contamination levels and allow the enumeration of the inoculum and one of the pathogenic strains (LM, EC, ST) at 3.0 log CFU/mL. Negative controls were considered flasks with BHI inoculated with each pathogen at 3.0 log CFU/mL and positive controls were considered flasks inoculated only with CM strains. Controls and treatments were conducted in triplicate.

Flasks were incubated at −1 °C for 28 days, 4 °C for 14 days and 25 °C for 48 h in a shaker (Rotamax 120, Heidolph, Schwabach, Germany) at 150 rpm. Pathogenic bacteria counts were performed using specific chromogenic media: RAPID'*L.mono,* RAPID'*E.coli* 2 and, RAPID'*Salmonella* (BioRad, Marnes, France). CM population was estimated as the difference between counts on plate count agar (PCA) (BioRad, Marnes, France) and chromogenic media. The plating was also conducted in triplicate.

2.2.2. Antimicrobial effect of CM in co-culture with the addition of EDTA

The influence of the addition of ethylenediaminetetraacetic acid (EDTA) (VWR, Radnor, USA) on the effect of CM against pathogens was investigated, since this chelating agent showed synergestic interaction with bacteriocins from gram-positive bacteria targeting gram-negatives (Mathur et al., 2017). Co-cultures were carried out in flasks containing BHI broth with EDTA 1.0 mM, incubated at 25 °C for 48 h, in a shaker at 150 rpm. Bacterial counts were performed following the same procedure described above. To find the concentration of EDTA that would not interfere in the bacterial growth by itself, a previous experiment was conducted. Serial concentrations of EDTA (1, 5, 10, 20 and 40 mM) were added to the broth medium with each pathogen (LM, EC, ST) (Bordignon-Junior et al., 2012). The concentration that showed no difference in growth compared to negative control, inoculated broth without EDTA, was selected.

2.2.3. Antimicrobial effect of CM cell-free supernatant (CFS) using agar well diffusion

To check if the antimicrobial effect of CM was mediated by the production of antimicrobial molecules in the culture supernatant, three tubes containing 10 mL of BHI broth were inoculated with each CM strain. The tubes were incubated at 25 °C for 48 h and centrifuged (Model Eppendorf Centrifuge 5804, Hamburg, Germany) at 16,000 *g* for 10 min. The supernatant of two tubes was treated with sodium hydroxide (NaOH) 1 M (VWR, Radnor, USA) until pH 6.5 to neutralize antimicrobial effect related to the undissociated form of organic acids potentially produced by CM. Finally, the supernatant of the last tube was filtered through 0.2 μm sterile Minisart syringe filters (Sartorius, Germany), resulting in a cell-free supernatant (CFS). The supernatants were inoculated in wells made in three PCA plates, previously spread with 100 μL of each pathogenic bacterium (LM, EC, ST) at 6.0 log CFU/mL. Four treatments were applied on each plate: (1) sterile BHI broth (blank), (2) centrifuged supernatant, (3) centrifuged supernatant treated with NaOH, and (4) centrifuged supernatant treated with NaOH and filtered (CFS). The halo of inhibition was measured after 48 h of incubation at 37 °C, and all treatments were performed in triplicate.

2.2.4. Minimum inhibitory concentration (MIC) of CM cell-free supernatant (CFS) towards LM

CM_B824, CM_B827 and CM_B829 at 6.0 log CFU/mL were incubated at two different growth conditions (at 25 °C for 48 h and 4 °C for 14 days) in co-culture with and without LM (3.0 log CFU/ mL). The combination of the different parameters resulted in 12 treatments: (1) CM_B824 at 25 °C (2) CM_B824 + LM at 25 °C; (3) CM_B824 at 4 °C, (4) CM_B824 + LM at 4 °C, (5) CM_B827 at 25 °C, (6) CM_B827 + LM at 25 °C; (7) CM_B827 at 4 °C, (8) CM_B827 + LM at 4 °C, (9) CM_B829 at 25 °C, (10) CM_B829 + LM at 25 °C; (11) CM_B829 at 4 °C, (12) CM_B829 + LM at 4 °C.

After co-culture incubation, CFS of different treatments was obtained as described previously. CFS obtained from co-cultures and LM at 6.0 log CFU/mL (10 μL), grown in BHI broth, were inoculated in 24-well plates at different concentrations: 62.5, 50, 37.5 and 18.75%, that were composed by the combination *v*/v of CFS and sterile BHI broth. Plates were incubated at 37 °C for 24 h at 150 rpm, in triplicate. The growth of LM was verified visually. The minimum inhibitory concentration (MIC) was considered as the lowest concentration of the supernatant where no visible growth was observed (CLSI, 2012).

2.3. Cooked ham essay

The meat matrix essay was performed considering the results obtained in the *in vitro* essay. Therefore, the bioprotective effect of CM strains was assessed in a meat matrix stored at refrigeration against *Listeria* sp. A pool of L. *innocua* (LI) was used as surrogate for LM in this

essay.

