Application of *Carnobacterium maltaromaticum* as a feed additive for weaned rabbits to improve meat microbial quality and safety

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ABSTRACT

This study addresses the improvement of meat microbial quality by enriching the diet of farm animals with a protective culture. Weaned Grimaud rabbits were divided into two experimental groups: a control and a diet supplemented with Micocin® (*Carnobacterium maltaromaticum* CB1; 8 Log_{10} CFU/kg of feed). Overall, meat quality was not affected substantially by the treatment. Total Aerobic Mesophilic (TAM), *Escherichia coli* and other coliforms, *Enterobacteriaceae*, *Staphylococcus aureus*, *Pseudomonas* spp., *Listeria* spp. and presumptive lactic acid bacteria counts were evaluated on whole thighs stored under aerobic (0, 3, 6, 8 days) and anaerobic (0, 5, 10, 15, 20 days) conditions at 4 °C. The results demonstrated that the microflora on refrigerated thighs was modulated by the addition of Micocin® ($P < 0.05$) and that the most effective reduction of *Listeria monocytogenes* growth was observed with ground meat stored under anaerobic conditions at 4 °C with a 2 Log difference at the end of a 15-day storage ($P = 0.025$).

Keywords: *Carnobacterium maltaromaticum* CB1; *Listeria monocytogenes;* meat contamination; meat safety; rabbit meat; shelf life.

1. Introduction

Nowadays, importance of healthy foods, including meat, continues to be a concern for the consumer (Fread, 2015). Rabbit meat often stands for its healthier characteristics due to its higher protein content, low unsaturated fats, richer in polyunsaturated ones, absence of uric acid and purines, compared to pork or beef meat (Dalle-Zotte, 2004; Ramírez et al., 2005, Hernández, 2006; Nistor et al., 2013). However, its annual consumption remains limited worldwide to 0.30 kg per capita (Gidenne, 2006) in comparison to beef (6.4 kg), pork (12.5 kg) and poultry (13.5 kg, OECD, 2015). According to the Codex Alimentarius Commission (CAC, 2005) and the FAO (2005), meat is traditionally viewed as a potential vehicle for the transmission of foodborne disease with *Campylobacter* spp., *Salmonella enterica* serotypes, *Listeria monocytogenes* and *Escherichia coli* being the most frequently reported culprits (Newell et al., 2010). Meat is the most frequently implicated food in Canada, and fish in the USA (Bélanger et al., 2015). Foodborne diseases have economic consequences evaluated at 3.7 billion $CAN (PHAC, 2012a) and 10-83 billion $USD (Nyachua, 2010) per year in Canada and the USA, respectively, whereas in the European Union, 3 billion € is accounted for annually for *Salmonella* infections alone (DeWaal, & Robert, 2005). Even when meat is produced under strict hygienic conditions, surface contamination by spoilage and pathogenic microorganisms is to be expected. Even healthy animals may constitute a reservoir for foodborne pathogens (PHAC, 2012b). Therefore, new strategies must be investigated for microbial control as the use of chemical additives is no longer a viable option in terms of consumers' demands (Ricke, 2003). More natural interventions have been widely studied by the food processing industry including lactic acid bacteria (LAB), which act as protective cultures in functional meat (Vamanu, & Vamanu, 2010). Some of them improved shelf life during

food and meat storage and it is due, at least in part, to the production of inhibitory substances such as organic acids, ethanol, diacetyl, bacteriocins and hydrogen peroxide (Kandler, & Weiss, 1986) that limit the growth of other organisms, including pathogens (Leroy, & De Vuyst, 2004; Castellano et al., 2008). In the meat industry, the prevalence of LAB is achieved through a competitive exclusion to extend the shelf life of meats notably under modified atmosphere packaging (Saucier, 1999).

Micocin® is a dry-formulated live culture of *Carnobacterium maltaromaticum* CB1 which produces bacteriocins and other antimicrobial metabolites. It was designed to be used for ready-to-eat meats where this LAB species forms a major part of the microbial population. It has been approved for use in Canada, Mexico, Costa Rica, Colombia and the United States (Health Canada, 2010; Marketwire, 2011). It has the ability to control the growth of spoilage and pathogenic bacteria during the storage of vacuum packaged meat products (Goktepe, 2006; Gálvez et al., 2008). *C. maltaromaticum* is an atypical heterofermentative, tolerant to freezing, thawing, high pressure and it can grow at temperatures as low as 0 °C (Caplice, & Fitzgerald, 1999; Hammes, & Hertel, 2003; Leisner et al., 2007). Strain CB1 produces three bacteriocins: carnocyclin A, piscicolin 126 and carnobacteriocin BM1, which have been proven to be effective to inhibit the growth of *Enterococcus faecalis, E. faecium, Pediococcus acidilactici, C. divergens, Lactococcus lactis* spp. *lactis, Lactobacillus curvatus, Lb. casei, Leuconostoc gelidum, Staphylococcus aureus, Clostridium botulinum,* and more particularly, *L. monocytogenes* (Laursen et al., 2005; Casaburi et al., 2011; Gonzalez, Yien, & Castrillon, 2013).

LAB have been successfully used in feed, as a probiotic supplement improving notably gastrointestinal health of the animal ingesting it (Collins, & Gibson, 1999). Studies in rabbits have shown reduced gut colonization of *E. coli* and other enteric

100 pathogens, higher average daily weight gain, better feed conversion ratio and enhanced
101 absorption of the intestinal mucosa (Kritas et al., 2008; Coperland et al., 2009; Ezema,
102 & Eze, 2012; Seyidoglu, & Peker, 2015). However, to our knowledge, no studies have
103 investigated the effect of such probiotic feed additives with respect to meat quality and
104 safety. Therefore, the aim of this study was to demonstrate that the use of a positive
105 microflora, such as Micocin®, as a feed additive in rabbit rations, can modulate carcass
106 contamination in order to improve meat microbial quality and safety.

107 **2. Materials and Methods**

108 *2.1. Animal housing and feeding*

109 Animal care and handling procedures were approved by Université Laval's Animal
110 Use and Care Committee, which strictly adheres to the Guidelines of the Canadian
111 Council on Animal Care (CCAC, 2009). A total of 144, 35-day-old weaned female
112 Grimaud breed rabbits were obtained from a commercial farm (Laprodéo, Saint-Tite,
113 Quebec, Canada) and were maintained in conventional commercial cages. Rabbits were
114 individually weighted upon arrival and assigned immediately either to the experimental
115 or the control group. Rabbits were placed six per cage (0.37 m^2 per rabbit) in order to
116 have homogeneous weight per cage and within groups; the cage constituted the
117 experimental unit. Twelve cages were analyzed per experimental group. In order to
118 make sure that the control group does not get contaminated by the microbial culture
119 (Micocin®, Griffith Foods, Toronto, ON, Canada) given to the experimental one, the
120 animals had to be housed in two different but similar rooms and strict biosecurity
121 measures were observed. On a daily basis, control group were always visited first and
122 the personnel changed clothes, mask, hair net and gloves between each group. If the
123 control group needed to be revisited, personnel had to shower first. A cycle of 12 h of

124 light (starting at 9:00 am) and 12 h of dark was used throughout the experiment,
125 temperature was at 20.1 ± 0.4 °C and humidity level at 33 ± 4 %.

126 The experimental group was fed the ration supplemented with the protective culture
127 Micocin® containing *C. maltaromaticum* CB1 at a final concentration of 8 Log_{10} CFU
128 (Colony-Forming Unit) per kg of feed. Micocin® was provided to us as a concentrate
129 containing 10 Log_{10} CFU/g which was added during the commercial pelleting process
130 (Table 1). Feed was manufactured in a commercial facility in separate 600 kg batches
131 (Belisle Solution Nutrition, St-Mathias-sur-Richelieu, Quebec, Canada). The feed
132 supplemented with Micocin® was manufactured last to avoid contaminating the
133 equipment. Animals were fed *ad libitum* until a minimal target slaughter weight of
134 2,200 g was reached, which took 21 to 28 days. They were weighed and the feed intake
135 was measured weekly during the experimental period to determine body weight (BW),
136 average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio
137 (FCR).

138 To make sure truck and slaughter line was not contaminated by
139 *C. maltaromaticum* CB1, the two groups had to be slaughtered on two different days,
140 the one without supplement first, to avoid cross contamination. They were fasted 15 h
141 before slaughter, including transport and lairage time, according to the current
142 commercial practices to reduce transport-related sickness (Bianchi et al., 2008). They
143 had access to water at all times prior to transport. The length of transport to the abattoir
144 was 30 min, and animals were allowed a waiting period of 30 min before slaughter.
145 They were the first rabbits to be slaughtered at those two dates in order to standardize
146 contamination coming from the slaughter house. Animals were slaughtered in a
147 provincially inspected establishment according to regulations in Quebec, Canada
148 (DGSAIA, 2011).

149 *2.2. Meat quality measurement*

150 For meat quality measurement, one rabbit per cage was randomly analyzed. The
151 muscular pH of the *Biceps femoris* (BF) and the *Longissimus lumborum* (LL) muscles
152 were measured *post-mortem* after 1 (pH 1) and 24 h (pHu; Blasco, & Ouhayoun, 1996)
153 using a portable pH meter (ROSS, Orion Star A221, Thermo Scientific, Beverly, CA,
154 USA) combined with an Orion Kniphe electrode (ThermoFisher, Nepean, ON, Canada)
155 and a temperature compensation probe (928 007 MD, micro probes ATC, Maryland,
156 USA). Meat colour was evaluated 24 h after slaughter on the LL and the exposed
157 surface of the BF using a Chromameter (Chromameter CR 300 Minolta Ltd., Osaka,
158 Japan) equipped with a D65 light source and a 0° viewing angle geometry according to
159 the reflectance coordinates (L*, a*, b*; CIE, 1976), after exposing the muscle surface
160 for 20 min blooming time (Faucitano, Chevillon, & Ellis, 2010). Meat exudate lost (%)
161 during cold storage was measured by weight difference of the thighs. Regarding drip
162 loss, the measure was taken from a piece of *Longissimus thoracis et lumborum* muscle
163 (LTL about 2 cm thick x 2.5 cm in diameter) also by weight difference, according to the
164 EZ-Driploss method (Rasmussen, & Anderson, 1996), where samples are stored at 4 °C
165 for 48 hours. Cooking loss was determined on a similar piece of LTL muscle (Pla,
166 1999) and is expressed as a percentage of the initial weight loss. Each sample was
167 placed into an 18 oz Whirl-Pak bag (Nasco Whirl-Pak®, USA) and immersed in a water
168 bath at 70 °C for 15 minutes after removing the air from the bag. The samples were then
169 removed from the bag, patted dry with filter paper and weighed (Vergara, Berruga, &
170 Linares, 2005; Apata et al., 2012).

