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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 ($P = 0.025$).

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 & Gondret, 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 (Organisation for Economic Co-operation and Development), 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, Tanguay, Hamel, & Phypers, 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 (DeWall & 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 (Castellano, Belfiore, Fadda, & Vignolo, 2008; Leroy & De Vuyst, 2004). 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

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Table 1
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] Analyzed values.
[c] Calculated values.

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 (Galvez, Lopez, Abriouel, Valdivia, & Omar, 2008; Goktepe, 2006). *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, Laursen, Prévost, Drider, & Dalgaard, 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* (Casaburi et al., 2011; González, Yien, Castrillon, & Ortega, 2013; Laursen et al., 2005).

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

2. Materials and methods

2.1. Animal housing and feeding

Animal care and handling procedures were approved by Université Laval's Animal Use and Care Committee, which strictly adheres to the Guidelines of the Canadian Council on Animal Care (CCAC, 2009). A total of 144, 35-day-old weaned female Grimaud breed rabbits were obtained from a commercial farm (Laprodéo, Saint-Tite, Quebec, Canada) and were maintained in conventional commercial cages. Rabbits were individually weighted upon arrival and assigned immediately either to the experimental or the control group. Rabbits were placed six per cage (0.37 m^2 per rabbit) in order to have homogeneous weight per cage and within groups; the cage constituted the experimental unit. Twelve cages were analyzed per experimental group. In order to make sure that the control group does not get contaminated by the microbial culture (Micocin®, Griffith Foods, Toronto, ON, Canada) given to the experimental one, the animals had to be housed in two different but similar rooms and strict biosecurity measures were observed. On a daily basis, control group were always visited first and the personnel changed clothes, mask, hair net and gloves between each group. If the control group needed to be revisited, personnel had to shower first. A cycle of 12 h of light (starting at 9:00 am) and 12 h of dark was used throughout the experiment, temperature was at 20.1 ± 0.4 °C and humidity level at 33 ± 4%.

The experimental group was fed the ration supplemented with the protective culture Micocin® containing *C. maltaromaticum* CB1 at a final concentration of 8 Log_{10} CFU (Colony-Forming Unit) per kg of feed. Micocin® was provided to us as a concentrate containing 10 Log_{10} CFU/g which was added during the commercial pelleting process (Table 1). Feed was manufactured in a commercial facility in separate 600 kg batches (Belisle Solution Nutrition, St-Mathias-sur-Richelieu, Quebec, Canada). The feed supplemented with Micocin® was manufactured last to avoid contaminating the equipment. Animals were fed ad libitum until a minimal target slaughter weight of 2200 g was reached, which took 21 to 28 days. They were weighed and the feed intake was measured weekly during the experimental period to determine body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

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

2.2. Meat quality measurement

For meat quality measurement, one rabbit per cage was randomly analyzed. The muscular pH of the *Biceps femoris* (BF) and the *Longissimus lumborum* (LL) muscles were measured *post-mortem* after 1

(pH 1) and 24 h (pHu; Blasco & Ouhayoun, 1996) using a portable pH meter (ROSS, Orion Star A221, Thermo Scientific, Beverly, CA, USA) combined with an Orion Kniphe electrode (ThermoFisher, Nepean, ON, Canada) and a temperature compensation probe (928 007 MD, micro probes ATC, Maryland, USA). Meat colour was evaluated 24 h after slaughter on the LL and the exposed surface of the BF using a Chromameter (Chromameter CR 300 Minolta Ltd., Osaka, Japan) equipped with a D65 light source and a 0° viewing angle geometry according to the reflectance coordinates (L*, a*, b*; CIE, 1976), after exposing the muscle surface for 20 min blooming time (Faucitano, Chevillon, & Ellis, 2010). Meat exudate lost (%) during cold storage was measured by weight difference of the thighs. Regarding drip loss, the measure was taken from a piece of *Longissimus thoracis et lumborum* muscle (LTL about 2 cm thick × 2.5 cm in diameter) also by weight difference, according to the EZ-Dripless method (Rasmussen & Anderson, 1996), where samples are stored at 4 °C for 48 h. Cooking loss was determined on a similar piece of LTL muscle (Pla, 1999) and is expressed as a percentage of the initial weight loss. Each sample was placed into an 18 oz. Whirl-Pak bag (Nasco Whirl-Pak®, USA) and immersed in a water bath at 70 °C for 15 min after removing the air from the bag. The samples were then removed from the bag, patted dry with filter paper and weighed (Apata, Koleosho, Apata, & Okubanjo, 2012; Vergara, Berruga, & Linares, 2005).