2.3.1. Effect of CM against LI in sliced cooked ham

Cooked ham was processed by a medium scale producer in Southern Brazil with pork ham muscles (*M. semimembranosus, M. semitendinosus* and *M. biceps femoris)* (4% fat). Muscles were injected with a brine solution (25 g brine/100 g ham meat), containing the following ingredients (g/ Kg): water, 100; salt, 20; sodium tri-polyphosphate, 3.0; sodium pyrophosphate 1.0; $NaNO_2$, 0.130; $NaNO_3$, 0.260; sodium erythorbate, 0.5; monosodium glutamate, 2; natural carmine dye, 0.3. After injection, meat was tumbled under vacuum at 4 °C. After tumbling, the product was stuffed in multilayer shrinkable plastic casings (Schur, Barueri, São Paulo, Brazil) and placed in stainless steel molds (110 mm width, 120 mm height, 230 mm length). The product was then cooked in water bath at 85 °C until core temperature of 72 °C was reached. After cooking the product was pre-cooled in an ice bath until the core temperature was 50 °C and then cooled in a chilling room at 0 °C until reaching 4 °C. After a minimum of 24 h of cooling, the mold was removed, and the product was sliced (dimensions 3 mm × 105 mm width × 115 mm height) and packaged in expanded polystyrene trays and covered with low density polyvinyl chloride film.

Cooked ham slices from the same batch, with a shelf-life of 7 days, were purchased at the producer store. Slices were sterilized at 121 °C for 15 min (Alves, Martinez, Lavrador, & De Martinis, 2006) to avoid the interference of the natural microbiota and chilled overnight at 4 °C.

LI cold adaptation was performed as follows: strains were inoculated in BHI at 37 °C until stationary phase and subsequently cultured in BHI broth at 4 °C until stationary phase, which was confirmed by colony count in PCA agar plates. Equal volumes of each LI strain were mixed in a sterile flask to form a pool at the concentration of 3.0 log CFU/ mL. Then, three slices were distributed into eight treatments: (1) NC (negative control); (2) CM_B824; (3) CM_B827; (4) CM_B829; (5) LI; (6) CM_B824 + LI; (7) CM_B827 + LI; and (8) CM_B829 + LI.

Each side of ham slice was inoculated with 50 μL of the LI pool at 3.0 log CFU/ mL, which was spread evenly with a sterile Drigalski spatula. NC was inoculated with 50 μL 0.1% peptone water. To allow the absorption of the inoculum, the slices were kept in a laminar flow cabinet for 1 h at room temperature. Then, the slices were inoculated with 50 μL of each CM strain at 5.0 log CFU/ mL, except treatment 5 (LI). After 1 h, the slices were packed in sterile expanded polystyrene trays and covered with polyvinyl chloride film (permeability of 1400 cm^3 O_2/ m^2/ 24 h/ 22.8 °C). The trays were stored in a low temperature incubator (Fanem 347 CD, São Paulo, Brazil) at 4 °C for 7 days. Samples were withdrawn at 0, 2, 5 and 7 days of storage for the determination of pH, instrumental color and bacterial counts.

2.3.2. Determination of instrumental color

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

2.3.3. Determination of pH

For pH determination, 3 g of each sample were homogenized in 30 mL of deionized water for 1 min in a stomacher blender (Model Masticator Basic 2000, IUL, Barcelona, Spain). The pH value of the suspension was determined in triplicates using a pH-meter (Model HI 99163, Hanna, Póvoa de Varzim, Portugal) calibrated with buffer solutions at pH 4.0 and 7.0.

2.3.4. Microbiological analysis

For microbiological analysis, the method adopted agreed with normative instruction number 62 from Ministry of Agriculture, Livestock and Food Supply (Brazil, 2003). 10 g of each sample were homogenized with 90 mL of sterilized peptone-water (0.1% *w/v*) (Kasvi, São José dos Pinhais, Brazil) for 2 min using a stomacher blender (Masticator Basic 2000, IUL, Barcelona, Spain). Serial tenfold dilutions were performed, and 100 μL of the selected dilution was inoculated on PCA for CM and PCA overlaid with 10 mL of melted PALCAM agar (Sigma Aldrich, MO, USA) for LI. CM population was estimated as the difference between counts on PCA and PCA overlaid with PALCAM agar. Moreover, the colonies of CM and LI were differentially counted in PCA agar based on colony size (colonies of CM being smaller). The experiment was carried out in duplicate. The plates were incubated at 37 °C for 48 h (LI) and at 25 °C for 48 h (CM).

2.4. Statistical analysis

The *in vitro* effect of CM on the growth of EC, LM and ST at different temperatures (−1 °C, 4 °C and 25 °C) and with the adittion of EDTA at 25 °C was analyzed by one-way ANOVA and means were compared by Tukey test ($P < .05$), using VassarStats online.