171 *2.3. Muscle Sampling*

172 One leg per animal was packaged aerobically in a styrofoam tray
173 (14*w* x 24*l* x 4.5*h* cm) with an absorbent pad, sealed with an oxygen-permeable

polyethylene film (35 ga; oxygen transmission 825 cc/100 sq. in. per 24 h at 23 °C; water vapor transmission rate 24 g/100 sq. in. per 24 h at 38 °C and 90% RH) obtained from a local food equipment distributor (Emballage L. Boucher, Quebec, QC, Canada) and stored at 4 °C for 0, 3, 6 or 8 days. The other leg was vacuum packaged (Sipromac, St-Germain, QC, Canada) in bags (nylon [23%] and polyethylene [77%; seven multilayered] of 300 ga; oxygen transmission 3.3 cc/100 sq. in. per 24 h at 23 °C; water vapor transmission rate 0.5 g/100 sq. in. per 24 h at 38 °C and 90% RH; Sealed Air Co, Mississauga, ON, Canada) and also stored at 4 °C for 0, 5, 10, 15 or 20 days. The rest of the carcass was deboned and the meat was ground (Electric meat Grinder, No RE50255, IPNO IPXI, China) and stored at -30 °C.

2.4. Proximate analysis

Samples (100 g) were lyophilized (freeze dryer Model 6203-3005-OL, Virtis Co., Gardiner, NY, USA) for 7 days. The fat content was measured using a Tecator extraction unit (Soxtec system HT 1043, Hoganas, Sweden) by the procedure 991.36 of the Association of Official Analytical Chemists (AOAC, 1995). Total proteins were quantified using the procedure 992.15 of the AOAC (1995) with a protein analyzer LECO® (model FP-2000, Leco Corp., St. Joseph, MO, USA). Fat and protein contents are expressed on the wet weight basis and the analysis was performed in triplicate.

2.5. Determination of muscle antioxidant status

2.5.1. Total phenol content

Total phenol content was measured using the method of Jang et al. (2008). Each raw ground meat sample (5 g) was homogenized in distilled water (15 ml) and chloroform (9 ml) and then centrifuged at $3000 \times g$ for 5 min at room temperature (21 °C). Chloroform was added to remove the lipids. The total phenol content in the aqueous supernatant was estimated by the Folin-Ciocalteu method (Subramanian,

199 Padmanaban, & Sarma, 1965). Diluted sample aliquots of 1 ml (1:4, v/v) were added to
200 2N Folin-Ciocalteu's phenol reagent (500 ml; Sigma-Aldrich, St. Louis, MO, USA)
201 followed by addition of 10% $NaCO_3$ (1 ml). Reaction mixture was vortexed and the
202 absorbance was measured with a spectrophotometer (Varioskan™ Microplate
203 instrumentation Thermo Electron Corporation, Vantaa, Finland) at 700 nm after
204 incubating for 1 h at room temperature (21 °C). Quantification was based on a standard
205 curve generated with gallic acid. The results are expressed in GAE (gallic
206 acid equivalent per g of meat, µg GAE/g). All measurements were performed in
207 triplicate.

208 *2.5.2. Lipid oxidation*

209 Lipid oxidation products were measured in ground meat stored at -30 °C,
210 quantitated using the thiobarbituric acid reactive substances (TBARS) method and are
211 expressed as malondialdehyde (MDA) equivalents according to the method of Ermis et
212 al. (2005) with the following modifications. Briefly, 10 g of minced meat was
213 homogenized with 10 ml of Phosphate Buffered Saline solution (PBS, Sigma-Aldrich,
214 St. Louis, MO, USA). After centrifugation (3,000 × g for 15 min at 4 °C), 12.5 µl of
215 butylated hydroxytoluene (BHT) solution was added to 500 µl of supernatant and
216 vortexed. Then, 250 µl of trichloroacetic acid (TCA) was added to the mixture and
217 placed on ice for 30 min. After centrifugation (3000 × g for 10 min at 4 °C), 500 µl of
218 the supernatant was added to 37.5 µl of ethylenediaminetetraacetic (EDTA) and 125 µl
219 of thiobarbituric acid in 0.05 N NaOH followed by 15 min in boiling water (100 °C) to
220 allow the colour reaction to develop. After heating, the samples were cooled at room
221 temperature (5 min) and centrifuged for 10 min at 3,000 × g and 4 °C. Absorbance
222 (100 µl) was measured at 530 nm using a spectrophotometer (Varioskan™). The results

223 are expressed in nanomoles of MDA per g of meat. Measures were performed in
224 triplicate for each meat sample.

225 *2.5.3. Carbonyl content*

226 Protein carbonyl groups were evaluated on 5 g of ground meat using an assay kit
227 from Cayman Chemical Company (Item No. 10005020, Ann Arbor, MI, USA). Nucleic
228 acids were removed according to the manufacturer's instructions. Absorbance was
229 measured at 370 nm (Varioskan^{TM}) and the results are expressed as nanomoles of
230 2.4-dinitrophenylhydrazine (DNPH) fixed per mg of protein. All measurements were
231 performed in triplicate.

232 *2.6. Microbial Analysis*

233 For microbial enumeration on the thighs, a sampling procedure similar to the one for
234 whole poultry carcasses described by Brichta-Harhay et al. (2007) was used. One leg
235 from the five remaining rabbits per cage was randomly taken at each sampling time.
236 Each cage was sampled at every sampling time and conditions (aerobic and anaerobic).
237 Thigh was aseptically placed in a sterile Stomacher bag (Stomacher® 400C, Seward
238 Laboratory Systems Inc., London, UK), weighted (measure was also used to evaluate
239 meat exudate in section 2.2) and sealed after 300 ml of 0.1% (wt/vol) peptone water
240 were added (Bacto peptone, Difco Laboratories, Inc., Detroit, MI, USA). The bag was
241 placed on a rotary shaker (Boekel Scientific Orbitron Rotator II, model 260250, New
242 York, USA) for one minute on each side and then manually massaged for 30 sec to
243 remove microorganisms from the surface. When ground meat was analyzed, 25 g was
244 homogenized in 225 ml of peptone water for 2 min at 230 rpm in a stomacher
245 (Stomacher® 400 circulator, Seward, England). Ten-fold dilutions were carried out in
246 0.1% peptone water for enumeration on appropriate agar plates (Saucier, Gendron, &

247 Gariépy, 2000). Total Aerobic Mesophilic (TAM) counts were performed on Plate
248 Count Agar medium (PCA; Difco Laboratories Inc.) incubated at 35 °C for 48 h
249 (MFHPB-18; Health Canada, 2001). Presumptive Lactic Acid Bacteria (LAB) were
250 enumerated on deMan, Rogosa and Sharp (MRS; Difco Laboratories Inc.; Saucier,
251 Gendron, & Gariépy, 2000) and on All Purpose Tween (APT; Difco of Becton,
252 Dickinson) agar plates since *Carnobacterium* is not particularly acid-tolerant and grow
253 poorly on MRS. The plates were incubated anaerobically for 48 h at 25 °C using
254 anaerobic jars with an envelope generator of H_2 and CO_2 (AnaeroGen^{TM}2.5L,
255 AN0025A, Oxoid Company, Nepean, ON, Canada). Presumptive *Pseudomonas* spp.
256 were determined on Cetrimide-Fucidin-Cephalosporin (CFC) agar (supplement
257 No.SR0103E, Oxoid) and plates were incubated at 25 °C for 48 h (Mead, & Adams,
258 1977; Gill, & Greer, 1993). Coliform and *E. coli* counts were determined using 3M
259 Petrifilm^{TM} plates after incubation at 35 °C for 18-24 h (MFHPB-34; Health Canada,
260 2013). Presumptive *S. aureus* strains were evaluated on 3M Petrifilm^{TM} plates incubated
261 at 37 °C for 26 h (MFLP-21; Health Canada, 2004). *Enterobacteriaceae* counts were
262 performed on 3M Petrifilm^{TM} (MFLP-09; Health Canada, 2007) after incubation at
263 37 °C for 24 h. Presumptive *Listeria* spp. were determined on PALCAM medium
264 (PALCAM *Listeria* Agars Base; Merck, Germany) without supplements, while plates
265 were incubated at 30 °C for 48 h. Regarding *L. monocytogenes,* counts were performed
266 using PALCAM *Listeria* selective supplement (No. 1. 12122.001; EMD, NJ, USA),
267 plates were put in a 30 °C incubator for 48 h (MFHPB-30; Health Canada, 2011).
268 Measurements were performed in duplicate. All bacterial counts were transformed to a
269 Log_{10} value of colony-forming units per gram of thigh weight (Log_{10} CFU/g) prior to
270 statistical analysis according to Gill (2000). Except for presumptive *S. aureus,* coliform,
271 *E. coli*, *Enterobacteriaceae* counts, which were transformed to a Log_{10} value of colony

272 forming units per ten grams of thigh weight (Log_{10} CFU/10g). For counts on PCA,
273 MRS, APT, CFC and Palcam, detection level was 1.76 Log_{10} CFU/10g, and
274 1.32 Log_{10} CFU/10g for presumptive *S. aureus,* coliforms, *E. coli* and
275 *Enterobacteriaceae* counts.

276 Microbial analysis was also performed on the faeces during the feeding period. They
277 were collected (500 g) from the pan underneath the 12 cages and were analyzed once a
278 week for the presence of *C. maltaromaticum* CB1 and enumeration of TAM,
279 presumptive LAB on MRS and APT, coliforms and *E. coli*, and *Enterobacteriaceae* as
280 described above. The samples were stored at 4 °C and were analyzed within 24 h. A
281 25 g sample of faeces was homogenized in 225 ml of peptone water and dilution plated
282 on appropriate media similarly to ground meat described above.

283 *2.7. Experimental inoculation of ground meat with L. monocytogenes*

284 *2.7.1. Bacterial cultures and growth conditions*

285 A cocktail of five *L. monocytogenes* strains, namely 1043 (1/2a), 2371, 2558 (1/2b),
286 2739, 2812 (1/2a), were used in this study. They were all isolated from meat products
287 and kindly provided by Health Canada (Ottawa, ON, Canada). Stock cultures were
288 stored at -80 °C in Brain Heart Infusion (BHI; BBL-Becton Dickinson, Mississauga,
289 Ontario, Canada) supplemented with 20% glycerol (FisherBiotech, Fairlawn, NJ, USA).
290 Prior to experimental use, working cultures were individually thawed and subcultured
291 (1%) daily in BHI broth for a minimum of two and a maximum of seven consecutive
292 days. Cultures were incubated at 30 °C for 24 h. *L. monocytogenes* inoculum was
293 prepared by mixing equal volume of strains grown separately to stationary phase. Cell
294 suspensions were harvested by centrifugation (5,000 x g for 10 min at 4 °C), washed
295 ones and resuspended in 12.5 ml of peptone water. Cell suspension was diluted a 100

296 fold and meat was inoculated with 100 µL in order to obtain a final concentration of
297 4 Log_{10} CFU/g of meat.