2.3. Muscle sampling

One leg per animal was packaged aerobically in a styrofoam tray (14 w × 24 l × 4.5 h cm) with an absorbent pad, sealed with an oxygen-permeable polyethylene film (35 ga; oxygen transmission 825 cm^3/100 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 cm^3/100 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 × *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, Padmanaban, & Sarma, 1965). Diluted sample aliquots of 1 ml (1:4, v/v) were added to 2 N Folin-Ciocalteu's phenol reagent (500 ml; Sigma-Aldrich, St. Louis, MO, USA) followed by addition of 10% $NaCO_3$ (1 ml). Reaction mixture was vortexed and the absorbance was measured with a spectrophotometer (Varioskan™ Microplate instrumentation Thermo Electron Corporation, Vantaa, Finland) at 700 nm after incubating for 1 h at room temperature (21 °C). Quantification was based on a standard curve generated with gallic acid. The results are expressed in GAE (gallic acid equivalent per g of meat, μg GAE/g). All measurements were performed in triplicate.

2.5.2. Lipid oxidation

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

2.5.3. Carbonyl content

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

2.6. Microbial analysis

For microbial enumeration on the thighs, a sampling procedure similar to the one for whole poultry carcasses described by Brichta-Harhay et al. (2007) was used. One leg from the five remaining rabbits per cage was randomly taken at each sampling time. Each cage was sampled at every sampling time and conditions (aerobic and anaerobic). Thigh was aseptically placed in a sterile Stomacher bag (Stomacher® 400C, Seward Laboratory Systems Inc., London, UK), weighted (measure was also used to evaluate meat exudate in Section 2.2) and sealed after 300 ml of 0.1% (wt/vol) peptone water were added (Bacto peptone, Difco Laboratories, Inc., Detroit, MI, USA). The bag was placed on a rotary shaker (Boekel Scientific Orbitron Rotator II, model 260,250, New York, USA) for 1 min on each side and then manually massaged for 30 s to remove microorganisms from the surface. When ground meat was analyzed, 25 g was homogenized in 225 ml of peptone water for 2 min at 230 rpm in a stomacher (Stomacher® 400 circulator, Seward, England). Ten-fold dilutions were carried out in 0.1% peptone water for enumeration on appropriate agar plates (Saucier, Gendron, & Gariépy, 2000). Total Aerobic Mesophilic (TAM) counts were performed on Plate Count Agar medium (PCA; Difco Laboratories Inc.) incubated at 35 °C for 48 h (MFHPB-18; Health Canada, 2001). Presumptive Lactic Acid Bacteria (LAB) were enumerated on deMan, Rogosa and Sharp (MRS; Difco Laboratories Inc.; Saucier et al., 2000) and on All Purpose Tween (APT; Difco of Becton, Dickinson) agar plates since *Carnobacterium* is not particularly acid-tolerant and grow poorly on MRS. The plates were incubated anaerobically for 48 h at 25 °C using anaerobic jars with an envelope generator of H_2 and CO_2