Microbiological and physicochemical data obtained from cooked ham essay were analyzed using a random block design, considering a mixed linear model including treatment and storage time as fixed effects and replication as a random effect. Means were compared by Tukey test ($P < .05$). Cooked ham assay was performed two times independently and differences between replicates were not significant ($P < .05$). The microbiological analyses were conducted in duplicate ($n = 4$) and physicochemical analyses in triplicate ($n = 6$). For statistical analysis, bacterial counts with values below the limit of detection were considered as 0.69 log CFU/g. The analysis was performed using the software Statgraphics® Centurion XVI version 16.1.11 (Statpoint Technologies, Warrenton, Virginia, USA).

3. Results and discussion

3.1. In vitro essay

3.1.1. Antimicrobial effect in co-culture

LAB can inhibit spoilage and pathogenic microorganisms by competitive growth and synthesis of antagonistic compounds such as organic acids and bacteriocins (Gómez-Sala et al., 2016). When in co-culture at −1 °C, CM strains were able to reduce the population of LM from 6.6 (control) to < 1.0 (CM_B824), 2.3 (CM_B827), and 1.7 (CM_B829) log CFU/ mL (Fig. 1). However, EC and ST were not inhibited when co-cultured at −1 °C with any of the CM strains (data not shown). Moreover, at 4 °C, all CM isolates inhibited the growth of LM ($P < .05$), showing a count reduction of a least 5.5 log CFU/ mL as compared to LM alone (negative control) (Fig. 2). Still, EC and ST were not inhibited at the condition of 4 °C. The ability of CM, differently from others LAB, to grow under low temperatures (Leisner et al., 2007) allows a competition with other bacteria in this kind of environment.

At 25 °C, CM_B824 and CM_B827 showed a weak but significant inhibition effect towards LM ($P < .05$) when in co-cultures (Fig. 3 E). CM did not show any inhibitory effect when grown in co-culture with EC and ST at 25 °C (Fig. 3 A and 3C). However, when EDTA was added in the co-culture broth, all CM isolates reduced the growth of EC ($P < .05$) (Fig. 3 B), and CM_B824 and CM_B827 inhibited the growth of ST and LM ($P < .05$) (Fig. 3 D and 3F).

As *Carnobacterium* is phylogenetically related to other genera of LAB it is possible to compare these bacteria (Hammes & Hertel, 2006). Other authors also demonstrated the antilisterial activity of LAB. Rivas et al. (2014) showed the antilisterial activity of *Lactobacillus curvatus* and its purified bacteriocin, sakacin Q on cooked meat. Ho et al. (2018) found antilisterial activity in co-cultures of *Lactococcus lactis, Lact. raffinolactis, Leuconostoc mesenteroides, Leuc. pseudomesenteroides, Weissella soli*, and *W. viridescens*. Furthermore, Huang et al. (2016) demonstrated

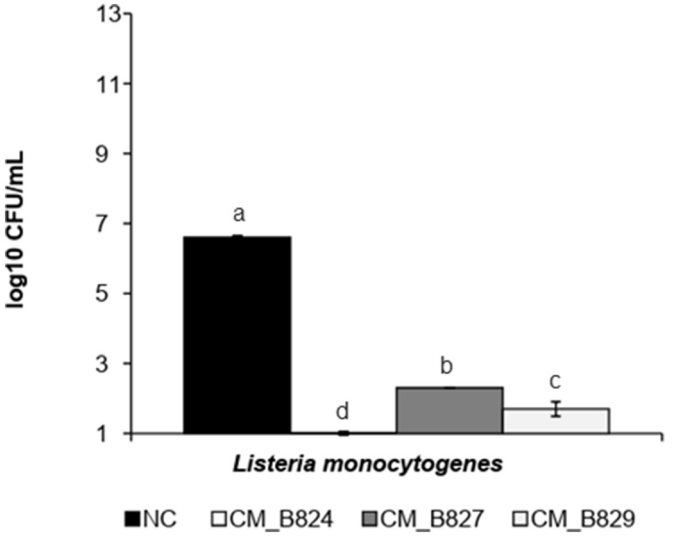

Fig. 1. Count of L. *monocytogenes* at co-culture with *C. maltaromaticum* isolates at −1 °C for 28 d.
NC = negative control; CM = *Carnobacterium maltaromaticum*.
No common superscript indicates that there is a significant difference among treatments ($P < .05$).