298 *2.7.2. Ground meat inoculation and incubation*

299 A total of four experimental ground meat groups were analyzed: uninoculated meat
300 from rabbit fed (1) the control ration without *C. maltaromaticum* and (2) from rabbit fed
301 with the ration supplemented with *C. maltaromaticum*; (3) *L. monocytogenes* inoculated
302 meat from rabbit fed the control ration without *C. maltaromaticum* and (4) from rabbit
303 fed with the ration supplemented with *C. maltaromaticum.* The control groups, not
304 inoculated with *L. monocytogenes,* were followed as well to study the effect of
305 *C. maltaromaticum* on indigenous microflora found in ground meat. It was placed in a
306 household mixer (KitchenAid®, Artisan®, Michigan, USA) and appropriate volumes of
307 the *L. monocytogenes* cocktail were added and mixed for 4 min; peptone water was used
308 for the none inoculated groups. The meat was then divided into thin layers of 25 g
309 samples and was packaged under aerobic conditions in sterile laboratories plastic bags
310 (Whirl-Pak®, B01009, Nasco, USA) or was vacuum packaged as described above, but
311 in smaller bags. Cell enumeration was performed after 0, 3, 6, 9, 12 and 15 days of
312 storage at 4 and 10 °C. Ground meat samples were analyzed as described above in
313 section 2.6.

314 *2.8. Presence of C. maltaromaticum CB1 on faeces, thighs and ground meat*

315 *2.8.1. Growth and culture conditions for indicator strains and bacteriocin production*

316 For use in these experiments, stock frozen cultures in 20% glycerol were subcultured
317 in 9 ml of APT broth incubated at 25 °C for *Carnobacterium* strains and MRS broth
318 incubated at 37 °C for *Pediococcus acidilactici* UL5. *P. acidilactici* UL5 and
319 *C. divergens* were used as indicator strains for the detection of bacteriocin production

by *C. maltaromaticum* CB1. *P. acidilactici* was kindly provided by the Department of Food Science, Université Laval. *Carnobacterium divergens* LV13 was obtained from Dr. B.G. Shaw (Institute of Food Research, Langford, Bristol, UK; culture is available from National Collection of Food Bacteria as strain 2855) and incubated at 25 °C for 24 h in anaerobiosis as described for the presumptive LAB enumeration in section 2.6. Strains were subcultured (1%) daily for a minimum of two and a maximum of seven consecutive days.

To determine presence and prevalence of *C. maltaromaticum* CB1 on thighs and in faeces, characteristic colonies from APT enumeration plates were subcultured in 1 ml of APT broth and incubated as described above. A 100 µl aliquot of each of those cultures were placed in U-bottom 96-well microtiter plates (Greiner bio-one CELLSTAR® 96 Well plate, VWR International, Alberta, CA). Using a 48-pin Microplate Replicator (2.54 cm Pin Length, V&P Scientific, San Diego, CA), aliquots were transferred onto APT plates and were let to dry under a biosafety cabinet. For early detection of bacteriocin production by *C. maltaromaticum,* a soft APT agar (7.5 ml and 7.5% agar) inoculated (1%) with the indicator organism was poured on those replicated plates (Ahn, & Stiles, 1990). They were then incubated at 25 °C under anaerobiosis as described for the presumptive LAB enumeration. Cultures with zones of inhibition were further characterized for detection of the carnocyclin gene.

2.8.2. Molecular characterization of C. maltaromaticum CB1

For faeces and thighs, selected strains exhibiting zones of inhibition were grown in 10 ml of APT broth and incubated for 24 h at 25 °C. Isolation of total DNA was performed from $2x10^9$ CFU of bacterial culture. For ground meat, a 25 g sample of minced beef was placed in a sterile stomacher bag with a filter membrane and was then homogenized in 225 ml of peptone water as for cell enumeration described above. The

liquid phase was transferred into four sterile tubes of 50 ml and placed at -20 °C for 15 min to promote the separation of fat from the meat. Using a sterile swab, the floating fat was removed from the liquid surface. Tubes were centrifuged at 15,000 g for 10 min at 4 °C. After discarding the supernatant, the pellets were stored at -20 °C and gene detection was performed on a loopful of each re-suspended in 1 mL of APT.

DNA extraction was performed using Dneasy blood and tissue kit (#69504, Qiagen, Toronto, Ontario, Canada) by following the protocol for Gram-positive bacteria according to the manufacturer's instructions. DNA purity and quantity were verified by a Nanodrop 2000 (Thermo Scientific, Wilmington, USA). The oligonucleotide primers used for the Polymerase Chain Reaction (PCR) were obtained from Integrated DNA Technologies (IDT, Iowa, USA; Table 2). Presence of *C. maltaromaticum* was determined by using three genes (Saucier et al., 2016). The *16S* DNA region, specific for *C. maltaromaticum* and *C. gallinarum,* was amplified with the primer set 27F and 16S-cpg. Interspacer region (ISR) primers are targeting a specific region of *C. maltaromaticum* located between the *16S* rDNA and *23S* rDNA. The amplification of carnocyclin A, (CclA; circular bacteriocin produced by *C. maltaromaticum*) was performed using the primers CclA-F and CclA-R. All polymerase chain reactions were performed in 25 µL reaction using a maximum of 8 µL DNA samples; primers are described in Table 2. PCR products were analyzed for each experiment by electrophoresis in a 2% (wt/vol) agarose gel (Life Technologies, catalog #15510-027; Table 2).

2.9. Statistical analysis

To determine the effect of treatment, time, and their interactions on the microbiological aspect of the study, data were assessed by an analysis of the variance (ANOVA) using the MIXED procedure of SAS software. The linear and quadratic

effects of time were determined by polynomial contrasts. With respect to data on ground meat, the temperature was added as the third effect with treatment and time. The two treatments were analyzed independently to determine the overall effect of supplementation with Micocin® versus the control one. For these analyzes, time of storage under aerobic conditions (0, 3, 6 and 8 d) and anaerobic conditions (0, 5, 10, 15 and 20 d) was taken into consideration (SAS Institute, Inc. 2002). Significant difference was declared at $P < 0.05$ and a tendency was declared at $P < 0.10$.

3. Results

3.1. Growth performance

Overall, there are no interaction and statistical differences on rabbit growth performances with respect to average daily weight gain, average daily feed intake and feed conversion ratio ($P > 0.05$; Table 3). However, the average daily feed intake was lower for the group supplemented with Micocin® compared to the control group on the third week of feeding ($P = 0.014$). Slaughter weight for the Micocin® group was 137 g heavier ($P = 0.0003$; data not shown) despite a lower initial weight (117 g) than the other group. Because the control group had to be slaughtered before to avoid cross contamination, heavier rabbits were assigned to that one in order to meet slaughter weight requirement. Therefore, body weight remained significantly higher for the control group during the 3-first feeding weeks ($P < 0.0001$). On average, both experimental groups met the 2.2 kg minimal weight requirement for commercialization.

3.2. Meat quality traits

Meat composition and quality parameters are presented in Table 4. Meat composition in terms of protein, lipid and moisture content was not influenced significantly by dietary treatment. In terms of muscle pH, it declined below 6 within 24 h after slaughter indicating limited incidence of DFD meat.

A significant difference was observed between the two experimental groups with reference to the pH in the LL muscle 1 h after slaughter ($P = 0.025$), but not in the BF muscle ($P > 0.05$). Furthermore, the pHu 24 h after slaughter was lower in BF from the control compared to the *C. maltaromaticum* CB1 supplemented one ($P = 0.004$), but no significant difference was observed in regard to the LL muscle ($P > 0.05$). Average pH variations were small and below 0.2 unit between the two experimental groups.

Colour parameters of the BF muscle, namely L* ($P = 0.034$), a* ($P = 0.015$) and b* ($P = 0.002$) were significantly higher in meat from the control group than with the Micocin® supplemented one. The meat from rabbit fed with Micocin® supplemented diet was darker, less red and less yellow than the control one. Colour parameters of the LL muscle were not affected by Micocin® supplementation.

In aerobic conditions, water loss for the Micocin® group was significantly smaller on day 3 and day 8 ($P = 0.021$, $P = 0.005$, respectively) and only on day 5 ($P = 0.003$) in anaerobic conditions, compared to the control (Table 4). Drip loss was not significantly different between the two experimental groups ($P > 0.05$) whereas cooking loss was greater with the Micocin® supplemented one by less than 5% ($P = 0.006$; Table 4). Supplementing the diet with *C. maltaromaticum* CB1 had no detrimental effect on total content in polyphenols and carbonyls, as well as on lipid oxidation in raw meat after slaughter ($P > 0.05$; Table 4).

3.3. Microbial analysis of rabbit thighs stored under aerobic or anaerobic conditions

Microflora evolution on rabbit thighs from animals fed rations supplemented with or without *C. maltaromaticum* CB1 when packaged under aerobic and anaerobic conditions is presented in figures 1 and 2, respectively; tables 5 and 6 list *P* values associated with these results. Linear and quadratic interactions of treatment with time were observed; concentration reached at the end of the storage period varied with the

420 microbial groups tested. Microbial analysis of refrigerated rabbit thighs reveals that for
421 all tests, under both aerobic and anaerobic storage conditions, the cell counts increased
422 significantly over time ($P = 0.001$), except for presumptive *S. aureus* which remained
423 at the same level during the whole storage period ($P > 0.05$). The various microbial
424 groups studied exhibited an exponential growth and even reached stationary phase in
425 some cases. Throughout the experiment, all *E. coli* counts remained below the detection
426 level (1.32 Log_{10} CFU/10g) under aerobic and anaerobic storage (data not shown)
427 indicating that appropriate hygienic food processing conditions were followed. At the
428 end of the storage period, cell count variations between the two experimental groups
429 were below 1 Log unit under aerobic conditions. Under anaerobic conditions, however,
430 presumptive LAB enumerated on MRS were 1 Log higher with thighs from the
431 Micocin® supplemented group while *Enterobacteriacea* and coliform counts were
432 1 Log lower. Presumptive *Listeria* was almost one Log lower at 0.93 under the same
433 conditions. Hence, a stronger and more positive microflora modulating effect of
434 Micocin® was observed under anaerobic conditions at 4 °C on the thighs.

435 *3.3.1. Aerobic conditions*

436 Under aerobic conditions, only presumptive *Pseudomonas* spp. ($P = 0.001$),
437 presumptive LAB (on MRS and APT; $P = 0.001$) and *Listeria* spp. ($P = 0.01$) counts
438 were significantly different amongst treatments during storage (control vs. Micocin®
439 groups; Table 5). On day 0, the initial coliform, *Enterobacteriaceae* and presumptive
440 *S. aureus* counts were below detection level (1.32 Log_{10} CFU/10g) for both
441 experimental groups; while presumptive *S. aureus* counts remained below
442 2 Log_{10} CFU/10g for both as well, during the whole experiment. The presumptive
443 *Pseudomonas* spp. counts varied from 1.05 to 7.50 CFU/g during the storage period and
444 remained the prevailing microflora. Considering that end of shelf life is reached when

445 cell count is at 7 Log_{10} CFU/g or higher, rabbit thighs reached that level after 8 days
446 when stored under aerobic conditions. Interestingly, thighs from the Micocin® group
447 had, on day 0, a presumptive *Pseudomonas* count of 1.17 Log above the control, but at
448 the end of the storage period, it was 0.5 Log below (Fig. 1B). A similar pattern was also
449 observed with TAM, but with a magnitude less than 1 Log unit (Fig. 1A). Under such
450 conditions, the various microbial counts performed were either similar or slightly above
451 for the Micocin® group, but all below 1 Log unit difference.

452 *3.3.2. Anaerobic conditions*

453 Overall, a significant treatment effect ($P \leq 0.01$; Table 6) was observed for the
454 dietary addition of *C. maltaromaticum* CB1, compared with the control diet when the
455 thighs were placed under anaerobic conditions during a 20-day storage period for all
456 microbial counts performed, except for the presumptive *Pseudomonas* spp. and
457 *S. aureus* ($P > 0.05$). Total aerobic mesophilic, presumptive LAB (on MRS and APT)
458 counts for the Micocin® supplemented group were above the control. As for
459 *Listeria* spp., coliform and *Enterobacteriaceae* counts, they were below at the end of the
460 storage period, with a Log difference reaching 0.93 to 1.19. As expected, the LAB
461 constitutes the main microflora under anaerobic conditions for both experimental
462 groups, and counts were higher ($P < 0.001$) for the *C. maltaromaticum* CB1
463 supplemented one.
464

3.4. Microbial analysis of rabbit ground meat stored under aerobic or anaerobic conditions at 4 or 10 °C

Modulation of the microflora by the presence of *C. maltaromaticum CB1* in the ration was also investigated in ground meat stored at 4 and 10 °C during 0, 3, 6, 9, 12 and 15 days under aerobic (Fig. 3) and anaerobic (Fig. 4) conditions. Tables 7 and 8 list *P* values associated with these results and, linear and quadratic interactions of temperature with time were observed in ground meat except for presumptive *S. aureus*. Overall, microbial growth was favoured at 10 compared to 4 °C over the storage period and shelf life was reduced by at least three days (Fig. 3 and 4). Microbial tests reveal cell growth during the storage period including presumptive *S. aureus* this time in ground meat ($P = 0.001$); but for *E. coli,* counts remained below detection level again (1.32 Log_{10} CFU/10g). Contrary to what was observed with thighs, no significant effect of treatment was revealed for ground meat stored under aerobic or anaerobic conditions ($P > 0.05$).

3.4.1. Aerobic conditions

On average, end of shelf life was reached after 6 days for meat stored at 10 °C compared to 9 days when at 4 °C under aerobic conditions. At the end of storage, variation in microbial counts performed with ground meat were all below 1 Log unit except for TAM which was 1.45 Log unit above for the Micocin® group at 10 °C. Presumptive LAB enumerated on APT with the Micocin® supplemented group were above the control and close to 1 Log unit (> 0.89) on day 3 and 6 at 4 °C, and on day 12 at 10 °C.

3.4.2. Anaerobic conditions

Anaerobic storage of ground meat from the Micocin® supplemented group increased shelf life between 12 to 15 days, but remained at 6 days for controls (Fig. 4). *C. maltaromaticum* CB1 grow well in these conditions as indicated by TAM and presumptive LAB counts on APT plates that are well above the control by 1 Log unit at the end of the storage period (Fig. 4A and D). This coincided with a cell concentration of *Enterobacteriacea*, coliforms and presumptive *S. aureus* of 1 Log unit below for the Micocin® supplemented group. In fact, Log difference greater than 1 Log unit (1.05-1.86) was observed throughout the anaerobic storage period at 10 °C for counts of presumptive *S. aureus*.

After 15 days of storage at 4 °C under anaerobic conditions, cell counts in ground meat were above those obtained on thighs; Log difference was as low as 0.29 for coliforms and reached 5.04 in the case of presumptive *S. aureus*. Indeed, growth of presumptive *S. aureus* was favoured in ground meat, but to a lesser extent with the Micocin® supplemented group (Fig. 2H and 4G).

3.5. Ground meat experimentally inoculated with L. monocytogenes and stored under aerobic or anaerobic conditions at 4 or 10 °C

Viable counts of *L. monocytogenes* inoculated (4 Log_{10} CFU/g) on rabbit ground meat samples stored at 4 and 10 °C during 0, 3, 6, 9, 12 and 15 days in aerobic and anaerobic conditions are presented in Fig. 5; Table 9 lists *P* values associated with these results. A linear treatment and time interaction was observed for the *L. monocytogenes* counts on inoculated ground meat stored under anaerobiosis ($P = 0.002$) whereas a temperature and time interaction ($P = 0.001$) was observed for both aerobic and anaerobic storage conditions. *L. monocytogenes,* being a well-recognized psychrotoph, grew to high numbers (6.74 to 10.05 CFU/g) in the inoculated control group at both

513 temperatures and under aerobic as well as anaerobic conditions. The effect of treatment
514 under anaerobiosis was significant ($P = 0.025$) for ground meat stored at 4 and 10 °C on
515 day 15. But greatest control of *L. monocytogenes* was observed for ground meat from
516 the Micocin® supplemented group stored at 4 °C under anaerobic conditions reaching a
517 2.1 Log unit difference compared to the control (Fig. 5). The effect of temperature and
518 treatment on *L. monocytogenes* growth in ground meat was also revealed by its growth
519 rate (Table 10). A temperature of 10 °C favours growth of *L. monocytogenes* under both
520 aerobic and anaerobic conditions, whereas the effect of supplementing the ration with
521 Micocin® led to a better control of this bacterium under anaerobic storage ($P < 0.0001$;
522 Fig. 5B). The effect of treatment in aerobiosis was significant only on day 15 ($P = 0.03$;
523 Fig. 5A) where the Micocin® supplemented group was 1.05 to 1.43 Log below the
524 control group at 4 and 10 °C, respectively. But under anaerobic conditions,
525 *C. maltaromaticum* reduced significantly *L. monocytogenes* stored at 4 and 10 °C
526 ($P = 0.0001$) with a reduction of more than 1.5 Log reaching 2.1 Log on day 15.

527 *3.6. Presence of carnocyclin-A producing C. maltaromaticum in the faeces during the*
528 *feeding period*

529 Faeces microbial analysis during the feeding period is presented in Table 11. The
530 female rabbit had just been weaned before their arrival (< 2 d). During the experiment,
531 the difference between the two experimental groups was below 1 Log unit. After one
532 week of feeding, all cell counts were fairly high (> 7.85 CFU/g). But, in weeks 2 and 3,
533 *Enterobacteriaceae*, coliform and *E. coli* counts were below 4.70 CFU/g, whereas TAM
534 and presumptive LAB on MRS and APT were above 5.62 CFU/g demonstrating a shift
535 in the faecal microflora towards a more desirable profile. Using PCR analysis of three
536 specific sequences, namely 16S-cpg, ISR, and CclA, the presence in the faeces of
537 *C. maltaromaticum* producing carnocyclin A was followed. Its presence was revealed

538 during the whole duration of the feeding period for the Micocin® supplemented group,
539 but only for the first week for the control (Table 12).

540 *3.7. Presence of carnocyclin-A producing C. maltaromaticum on thighs and in ground*
541 *meat*

542 Table 12 shows the presence/absence of *C. maltaromaticum* CB1 producing
543 carnocyclin A on rabbit thighs stored at 4 °C under aerobic and anaerobic conditions for
544 0, 3, 6 and 8 and for 5, 10, 15 and 20 days, respectively. *C. maltaromaticum* CB1
545 producing carnocycin A was detected in the Micocin® supplemented group after 0, 3
546 and 6 days of storage in aerobic conditions, but not on day 8. In the control group, under
547 the same aerobic storage conditions, *C. maltaromaticum* CB1 was absent at all sampling
548 time. Under anaerobic conditions, prevalence of *C. maltaromaticum* CB1 was
549 noticeable after 5 days of storage, but not to the same extent than after 15 or 20 days.

550 In order to improve detection of *C. maltaromaticum* producing carnocyclin A in
551 ground meat, PCR analysis was performed after total DNA extraction from the cell
552 pellet obtained with a 25 g meat sample. Prevalence of *C. maltaromaticum* producing
553 carnocyclin A was greater in ground meat coming from rabbits fed the ration
554 supplemented with Micocin® and during storage under anaerobic conditions (Table 13).
555 Indeed, it was absent on control ground meat incubated at 4 °C under aerobic conditions
556 (0/11). By feeding a ration supplemented with *C. maltaromaticum* CB1 (Micocin®), we
557 were able to modulate its presence in the faeces, on the thighs and in ground meat.

558 **4. Discussion**

559 *4.1. Growth performance and meat quality*

560 As expected, the effect on growth performance was limited when Micocin® was
561 added to the feed and, on average, both experimental groups reached the minimal
562 slaughter weight of 2.2 kg (Table 3). In order to follow the rabbit slaughter schedule at

563 the abattoir and to avoid cross contamination between the two experimental groups,
564 rabbits from the Micocin® supplemented group had to be slaughtered a week later. So,
565 lighter rabbits were therefore placed in the Micocin® group and remained as such for
566 the whole duration of the experiment except when slaughter weight was compared
567 ($P = 0.0003$). However, study with balanced groups with respect to weight will have to
568 be performed to confirm the beneficial effect on growth performance from the
569 supplementation. Amber, Yakout, & Hamed (2004) showed improved daily weight gain
570 and performance index with rabbits fed diet containing dried *Lactobacillus acidophilus*
571 (probiotics). Oso et al. (2013) reported a limited impact on the growth rate, but other
572 studies report positive effects with Bioplus 2B and *Bacillus cereus* var *toyoi* on rabbits
573 (Kritas et al., 2008; Trocino et al., 2005). Health status of the animals was followed on a
574 daily basis, and no detrimental effect was associated with the supplementation
575 whatsoever. Although the pHu after slaughter was lower in the BF, but not in the LL
576 muscle from the control group ($P = 0.004$, Table 4), a variation of less than 0.2 pH unit
577 is of little biological significance (Blasco, & Piles, 1990). Similarly to pHu, colour, only
578 for the BF, was affected by the supplementation with Micocin®; indeed, meat was
579 darker, less red and less yellow than the control meat ($P < 0.05$, Table 4). According to
580 Neffe-Skocińska et al. (2015), a decrease in the value of the yellow colour parameter b^*
581 may be a result of the lactic acid bacteria growth during meat products ripening. Colour
582 is generally accepted as one of the major attributes upon which consumers make
583 purchasing decisions (Font-i-Furnols, & Guerrero, 2014). Furthermore, the colour
584 parameters of meat are related to pHu, which influences the oxidation of the heme
585 pigments (Hulot, & Ouhayoun, 1999). According to Fraysse, & Darre, (1989), low pH
586 causes meat discolouration whereas high values give the meat a darker colour, but this
587 variation depends on the type of muscle and the state of the myoglobin (reddish; Hulot,

588 & Ouhayoun, 1999). The colour of BF muscle is different from that of the LL muscle
589 because of differences in metabolism and fibre type composition (Hulot, & Ouhayoun,
590 1999). Also, the lightness index (L* = 51.89 vs. 49.67) was significantly darker and the
591 red lower than the control group (a* = 0.85 vs. 2.16). For this parameter, our results are
592 different from those found by Worobo (1997) who indicates that inoculated meat with
593 *Leuconostoc gelidum* had a greater redness value compared with uninoculated one when
594 stored aerobically at 2 °C after vacuum storage at 4 °C for 45 days. However, the
595 studies of Dal Bosco, Castellini, & Bernardini (1997) demonstrated that discolouration
596 of meat is the result of an increase in oxidation of myoglobin (red) to metmyoglobin
597 (brown). Cooking loss of meat with Micocin® was significantly higher when compared
598 to the control group (27.43 vs. 24.37, Table 4) and according to Hughes et al. (2014),
599 the increase of the water loss during cooking is due to protein denaturation, but the
600 influence of Micocin® on this process was not evaluated here.