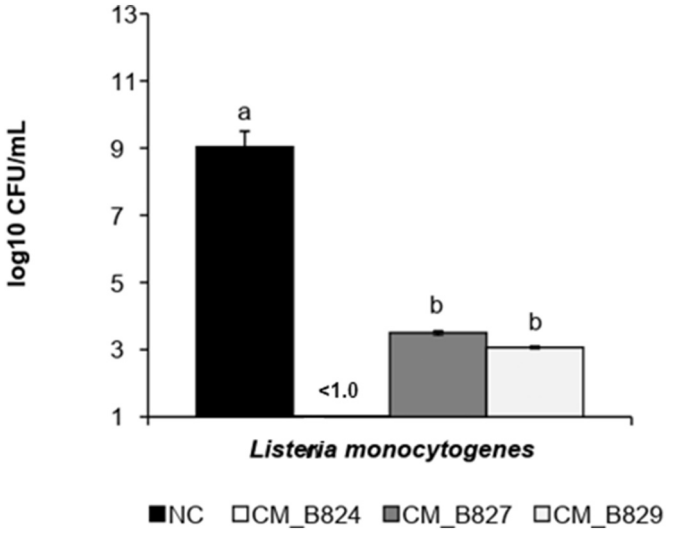

Fig. 2. Count of *L. monocytogenes* at co-culture with *C. maltaromaticum* isolates at 4 °C for 14 d.
NC = negative control; CM = *Carnobacterium maltaromaticum*.
No common superscript indicates that there is a significant difference among treatments ($P < .05$).

the inhibition of LM when in co-culture with *Enterococcus faecium* B1/B2.

Regarding the genus *Carnobacterium,* Alves et al. (2005) reported the antilisterial activity of a strain of CM (formerly *Carnobacterium piscicola*) isolated from Brazilian smoked fish. Dos Reis et al. (2011) also observed the antilisterial activity of CM in fish models. Moreover, Hammi et al. (2016) demonstrated an anti-*Listeria* activity by a new class IIa bacteriocin, termed maltaricin CPN, produced by a CM strain isolated from mold-ripened cheese.

Other authors observed inhibition of Gram-negative pathogens when bacteriocins from Gram-positive bacteria were analyzed in the presence of EDTA (Camargo, de Paula, Todorov, & Nero, 2016; Field et al., 2017; O'Connor, Ross, Hill, & Cotter, 2015; Prudêncio, Vanetti, & Prieto, 2015). A bacteriocin produced by CM UAL307, termed carnocyclin A, showed an antimicrobial activity against Gram-negative bacteria including EC and *Pseudomonas aeruginosa*, when incubated with EDTA (Martin-Visscher, Yoganathan, Sit, Lohans, & Vederas, 2011). Bacterial metabolites can become more effective biopreservatives when used in combination with other hurdles such as chelating agents. This combination is a strategy to increase the activity of the bacteriocins produced by Gram-positive bacteria. As the composition of membranes from Gram-positive bacteria and Gram-negative bacteria are different, multiple approaches for increasing the activity of a bacteriocin or other bacterial metabolites are necessary (Hwanhlem, Ivanova, Haertlé, Jaffrès, & Dousset, 2017). The chelating capacity of EDTA, which acts by removing Mg2+ and Ca2+, promotes the destabilization of the outer membrane from Gram-negative bacteria, allowing metabolites to access the cytoplasmic membrane (Field et al., 2017; Mathur et al., 2017).

3.2. Antimicrobial effect of CFS

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

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

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

Each isolate of each species appears to have an exclusive optimal condition to produce bacteriocins, and these conditions should be determined for each parameter and producer isolate (Masuda, Perez, Zendo, & Sonomoto, 2016; Pérez, González, Agrasar, & Guerra, 2013; Sidooski, Brandelli, Bertoli, Souza, & Carvalho, 2018).

3.3. Cooked ham essay

3.3.1. Microbiological analysis

The use of LAB as protective cultures to improve safety and prolong the shelf life of the meat products, including cooked meat products, is a concept that has been suggested by many authors (Comi, Andyanto, Manzano, & Iacumin, 2016; Metaxopoulos, Mataragas, & Drosinos, 2002; Vermeiren, Devlieghere, & Debevere, 2004).

There was significant interaction between 'treatment' and 'storage time' for the count of CM in cooked ham ($P < .05$) (Table 3). For LI count, there was no interaction between the fixed effects, but there was independent effect of 'treatment' and 'storage time' ($P < .05$).