601 Before firm conclusion can be made, more research should be done to confirm
602 whether the addition of probiotic bacteria, or certain species, improves the stability of
603 meat colour and cooking loss. Overall, the feed supplementation effect with Micocin®
604 on meat quality parameters is limited and the small variations observed may be, at least
605 in part, the results of rabbit individual variations.

606 *4.2. Modulation of the microflora*

607 Micocin® is a protective culture (*C. maltaromaticum* CB1) autorized in Canada, in
608 the US and many other countries for applications in ready-to-eat meat products (Health
609 Canada, 2010). It was used as a feed additive in this study, since it is easy to track with
610 a set of three genes including the one for carnocyclin A. It was isolated originally from
611 pork and has not been genetically modified according to the manufacturer's official
612 information (FDA, 2009). Hence, it is most likely widely distributed in the meat

613 production/processing environment (Health Canada, 2010). In addition, it may
614 contribute, at least in part, to the sporadic detection of *C. maltaromaticum* producing
615 carnocyclin A in the control group along with possible cross contamination despite strict
616 biosecurity measures. Its absence on rabbit thighs stored at 4 ºC under aerobic
617 conditions for 8 days and under anaerobic conditions for 15 and 20 days may reflect a
618 better ability of other indigenous microbes to prevail in such conditions. Furthermore,
619 detection was done on single colonies isolated from the APT agar plate with the thighs
620 where it was done on the whole cell pellet from the meat homogenate for ground meat
621 in order to improve detection. *C. maltaromaticum*, a facultative anaerobe, is expected to
622 exert a competitive exclusion effect that will vary according to the different strains
623 constituting the indigenous microflora and this may explain the various differences
624 observed on the thighs compared to ground meat. During storage, all microbial counts
625 increased more rapidly at 10 than at 4 °C and the extent vary with the ability of
626 microbial groups tested to grow at such temperature.

627 *C. maltaromaticum* producing carnocyclin A was detected in the faeces collected
628 from the Micocin® supplemented group (Table 12) suggesting that the organism
629 survived the GI passage. It is not known to be particularly resistant to low stomach pH,
630 but being imbedded within the pellet, the feed matrix may have provided a protective
631 effect. However, because the faeces were collected in the pan underneath the cages, part
632 of the contamination may have come from the feed falling onto them as well. Incidence
633 of *C. maltaromaticum* producing carnocyclin A was definitely higher on thighs and in
634 ground meat from the Micocin® supplemented group more so in anaerobic conditions
635 (Saucier et al., 2016) confirming that microorganisms in the feed can end up on the
636 meat either by contamination from the environment or the faeces (Huffman, 2002).

637 *Pseudomonas* is known to prevail on meat stored under aerobic storage conditions
638 whereas LAB does under anaerobic ones (Dainty, & Mackey, 1992; Saucier, 1999). So,
639 it was not surprising to see *C. maltaromaticum* producing carnocyclin A more
640 predominantly under anaerobic conditions (Table 12). Colonies picked from APT plates
641 obtained during microbial analysis of the thighs were used to determine the presence of
642 *C. maltaromaticum* producing carnocyclin A; and reduction of their detection during
643 storage suggests that other strains are better adapted to grow under the conditions used
644 here. Nonetheless, supplementing the feed with Micocin® had a positive reduction
645 effect on coliform, *Enterobacteriacea* and *Listeria* spp. counts for thighs (Fig. 2,
646 Table 6), as well as on presumptive *S. aureus* found in ground meat (Fig. 4, Table 8)
647 stored under anaerobic conditions. *S. aureus* is not a good competitor, notably in fresh
648 meat, where salt and other preservatives are not present (De Buyser et al., 2001).
649 Microbial counts for TAM, as well as presumptive LAB either on MRS or APT, were
650 higher in the Micocin® supplemented group under aerobic and anaerobic conditions,
651 most likely resulting from *C. maltaromaticum* addition in feed.

652 *4.3. Meat Safety*

653 The most convincing evidence that the feeding strategy described here is a valuable
654 and promising approach to better control microbial contamination and growth on meat
655 comes from the 2.1 Log difference obtained in ground meat stored under anaerobic
656 conditions à 4 °C and experimentally inoculated with a five strain cocktail of
657 *L. monocytogenes* (Fig. 5, Table 9). The inhibition effect observed in ground meat from
658 the Micocin® supplemented group directly supports our hypothesis that feeding
659 desirable microorganisms to farm animals can lead to safer products, including meat.
660 According to Ammor & Baltasar (2007), LAB are generally added to food in order to
661 meet safety, shelf life, technological effectiveness and economic feasibility criteria.

662 Many LAB associated with meat, including *C. maltaromaticum,* are known for their
663 bactericidal or bacteriostatic activity against other strains, species or genera of bacteria
664 (Imazaki et al., 2015). Bacteriocins alone are usually ineffective against gram-negative
665 bacteria because of the outer membrane that acts as a barrier to these inhibitory peptides
666 (Vaara., 1992; Gänzle, Hertel, & Hammes, 1999). According to Martin-Visscher et al.
667 (2008, 2011), even if carnobacteriocin BM1 and piscicolin 126 have a potent activity
668 against *L. monocytogenes*, the antimicrobial effect is primarily due to carnocyclin A.
669 These conclusions were also supported by those of Liu et al. (2014) who confirmed that
670 carnocyclin A is the active compound in Micocin® with strong anti-listerial activity.
671 However, Jack et al. (1996) has demonstrated that piscicolin 126 is effective against
672 *L. monocytogenes* in a commercial ham for up to 14 days of storage at 10 °C. Although
673 the CclA gene was used in this study to track the presence of *C. maltaromaticum* CB1
674 on meat, it also most probably, at least in part, contributes to the microbial inhibition
675 and the competitive exclusion observed, along with the two other bacteriocins produced.
676 Nevertheless, these antimicrobial peptides are ideal candidates for strategic use against
677 *L. monocytogenes* and further research is necessary to find microorganisms with a
678 broader and stronger antimicrobial activity, especially for meat stored under aerobic
679 conditions where LAB do not prevail readily.

680 **5. Conclusion**

681 This study demonstrates that it is possible to positively modulate carcass and meat
682 contamination by the introduction of a desirable microflora, here
683 *C. maltaromaticum* CB1, into the feed of weaned rabbits until they reached slaughter
684 weight. The results show that dietary supplementation with *C. maltaromaticum* CB1
685 increased its prevalence on meat, compared to the unsupplemented group, and led to a
686 competitive exclusion towards undesirable organisms namely coliforms,

687 *Enterobacteriaciae, Listeria* and presumptive *S. aureus*. The improvement of meat
688 safety by such feeding strategy was demonstrated by the inhibition of a
689 *L. monocytogenes* cocktail experimentally introduced into the ground meat from control
690 compared to the Micocin® supplemented group, especially during storage under
691 anaerobic and low temperature conditions (4 °C). *L. monocytogenes* numbers were
692 lower by more than 1 Log_{10} CFU/g and the anti-listerial effects of
693 *C. maltaromaticun* CB1 may be attributed, at least in part, to the bacteriocins it can
694 produce. Future experiments should examine the effect of Micocin® on
695 *L. monocytogenes* when the latter is present in very low initial numbers (< 100 CFU/g).
696 Now that the proof of concept has been established with *C. maltaromaticun* CB1, it is
697 important to continue exploring other microorganisms, or mix of them, with a broader
698 and stronger antimicrobial activity, to be introduced into the feed to better control
699 microbial contamination on meat especially under aerobic conditions and at higher
700 temperatures (7-10 °C). Improving the transit of those organisms, notably through the
701 acidic environment of the stomach, may require their encapsulation, although the
702 present results suggest that they survived through the gastrointestinal tract when
703 included in feed. Moreover, other experiments are also needed to establish if the
704 desirable microorganisms must be introduced throughout the growing and finishing
705 periods or if a shorter supplementation before slaughter would be sufficient.

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1001 **Table 1**

1002 Nutritional values and composition[a] of the commercial diets.

	Control		Micocin®	
	As fed basis	Dry basis	As fed basis	Dry basis
Dry matter %[b]	90.75 ± 0.07		90.70 ± 0.01	
Crude protein %[b]	16.00	16.52 ± 0.30		16.49 ± 0.07
Crude fat matter %[b]	4.60	3.67 ± 0.02		3.68 ± 0.01
Crude fiber %[c]	18.1	19.9		
Calcium %[c]	1.00	1.10		
Phosphorous %[c]	0.44	0.48		
Sodium %[c]	0.30	0.33		
Vitamin A UI/kg[c]	6034	6649		
Vitamin D UI/kg[c]	1018	1122		
Vitamin E UI/kg[c]	40.0	44.08		
Total selenium mg/kg[c]	0.19	0.21		
Added selenium mg/kg[c]	0.10	0.11		

[a]**Composition**: Alfalfa, beet pulp, wheat, soybean meal, canola meal, corn gluten feed, molasses, mineral and vitamin premix.
[b]Analysed values.
[c]Calculated values.

1003

1004 **Table 2**

1005 Primer sequences, directions, annealing temperature and size of the candidate products used to
1006 detect *Carnobacterium maltaromaticum* on thighs, faeces and ground rabbit meat by quantitative
1007 reverse transcription-polymerase chain reaction.

PCR primers	Primers sequence (5' to 3') and position	Annealing temperature	Product size (bp)	References
16S-cpg	27F (Forward AGAGTTTGATCCTGGCTCAG) 16-cpg (Reverse GAATCATGCGATTCCTGAAAC)	60	197	Barakat, Griffiths, & Harris (2000)
ISR	Cpis (Forward TTTATTTTTAATTAAATACCC) 23S-7 (Reverse GGTACTTAGATGTTTCAGTTC)	46	623	Rachman et al. (2004) Cailliez-Grimal et al. (2007)
CclA	CclA-F (Forward GCATATGGTATCGCACAAGGTACAGC) CclA-R (Reverse GCTGTGAAGACACCTGATAAACCG)	65	124	Socholotuik et al. (2012)

1008

Table 3

Growth performance of weaned rabbits fed either a control or a supplemented diet with Micocin®.

	Control	*C. maltaromaticum* CB1	SEM	*P* value
Initial body weight[a], g	1109.78	992.51	12.96	***P* < 0.0001**
Week 1				
ADG, g/j	57.37	56.13	0.92	NS
ADFI, g/j	139.75	136.43	2.94	NS
FCR	2.44	2.46	0.03	NS
Body weight, g	1568.77	1446.05	15.46	***P* < 0.0001**
Week 2				
ADG, g/j	53.68	50.36	1.04	NS
ADFI, g/j	153.68	152.66	2.32	NS
FCR	2.88	3.08	0.12	NS
Body weight, g	1950.04	1815.78	17.13	***P* < 0.0001**
Week 3				
ADG, g/j	47.11	48.05	1.1	NS
ADFI, g/j	172.45	161.35	2.34	**0.014**
FCR	3.69	3.42	0.11	NS
Body weight, g	2284.11	2166.47	17.54	***P* < 0.0001**
Week 4				
ADG, g/j	-	34.74	1.71	-
ADFI, g/j	-	172.45	4.93	-
FCR	-	5.03	0.14	-
Body weight, g	-	2421.49	28.05	-

[a] Because the control group had to be slaughtered before the Micocin® one to avoid cross contamination, heavier rabbits were placed in the control. SEM: standard error of the mean; n = 12 cages, a cage of six rabbits is the experimental unit. BW: body weight; ADG: average daily weight gain; ADFI: average daily feed intake; FCR: feed conversion ratio. NS: not significant. *P* value in bold is significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 4

Effect of *Carnobacterium maltaromaticum CB1* diet supplement on physicochemical analyses, meat quality parameters and antioxidant status of rabbit meat.

Quality parameters	Control	*C. maltaromaticum* CB1	SEM	*P* value
Proximate composition				
% Protein	18.03	17.90	0.28	NS
% Lipid	11.11	11.31	0.60	NS
% Moisture	70.44	69.88	0.47	NS
% Drip loss	1.01	1.06	0.16	NS
% Cooking loss	24.37	27.43	0.70	**0.006**
% Meat exudate loss aerobic 3-8 days				
D 3	0.72	0.16	0.15	**0.021**
D 6	1.14	0.90	0.27	NS
D 8	1.35	0.51	0.19	**0.005**
% Meat exudate loss anaerobic 5-20 days				
D 5	0.88	0.31	0.12	**0.003**
D 10	0.38	0.60	0.28	NS
D 15	0.82	1.09	0.38	NS
D 20	0.16	1.50	0.54	0.09
pH of BF muscle				
1 h	6.18	6.07	0.07	NS
24 h	5.42	5.62	0.04	**0.004**
pH of LL muscle				
1 h	6.01	5.82	0.05	**0.025**
24 h	5.39	5.40	0.03	NS
Colour of BF muscle				
L*	51.89	49.67	0.69	**0.034**
a*	2.16	0.85	0.35	**0.015**
b*	2.39	1.66	0.14	**0.002**
Colour of LL muscle				
L*	53.34	52.16	0.73	NS
a*	2.29	2.17	0.36	NS
b*	2.95	2.70	0.22	NS
Total phenols (µg GAE/g)	9.62	9.59	0.06	NS
TBARS[a] (nmol/g MDA)	2.16	2.30	0.12	NS
Carbonyls (nmol/mg protein)	2.45	2.50	0.64	NS

Each value represents the mean of twelve samples with SEM: standard error of the mean; n = 12 cages, a cage of six rabbits is the experimental unit.

[a]All lipid oxidation data are presented as mean of Malondialdehyde (MDA) values from three analyses performed in triplicate. TBARS: thiobarbituric acid reactive substances, SEM: standard error of the mean, NS: not significant. GAE: gallic acid equivalent. *P* values in bold are significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 5

Different *P* values of microbial counts on thigh samples stored at 4 ºC in aerobic conditions.

		Time		Treatment × time	
	Treatment	Linear	Quadratic	Linear	Quadratic
TAM	NS	**0.001**	**0.001**	**0.001**	NS
Presumptive *Pseudomonas*	**0.001**	**0.001**	**0.001**	**0.001**	0.09
LAB on MRS	**0.001**	**0.001**	**0.001**	**0.003**	NS
LAB on APT	**0.001**	**0.001**	**0.001**	NS	**0.01**
Listeria spp.	**0.01**	**0.001**	**0.001**	**0.03**	0.07
Enterobacteriacea	0.06	**0.001**	**0.001**	**0.002**	**0.002**
Coliforms	NS	**0.001**	**0.001**	**0.006**	**0.005**
Presumptive *S. aureus*	NS	NS	NS	**0.01**	NS

TAM: Total aerobic mesophilic, LAB: Lactic acid bacteria.

NS: not significant. *P* values in bold are significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 6

Different *P* values of microbial counts on thigh samples stored at 4 ºC in anaerobic conditions.

		Time		Treatment × time	
	Treatment	Linear	Quadratic	Linear	Quadratic
TAM	**0.001**	**0.001**	**0.001**	**0.003**	**0.001**
Presumptive *Pseudomonas*	NS	**0.001**	**0.001**	**0.001**	**0.001**
LAB on MRS	**0.001**	**0.001**	**0.001**	**0.003**	**0.007**
LAB on APT	**0.001**	**0.001**	**0.001**	NS	**0.02**
Listeria spp.	**0.01**	**0.001**	**0.001**	**0.001**	**0.02**
Enterobacteriacea	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
Coliforms	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
Presumptive *S. aureus*	NS	NS	NS	NS	NS

TAM: Total aerobic mesophilic, LAB: Lactic acid bacteria.

NS: not significant. *P* values in bold are significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 7

Different *P* values of microbial counts on uninoculated ground meat samples stored at 4 and 10 ºC in aerobic conditions.

		Time		Temperature × time	
	Temperature	Linear	Quadratic	Linear	Quadratic
TAM	**0.001**	**0.001**	NS	**0.001**	**0.001**
Presumptive *Pseudomonas*	**0.001**	**0.001**	NS	**0.005**	**0.001**
LAB on MRS	**0.001**	**0.001**	NS	**0.001**	**0.006**
LAB on APT	**0.001**	**0.001**	0.074	**0.006**	**0.004**
Enterobacteriacea	**0.004**	**0.001**	NS	**0.001**	**0.001**
Coliforms	**0.001**	**0.001**	NS	**0.001**	**0.001**
Presumptive *S. aureus*	NS	**0.001**	NS	NS	NS

TAM: Total aerobic mesophilic, LAB: Lactic acid bacteria.

NS: not significant. Other interactions and the treatment effect are not significant ($P > 0.05$).

P values in bold are significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 8

Different *P* values of microbial counts in uninoculated ground meat samples stored at 4 and 10 ºC in anaerobic conditions.

		Time		Temperature × time	
	Temperature	Linear	Quadratic	Linear	Quadratic
TAM	**0.008**	**0.001**	**0.007**	**0.001**	**0.006**
Presumptive *Pseudomonas*	**0.001**	**0.001**	**0.016**	**0.001**	**0.004**
LAB on MRS	**0.003**	**0.001**	**0.014**	**0.001**	**0.001**
LAB on APT	**0.001**	**0.001**	0.098	**0.001**	**0.001**
Enterobacteriacea	**0.001**	**0.001**	**0.002**	**0.001**	**0.001**
Coliforms	**0.001**	**0.001**	**0.029**	**0.001**	**0.001**
Presumptive *S. aureus*	0.078	**0.001**	NS	0.055	0.067

TAM: Total aerobic mesophilic, LAB: Lactic acid bacteria.
NS: not significant. Other interactions and the treatment effect are not significant ($P > 0.05$).
P values in bold are significant ($P < 0.05$), underlined values describe a tendency ($P < 0.10$).

Table 9

P values of microbial counts on inoculated ground meat samples with a cocktail of five strains of *Listeria monoctogenes* stored at 4 and 10 ºC in aerobic and anaerobic conditions.

	Temperature	Treatment	Time		Temperature × time		Treatment × time	
			Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
Aerobic conditions	**0.005**	NS	**0.001**	NS	**0.001**	NS	NS	NS
Anaerobic conditions	**0.005**	**0.025**	**0.001**	**0.022**	**0.001**	**0.001**	**0.002**	NS

NS: not significant. Other interactions under aerobic and anaerobic conditions are not significant ($P > 0.05$). *P* values in bold are significant ($P < 0.05$).

Table 10

Growth rate (CFU/g.day) of *Listeria monoctogenes* on inoculated ground meat samples with a cocktail of five strains of *Listeria monoctogenes* stored at 4 and 10 ºC in aerobic and anaerobic conditions.

	Control	Micocin®	SEM	*P* value	
				Temperature	Treatment
Aerobic conditions					
4 ºC	0.16	0.10	0.02	**0.001**	NS
10 ºC	0.31	0.28	0.02		
Anaerobic conditions					
4 ºC	0.23	0.09	0.01	**0.0001**	**0.0001**
10 ºC	0.34	0.26	0.01		

NS: not significant. No significant interactions under aerobic and anaerobic conditions were observed ($P > 0.05$);
P values in bold are significant ($P > 0.05$). Each value represents the mean of slopes from three repetitions (Fig. 5); best-fit curves were obtained using the Excel Software of Microsoft Office.

Table 11

Microbial enumeration of TAM, presumptive LAB on MRS, presumptive LAB on APT, coliforms, *Enterobacteriacea* and *Escherichia coli* in faeces during the feeding period.

	Week 1			Week 2			Week 3		
	Control	Micocin®	Reduction (Log unit)	Control	Micocin®	Reduction (Log unit)	Control	Micocin®	Reduction (Log unit)
TAM	9.44	9.03	0.41	6.64	7.18	-0.54	6.02	6.10	-0.08
LAB on MRS	8.45	8.64	-0.19	7.70	8.48	-0.78	5.62	5.96	-0.34
LAB on APT	8.81	9.12	-0.31	8.48	9.08	-0.60	6.58	6.95	-0.37
Enterobacteriaceae	8.44	8.29	0.15	3.08	3.20	-0.12	4.70	4.45	0.25
Coliforms	8.39	8.18	0.21	3.48	3.11	0.37	4.52	4.45	0.07
E. coli	8.35	7.85	0.50	3.15	2.60	0.55	4.34	3.90	0.44

TAM: Total aerobic mesophilic, LAB: Lactic acid bacteria.
Each value represents one fecal sample (500 g) collected from the pan underneath the cages and analyzed in duplicate.

Table 12

Presence of *Carnobacterium maltaromaticum* in faeces and rabbit thighs at 4 °C under aerobic and anaerobic conditions[a].

	Faeces (feeding weeks)				Thigh storage (days)						
					Aerobic				Anaerobic		
Days	1	2	3	4	0	3	6	8	5	15	20
Control	$1_{(20)}$	$0_{(30)}$	$0_{(21)}$	-	$0_{(24)}$	$0_{(11)}$	$0_{(34)}$	$0_{(24)}$	$1_{(34)}$	$0_{(23)}$	$1_{(24)}$
Micocin®	$1_{(20)}$	$1_{(17)}$	$2_{(22)}$	$1_{(10)}$	$4_{(20)}$	$4_{(20)}$	$1_{(25)}$	$0_{(24)}$	$8_{(24)}$	$1_{(24)}$	$1_{(24)}$

[a] Index number represents the number of colonies samples from APT plates for PCR analysis of three specific genes: 16S-cpg, ISR and CclA. Results are expressed as the number of colonies identified as *Carnobacterium maltaromaticum* by the PCR analysis.

Table 13

Presence of *Carnobacterium maltaromaticum* producing carnocyclin A in rabbit ground meat stored at 4 and 10 °C under aerobic and anaerobic conditions (0, 3, 6, 9, 12, 15 days) as determined by PCR analysis of three specific genes:16S-cpg, ISR and CclA[a].