During storage, the count of CM varied significantly, however,

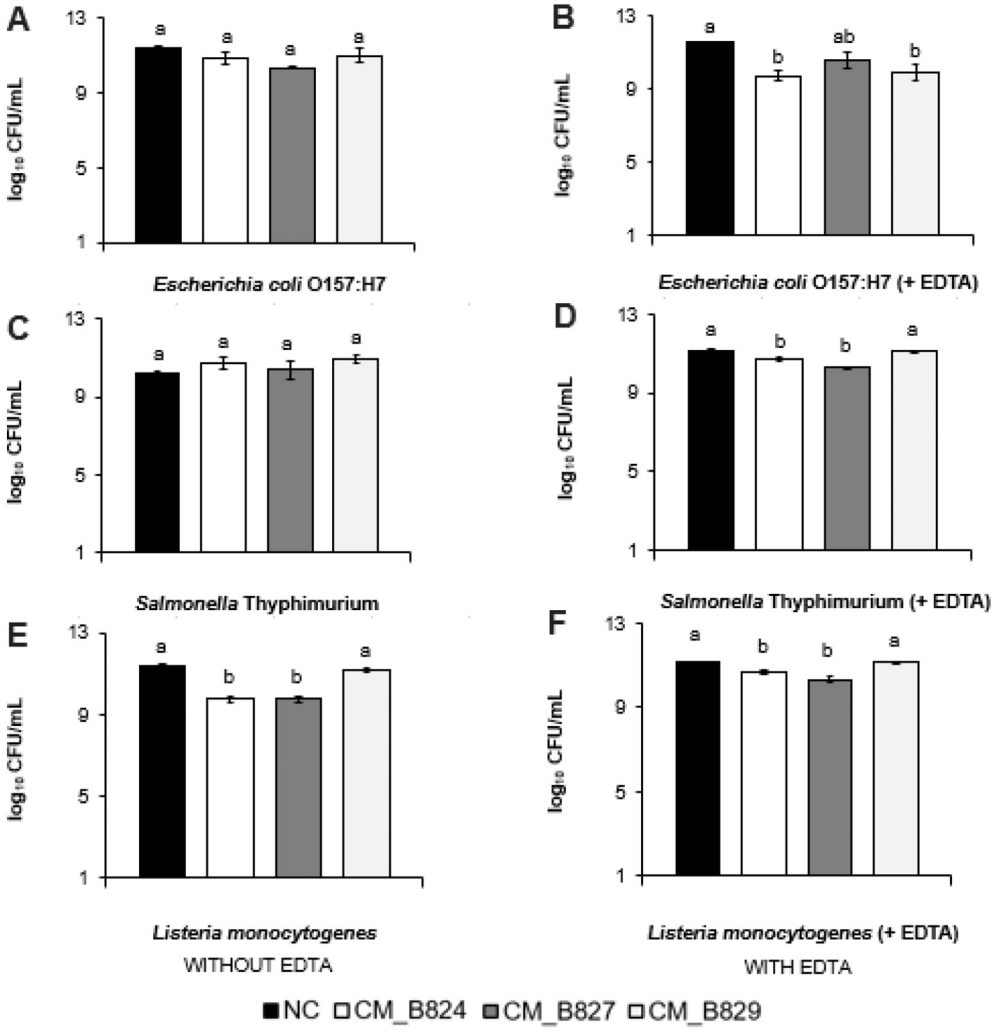

Fig. 3. Count of pathogenic bacteria (*E. coli* O 157:H7 (A-B), *S*. Thyphimurium (C-D), *L. monocytogenes* (*E*-F) in co-culture with *C. maltaromaticum* isolates at 25 °C for 48 h, with and without EDTA.
NC = negative control; CM = *Carnobacterium maltaromaticum.*

count differences were < 0.4 log CFU/g in all CM-treatments from day 1 to day 7 of storage, showing adaptation of CM strains to the cured cooked meat matrix. Conversely, the count of LI in the CM + LI treatments reduced significantly during storage, whereas the count of LI in the LI-treatment did not differ during storage ($P > .05$). At 2 days of storage, the count of LI in the CM + LI treatments decreased to non-detected level (< 0.69 log CFU/g) and the addition of CM_B829 reduced the count of LI > 2.0 log CFU/ g after 7 days of storage. CM_B824 and CM_B827 also caused a significant reduction of LI in ham during storage ($P < .05$). This inhibition of L. *innocua* may be attributed to the greater ability of *Carnobacterium* to grow and to adapt to this food matrix and storage conditions in comparison to *Listeria* (Amézquita; Brashears, 2002). This ability leads to a depletion of nutrients hindering the growth of the pathogen, as well as the occupation of the food matrix before the pathogen (Nilsson et al., 2005; Vermeiren et al., 2004).

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

3.3.2. Color and pH

There were significant interactions between 'treatment' and 'storage time' for pH and instrumental color coordinates of cooked ham ($P < .05$) (Tables 1 and 2). A slight increase in luminosity (L*) and a slight decrease in pH was observed in ham from all treatments during storage. The addition of CM strains in cooked ham did not cause significant changes in the pH of the product, which remained similar to NC and LI during storage. After 7 days of storage, CM_824 + LI showed lower pH than other treatments ($P < .05$), however, the pH values in all treatments were within the normal range for this type of meat product, *i.e.*, 5.6–6.2 (Arnau, Guerrero, Casademont, & Gou, 1995). Gao, Li, and Liu (2015) found that the addition of L. *sakei* C2 in vacuum packed sliced cooked ham decreased the pH value during the storage at refrigerated temperature. Conversely to other aciduric LAB genera such as *Lactobacillus*, *Leuconostoc* and *Pediococcus*, *Carnobacterium* is not a strong acid producer. Even if carbohydrate catabolism by carnobacteria appears to result in a diverse number of metabolites, these have