Experimental groups	Temperature	Storage days	Aerobic			Anaerobic		
			16S-cpg	ISR	CclA	16S-cpg	ISR	CclA
Control	4 ºC	0	-	-	-	-	-	-
		3	-	-	-	+	+	+
		6	-	-	-	-	-	-
		9	+	-	-	-	-	-
		12	+	-	-	-	-	-
		15	+	+	-	+	+	-
	10 ºC	3	-	-	-	-	-	-
		6	+	-	-	+	-	-
		9	+	-	-	+	+	-
		12	+	+	-	+	-	-
		15	+	+	-	+	-	-
	Total positive		7	3	0	6	3	1
Micocin®	4 ºC	0	+	+	+	+	-	-
		3	+	+	+	+	+	+
		6	+	+	+	+	+	+
		9	+	+	+	+	+	+
		12	+	+	-	+	+	+
		15	+	+	-	+	+	+
	10 ºC	3	+	-	-	+	+	+
		6	+	+	+	+	+	+
		9	+	+	+	+	+	+
		12	+	+	+	+	+	-
		15	+	+	+	+	+	+
	Total positive		11	10	8	11	10	9

[a] Number of positive gene identification out of 11 samples of ground meat for each storage conditions (n = 11; one sample per temperature and storage time).

List of figures

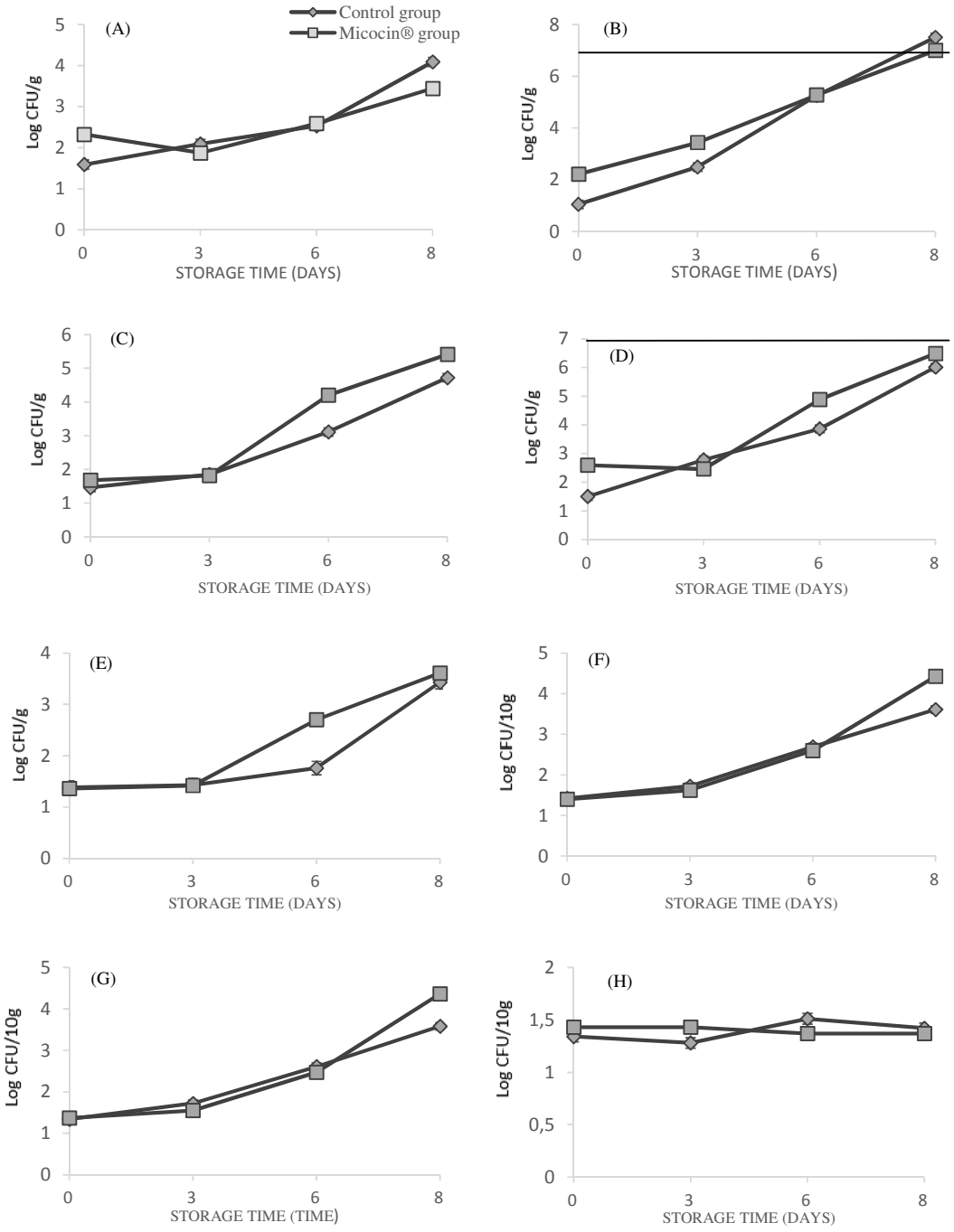
Fig. 1. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D) and *Listeria* spp. (E) counts in Log_{10} CFU/g, and *Enterobacteriaceae* (F), coliform (G) and presumptive *Staphylococcus aureus* (H) counts in Log_{10} CFU/10g on rabbit thighs between 0 and 8 days of storage at 4 °C under aerobic conditions. Bar represents standard error of the mean. Each point is a mean value of 12 cages with one thigh per cage analyzed at each sampling time. The cage of six rabbits is the experimental unit. Horizontal line indicates end of shelf life.

Fig. 2. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D) and *Listeria* spp. (E) counts in Log_{10} CFU/g, and *Enterobacteriaceae* (F), coliform (G) and presumptive *Staphylococcus aureus* (H) counts in Log_{10} CFU/10g on rabbit thighs between 0 and 8 days of storage at 4 °C under anaerobic conditions. Bar represents standard error of the means. Each point is a mean value of 12 cages with one thigh per cage analyzed at each sampling time. The cage of six rabbits is the experimental unit. Horizontal line indicates end of shelf life.

Fig. 3. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D), *Enterobacteriaceae* (E), coliform (F), and presumptive *Staphylococcus aureus* (G) counts in Log_{10} CFU/g on ground meat uninoculated rabbit between 0 and 15 days stored at 4 and 10 ºC in aerobic conditions. Bar represents standard error of the mean. Each point is a mean value of three repetitions. Horizontal line indicates end of shelf life.

Fig. 4. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D), *Enterobacteriaceae* (E), coliform (F), and presumptive *Staphylococcus aureus* (G) counts in Log_{10} CFU/g on ground meat uninoculated rabbit between 0 and 15 days stored at 4 and 10 ºC in anaerobic conditions. Bar represents standard error of the mean. Each point is a mean value of three repetitions. Horizontal line indicates end of shelf life.

Fig. 5. Growth of a cocktail of five *Listeria monocytogenes* strains inoculated at 4 Log_{10} CFU/g on ground rabbit meat from animals fed a control diet or a diet supplemented with Micocin® containing *Carnobacterium maltaromaticum* CB1 at a level of 8 Log_{10} CFU/kg of feed. Meat was stored under aerobic (A) or anaerobic (B) conditions at 4 or 10 °C. Each point represents the mean of three repetitions where, at each sampling time, one sample per cage was taken randomly and analyzed in duplicate for a total of twelve cages per experimental group. Bar represents standard error of the mean. $*P < 0.05$, $**P < 0.01$ represent the treatment effect at each sampling time and under the two conditions (aerobic and anaerobic).



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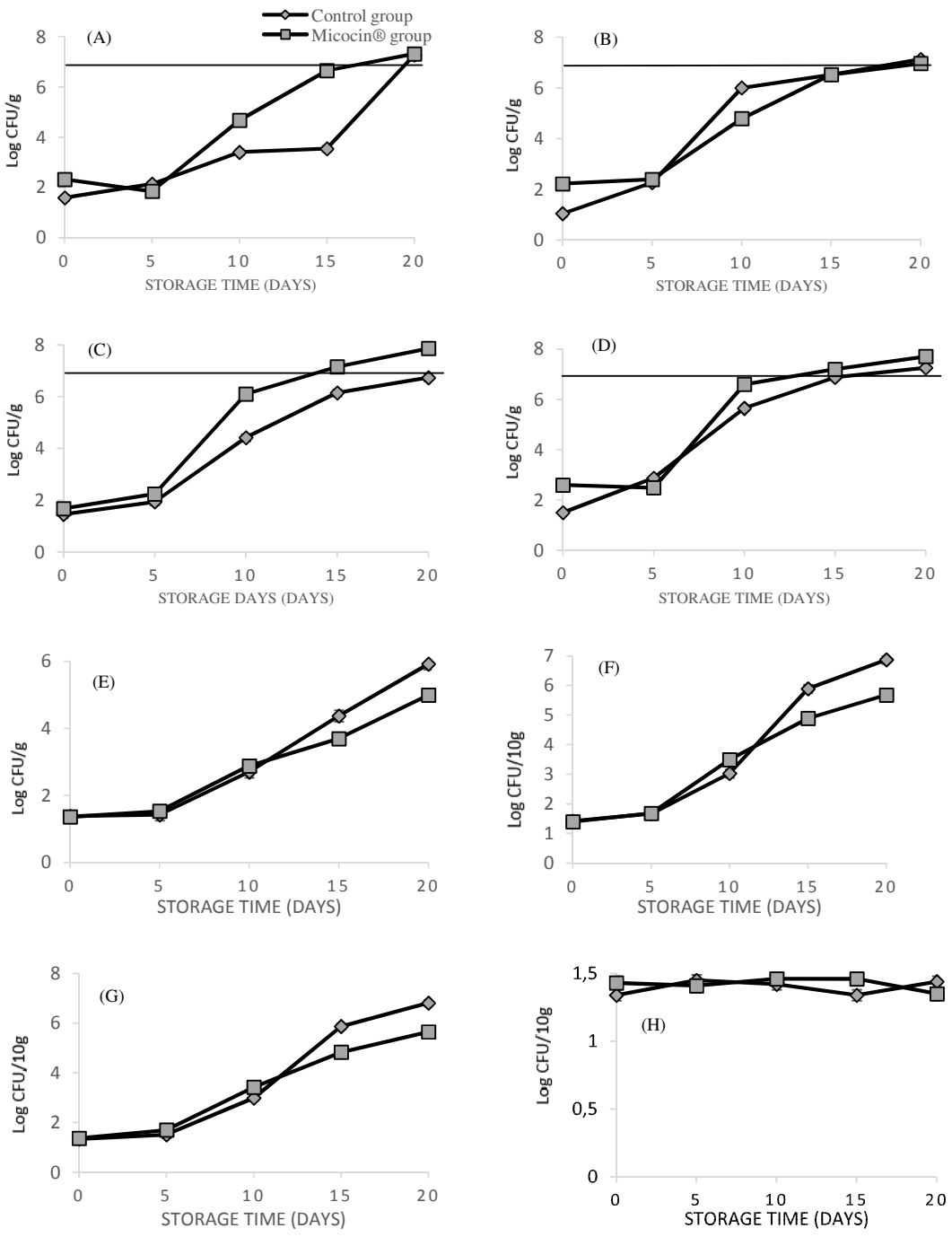