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

Treament	Storage days				
	0	2	5	7	*P* value
	L*				
NC	60.10 ± 0.66^{abB}	63.05 ± 0.27^{abA}	63.26 ± 0.57^{aA}	62.91 ± 0.62^{aA}	0.0017
LI	60.28 ± 0.71^{abB}	63.65 ± 0.39^{aA}	64.20 ± 0.24^{aA}	65.35 ± 0.94^{aA}	0.0001
CM_824	59.90 ± 0.66^{abB}	63.56 ± 0.23^{aA}	63.83 ± 0.34^{aA}	64.55 ± 0.36^{aA}	0.0000
CM_827	61.04 ± 0.41^{abC}	62.60 ± 0.48^{abBC}	63.52 ± 0.58^{aAB}	64.70 ± 0.59^{aA}	0.0006
CM_829	59.18 ± 0.85^{bB}	62.07 ± 0.47^{abA}	62.83 ± 0.40^{aA}	64.13 ± 0.28^{aA}	0.0000
CM_824 + LI	61.23 ± 0.70^{abA}	60.92 ± 0.59^{bcA}	63.10 ± 0.48^{aA}	62.97 ± 0.81^{aA}	0.0538
CM_827 + LI	61.99 ± 0.54^{abA}	59.71 ± 0.44^{cA}	61.03 ± 1.92^{aA}	63.17 ± 0.29^{aA}	0.1430
CM_829 + LI	62.32 ± 0.61^{aA}	63.11 ± 0.71^{aA}	61.20 ± 0.96^{aA}	63.98 ± 0.44^{aA}	0.4327
P value	0.0208	0.0000	0.1007	0.0454	
	a*				
NC	17.13 ± 1.01^{abA}	15.51 ± 0.32^{aA}	17.33 ± 0.32^{aA}	16.38 ± 0.56^{aA}	0.1822
LI	17.32 ± 0.64^{abA}	16.10 ± 0.54^{aA}	17.40 ± 0.30^{aA}	16.37 ± 0.56^{aA}	0.2248
CM_824	16.84 ± 0.57^{abA}	16.44 ± 0.42^{aA}	17.53 ± 0.42^{aA}	15.73 ± 0.88^{aA}	0.2321
CM_827	17.63 ± 0.57^{aA}	15.34 ± 0.15^{abB}	16.94 ± 0.26^{abA}	14.59 ± 0.27^{aB}	0.0000
CM_829	16.09 ± 0.62^{abAB}	16.73 ± 0.44^{aAB}	17.71 ± 0.43^{aA}	15.88 ± 0.18^{aB}	0.0374
CM_824 + LI	15.17 ± 0.21^{abA}	13.25 ± 0.70^{bB}	16.15 ± 0.50^{abA}	16.43 ± 0.37^{aA}	0.0005
CM_827 + LI	14.81 ± 0.59^{bBC}	13.27 ± 0.62^{bC}	16.79 ± 0.25^{abA}	15.96 ± 0.26^{aAB}	0.0002
CM_829 + LI	15.73 ± 0.63^{abA}	15.58 ± 0.53^{aA}	15.54 ± 0.45^{bA}	14.67 ± 0.31^{aA}	0.3356
P value	0.0159	0.0000	0.0024	0.0349	
	b*				
NC	12.31 ± 0.60^{bcA}	11.25 ± 0.40^{abA}	11.51 ± 0.61^{aA}	11.64 ± 0.34^{aA}	0.5059
LI	13.55 ± 0.65^{bcA}	11.50 ± 0.48^{abA}	11.92 ± 0.50^{aA}	12.73 ± 0.58^{aA}	0.0750
CM_824	10.94 ± 0.16^{cB}	11.75 ± 0.07^{aAB}	11.99 ± 0.62^{aAB}	12.71 ± 0.36^{aA}	0.0246
CM_827	14.56 ± 0.29^{abA}	11.33 ± 0.45^{abB}	11.44 ± 0.49^{aB}	12.04 ± 0.35^{aB}	0.0000
CM_829	17.19 ± 0.99^{aA}	11.59 ± 0.66^{abB}	11.13 ± 0.15^{aB}	12.30 ± 0.25^{aB}	0.0000
CM_824 + LI	11.52 ± 0.46^{cAB}	10.50 ± 0.33^{abB}	12.60 ± 0.34^{aA}	12.40 ± 0.44^{aA}	0.0048
CM_827 + LI	11.76 ± 0.75^{cA}	9.89 ± 0.13^{bB}	12.37 ± 0.31^{aA}	11.61 ± 0.36^{aAB}	0.0052
CM_829 + LI	11.75 ± 0.44^{cA}	12.34 ± 0.39^{aA}	11.60 ± 0.49^{aA}	12.30 ± 0.20^{aA}	0.5228
P value	0.0000	0.0071	0.3624	0.2701	

NC = negative control; CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua*. Means ± standard error. Different lowercase letters in the same column show significant differences among treatments ($P < .05$). Different uppercase letters in the same row show significant differences among storage time ($P < .05$).

generally a limited effect on the sensory attributes of foods (Leisner et al., 2007).

Regarding color parameters, there were small, but significant differences in instrumental color values between treatments during storage. However, those differences are likely due to intrinsic characteristics of the product itself than to the influence of the treatments.

The acceptability of cooked ham by the consumers is strongly related to the color of the product (Lloret, Picouet, Trbojevich, & Fernández, 2016). Changes in the color of a meat product caused by *Carnobacterium* was reported only by Peirson, Guan, and Holley (2003) who observed that a strain of *C. viridans* induced greening in cured bologna. However, this effect was detected only after the opening of the vacuum packages and took 2 days or less at 9 °C and 3 days at 4 °C. Nonetheless, the authors state that representative strains of most known species of *Carnobacterium* failed to cause discoloration in this type of cooked meat product.

As *Carnobacterium* are frequently predominant members of the LAB microbiota of non-spoiled raw meat and processed meat products, including ham and bacon, irrespective of whether products have been stored aerobically, vacuum packaged, or subjected to modified atmospheres, they normally do not cause changes in the physicochemical and sensory characteristics of the product (Li et al., 2018). Additionally, high concentrations of bacteria ($> 10^6$ - 10^7 CFU/g) in food are typically required before their activity is enough to influence the sensory properties of a product (Leisner et al., 2007). Gao et al. (2015) and Comi et al. (2016) reported that *Lactobacillus sakei* and *Lactococcus lactis* affected the growth of spoilage bacteria and L. *monocytogenes* and did not negatively affect the physicochemical properties of sliced cooked ham and cooked bacon, respectively. On the other hand, the addition of C. *maltaromaticum* in meat products as a protective culture has not been assessed. Thus, it could be considered that the addition of the CM strains in the present study did not negatively affect the physicochemical parameters of the product.

The *C. maltaromaticum* isolates used in this study present several properties that are desirable for biopreservative cultures: the isolates were able to growth and the count remained stable in the cooked ham during storage, the isolates did not cause significant changes in pH and color of the product and the fast inhibition of L. *innocua,* at the second day of storage, is another advantage of these isolates.

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

4. Conclusion

The three *C. maltaromaticum* strains tested showed an antilisterial potential *in vitro*, which was more important at −1 °C and 4 °C than at 25 °C. When applied in cooked ham, the antilisterial potential was confirmed, since the growth of *Listeria* spp. was inhibited by the addition of the strains of *C. maltaromaticum*, without affecting the physicochemical quality of the product. Therefore, *C. maltaromaticum* strains show potential to be used as bioprotective culture in cooked meat product to improve its safety.

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

Treatment	Storage days				
	0	2	5	7	*P value*
	C*				
NC	21.15 ± 1.10^{bcdA}	19.18 ± 0.20^{abA}	20.85 ± 0.26^{aA}	20.17 ± 0.33^{abA}	0.1266
LI	22.02 ± 0.71^{abcA}	19.80 ± 0.51^{aB}	21.12 ± 0.36^{aAB}	19.80 ± 0.25^{abB}	0.0100
CM_824	20.08 ± 0.52^{cdA}	20.22 ± 0.34^{aA}	21.25 ± 0.65^{aA}	19.70 ± 0.35^{abA}	0.1630
CM_827	23.23 ± 0.50^{abA}	19.09 ± 0.31^{abB}	20.43 ± 0.47^{aB}	18.92 ± 0.29^{bB}	0.0000
CM_829	24.61 ± 0.71^{aA}	20.37 ± 0.71^{aB}	20.93 ± 0.34^{aB}	20.09 ± 0.15^{abB}	0.0000
CM_824 + LI	18.76 ± 0.47^{dAB}	16.92 ± 0.69^{bcB}	20.51 ± 0.36^{aA}	20.44 ± 0.58B^{aA}	0.0003
CM_827 + LI	18.45 ± 0.26^{dB}	16.58 ± 0.45^{cC}	20.87 ± .026^{aA}	19.76 ± 0.18^{abA}	0.0000
CM_829 + LI	19.63 ± 0.56^{cdA}	19.82 ± 0.57^{aA}	20.00 ± .36^{aA}	19.15 ± 0.29^{abA}	0.6064
P value	0.0000	0.0000	0.4157	0.0335	
	h				
NC	35.77 ± 1.11^{bcA}	35.96 ± 1.42^{aA}	33.55 ± 1.76^{abA}	35.01 ± 1.60^{aA}	0.6609
LI	38.02 ± 1.45^{bcA}	35.55 ± 1.50^{aA}	34.37 ± 1.25^{abA}	40.13 ± 1.90^{aA}	0.0675
CM_824	33.02 ± 0.85^{cB}	35.60 ± 0.74^{aAB}	33.75 ± 1.14^{abB}	40.11 ± 2.09^{aA}	0.0048
CM_827	40.50 ± 1.59^{abA}	36.39 ± 1.09^{aAB}	33.86 ± 0.88^{abB}	39.60 ± 1.07^{aA}	0.0028
CM_829	46.01 ± 1.33^{aA}	34.58 ± 1.06^{aBC}	32.20 ± 0.81^{bC}	37.77 ± 0.77^{aB}	0.0000
CM_824 + LI	37.05 ± 0.92^{bcA}	38.56 ± 1.16^{aA}	38.02 ± 1.37^{aA}	37.00 ± 0.69^{aA}	0.6754
CM_827 + LI	37.18 ± 2.07^{bcA}	36.88 ± 1.55^{aA}	36.35 ± 0.85^{abA}	36.04 ± 1.11^{aA}	0.9463
CM_829 + LI	36.74 ± 0.74^{bcA}	38.22 ± 0.94^{aA}	37.54 ± 1.64^{abA}	39.99 ± 0.70^{aA}	0.2078
P value	0.0000	0.2915	0,0191	0.0461	
	pH				
NC	6.42 ± 0.02^{aA}	5.92 ± 0.02^{aB}	5.70 ± 0.02^{bC}	5.88 ± 0.02^{aB}	0.0000
LI	6.33 ± 0.11^{aA}	5.61 ± 0.01^{cC}	5.72 ± 0.05^{bBC}	5.87 ± 0.02^{aB}	0.0000
CM_824	5.93 ± 0.01^{bA}	5.64 ± 0.02^{cB}	5.61 ± 0.02^{bB}	5.87 ± 0.02^{aA}	0.0000
CM_827	6.46 ± 0.02^{aA}	5.85 ± 0.04^{abB}	5.61 ± 0.01^{bC}	5.89 ± 0.03^{aB}	0.0000
CM_829	6.47 ± 0.02^{aA}	5.94 ± 0.03^{aB}	5.62 ± 0.01^{bC}	5.93 ± 0.03^{aB}	0.0000
CM_824 + LI	5.83 ± 0.01^{bB}	5.93 ± 0.02^{aA}	5.89 ± 0.02^{aAB}	5.66 ± 0.02^{bC}	0.0000
CM_827 + LI	5.82 ± 0.01^{bAB}	5.94 ± 0.01^{aA}	5.90 ± 0.02^{aAB}	5.80 ± 0.06^{aB}	0.0196
CM_829 + LI	5.85 ± 0.03^{bA}	5.76 ± 0.07^{bcA}	5.90 ± 0.02^{aA}	5.91 ± 0.02^{aA}	0.0649
P	0.0000	0.0000	0.0000	0.0000	