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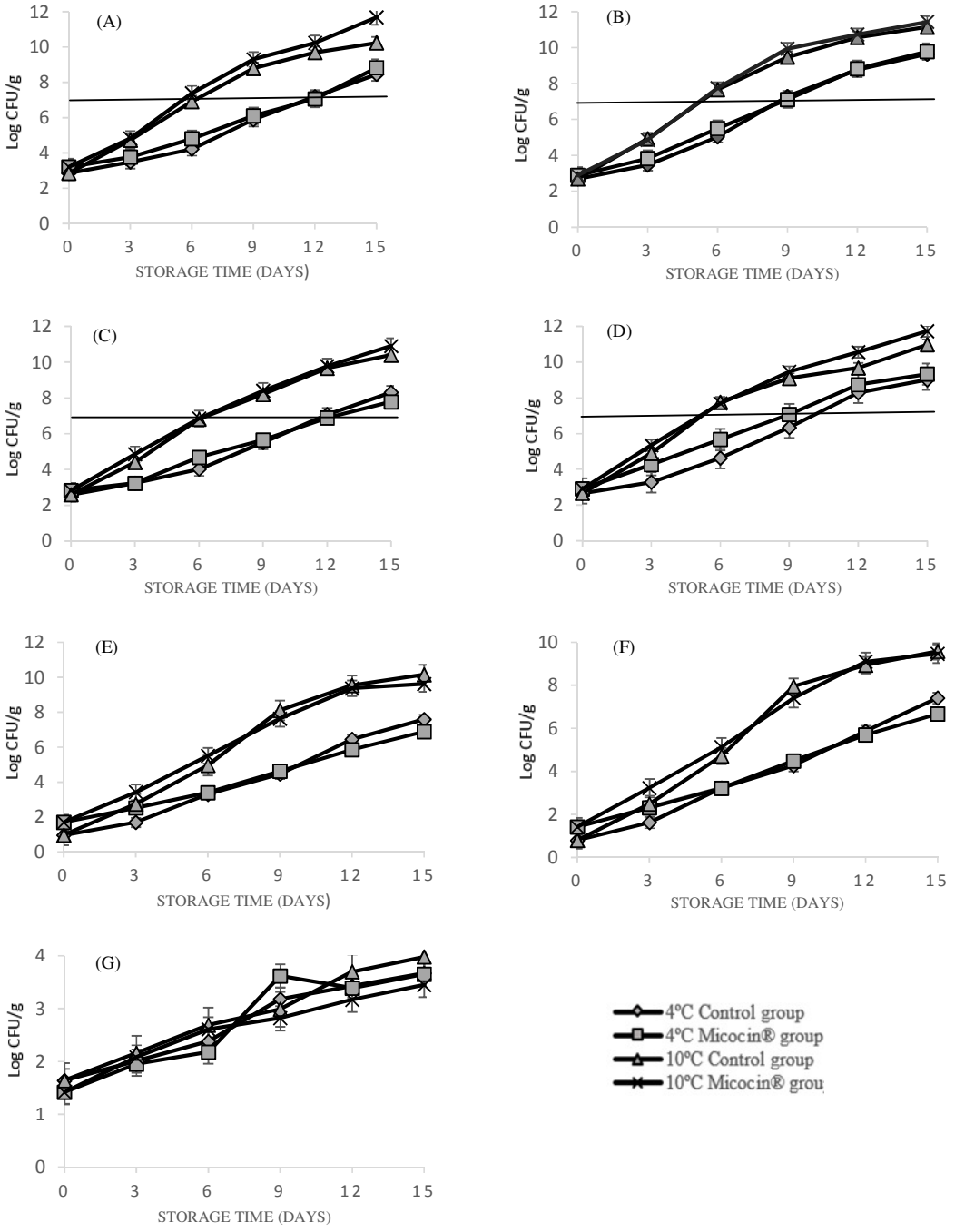

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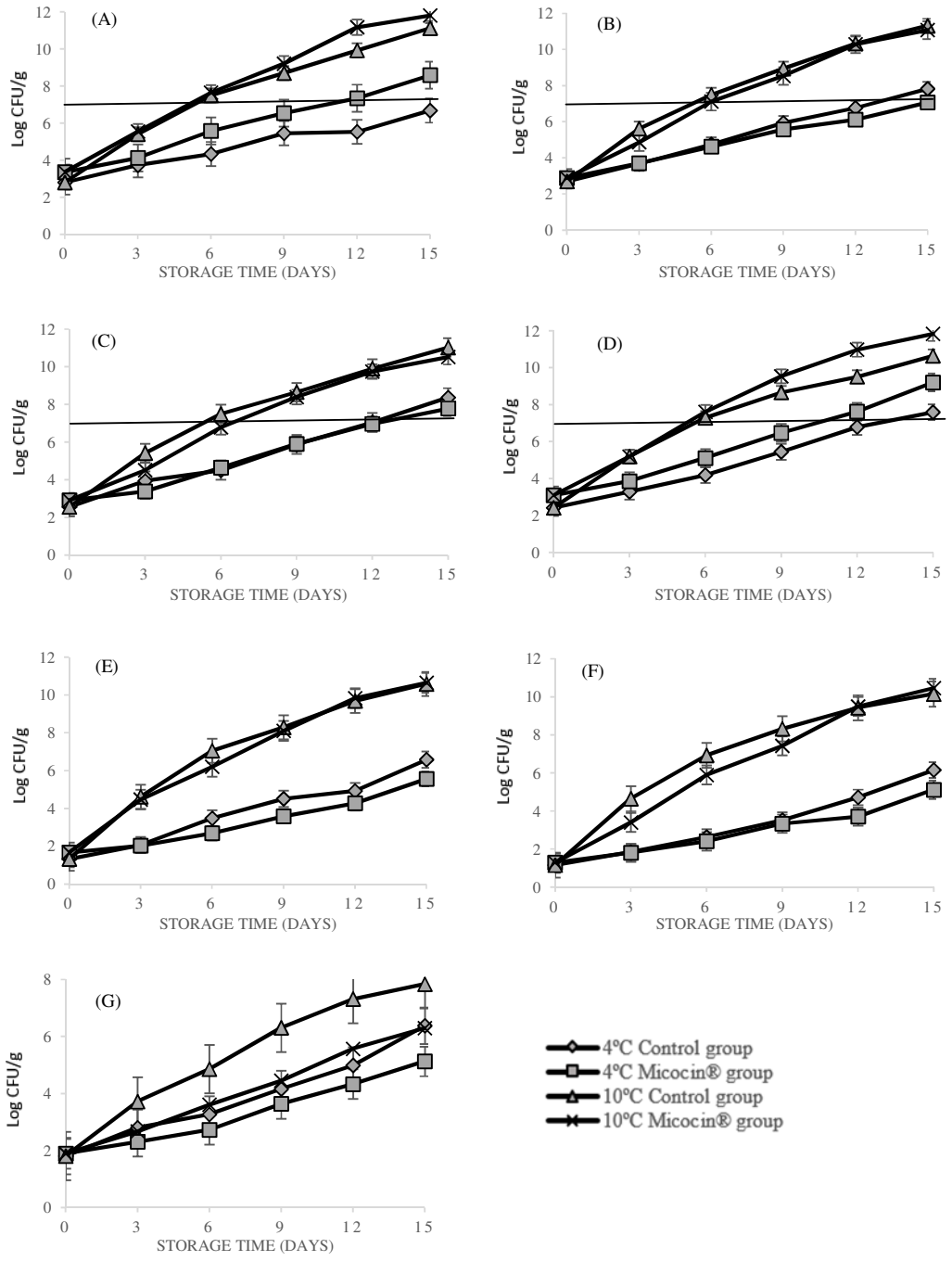

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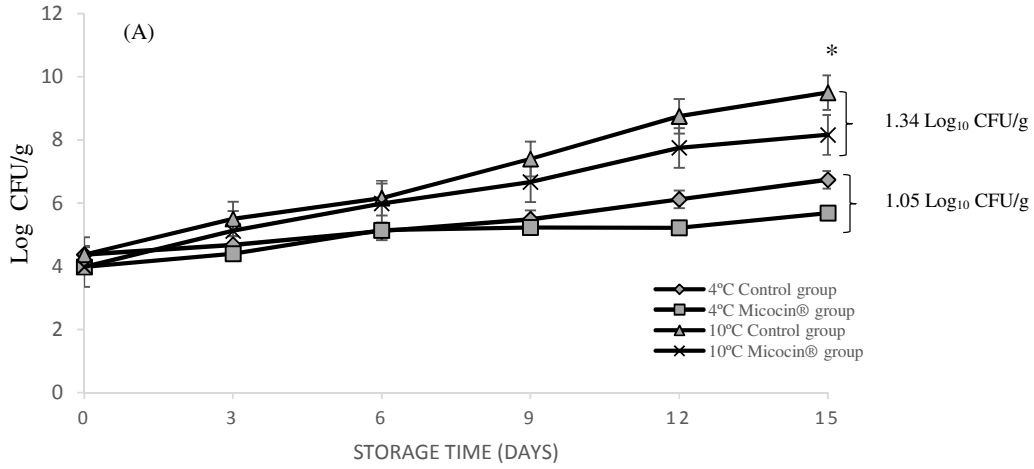

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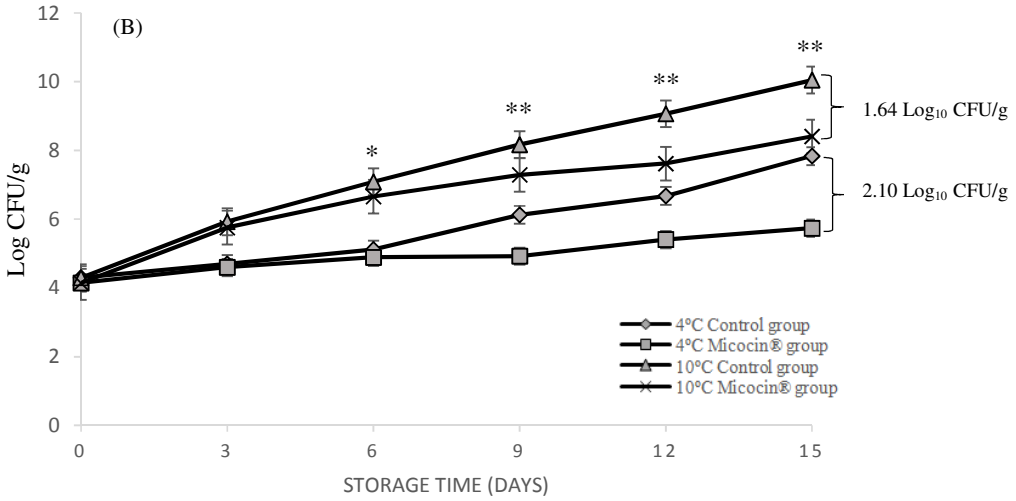

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