NC = negative control; CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua*. Means ± standard error. Different lowercase letters in the same column show significant differences among treatments ($P < .05$). Different uppercase letters in the same row show significant differences among storage time ($P < .05$).

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

Treatment	Storage days				
	0	2	5	7	*P*
	Count of *C. maltaromaticum* (log CFU/g)				
CM_B824	5.60 ± 0.02^{aA}	5.60 ± 0.02^{aAB}	5.26 ± 0.02^{abC}	5.33 ± 0.03^{aBC}	0.0120
CM_B827	5.42 ± 0.07^{aA}	5.41 ± 0.07^{aA}	5.08 ± 0.03^{bA}	5.40 ± 0.05^{aA}	0.0034
CM_B829	5.49 ± 0.05^{aA}	5.48 ± 0.05^{aA}	5.34 ± 0.08^{aA}	5.35 ± 0.03^{aA}	0.2163
CM_B824 + LI	4.75 ± 0.00^{bA}	4.75 ± 0.00^{bA}	4.64 ± 0.01^{cB}	4.31 ± 0.01^{cC}	0.0000
CM_B827 + LI	4.50 ± 0.14^{bA}	4.49 ± 0.13^{bA}	4.60 ± 0.01^{cA}	4.34 ± 0.02^{cA}	0.2174
CM_B829 + LI	4.46 ± 0.03^{bB}	4.46 ± 0.03^{bB}	4.71 ± 0.05^{cA}	4.54 ± 0.03^{bAB}	0.0222
P	0.0000	0.0002	0.0056	0.0075	
	Count of L. *innocua* (log CFU/g)				
LI	3.06 ± 0.02^{aA}	2.60 ± 0.11^{aA}	2.59 ± 0.50^{aA}	2.40 ± 0.09^{aA}	0.1190
CM_B824 + LI	2.15 ± 0.15^{bB}	ND^{bB}	ND ^{bB}	1.350 ± 0.50^{bAB}	0.0355
CM_B827 + LI	2.00 ± 0.00^{bB}	ND^{bB}	ND ^{bB}	ND^{bB}	0.0298
CM_B829 + LI	2.15 ± 0.15^{bB}	ND^{bB}	1.50 ± 0.50^{bAB}	ND^{bB}	0.0079
P	0.0067	0.0004	0.0042	0.0133	

CM = *Carnobacterium maltaromaticum*; LI – *Listeria innocua.*
ND = non-detected (limit of detection = 0.69 log CFU/g).
Means ± standard error. 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 ($P < .05$).

Declaration of Competing Interest

The *authors declare that there is no conflict of interest.* This statement is to certify that all authors have seen and approved the manuscript being submitted.

We warrant that the article is the authors' original work. We warrant that the article is not under consideration for publication elsewhere.

On behalf of all co-authors, the corresponding author shall bear full responsibility for the submission.

We attest to the fact that all authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to **Meat Science**.

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