(AnaeroGen™2.5 l, AN0025A, Oxoid Company, Nepean, ON, Canada). Presumptive *Pseudomonas* spp. were determined on Cetrimide-Fucidin-Cephalosporin (CFC) agar (supplement No.SR0103E, Oxoid) and plates were incubated at 25 °C for 48 h (Gill & Greer, 1993; Mead & Adams, 1977). Coliform and *E. coli* counts were determined using 3 M Petrifilm™ plates after incubation at 35 °C for 18–24 h (MFHPB-34; Health Canada, 2013). Presumptive *S. aureus* strains were evaluated on 3 M Petrifilm™ plates incubated at 37 °C for 26 h (MFLP-21; Health Canada, 2004). *Enterobacteriaceae* counts were performed on 3 M Petrifilm™ (MFLP-09; Health Canada, 2007) after incubation at 37 °C for 24 h. Presumptive *Listeria* spp. were determined on PALCAM medium (PALCAM *Listeria* Agars Base; Merck, Germany) without supplements, while plates were incubated at 30 °C for 48 h. Regarding *L. monocytogenes*, counts were performed using PALCAM *Listeria* selective supplement (No. 1. 12122.001; EMD, NJ, USA), plates were put in a 30 °C incubator for 48 h (MFHPB-30; Health Canada, 2011). Measurements were performed in duplicate. All bacterial counts were transformed to a Log_{10} value of colony-forming units per gram of thigh weight (Log_{10} CFU/g) prior to statistical analysis according to Gill (2000). Except for presumptive *S. aureus*, coliform, *E. coli*, *Enterobacteriaceae* counts, which were transformed to a Log_{10} value of colony forming units per ten grams of thigh weight (Log_{10} CFU/10 g). For counts on PCA, MRS, APT, CFC and Palcam, detection level was 1.76 Log_{10} CFU/10 g, and 1.32 Log_{10} CFU/10 g for presumptive *S. aureus*, coliforms, *E. coli* and *Enterobacteriaceae* counts.

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

2.7. Experimental inoculation of ground meat with L. monocytogenes

2.7.1. Bacterial cultures and growth conditions

A cocktail of five *L. monocytogenes* strains, namely 1043 (1/2a), 2371, 2558 (1/2b), 2739, 2812 (1/2a), were used in this study. They were all isolated from meat products and kindly provided by Health Canada (Ottawa, ON, Canada). Stock cultures were stored at − 80 °C in Brain Heart Infusion (BHI; BBL-Becton Dickinson, Mississauga, Ontario, Canada) supplemented with 20% glycerol (FisherBiotech, Fairlawn, NJ, USA). Prior to experimental use, working cultures were individually thawed and subcultured (1%) daily in BHI broth for a minimum of two and a maximum of seven consecutive days. Cultures were incubated at 30 °C for 24 h. *L. monocytogenes* inoculum was prepared by mixing equal volume of strains grown separately to stationary phase. Cell suspensions were harvested by centrifugation (5000 × *g* for 10 min at 4 °C), washed ones and resuspended in 12.5 ml of peptone water. Cell suspension was diluted a 100 fold and meat was inoculated with 100 µl in order to obtain a final concentration of 4 Log_{10} CFU/g of meat.

2.7.2. Ground meat inoculation and incubation

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

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

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

For use in these experiments, stock frozen cultures in 20% glycerol were subcultured in 9 ml of APT broth incubated at 25 °C for *Carnobacterium* strains and MRS broth incubated at 37 °C for *Pediococcus acidilactici* UL5. *P. acidilactici* UL5 and *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 2×10^9 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, Koné, Gagné, Cinq-Mars, & Guay, 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

Table 2
Primer sequences, directions, annealing temperature and size of the candidate products used to detect *Carnobacterium maltaromaticum* on thighs, faeces and ground rabbit meat by quantitative 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, and Harris (2000)
ISR	Cpis (Forward TTTATTTTTAATTAAATACCC) 23S-7 (Reverse GGTACTTAGATGTTTCAGTTC)	46	623	Rachman, Kabadjova, Valcheva, Prévost, and Dousset (2004)Cailliez-Grimal, Edima, Revol-Junelles, and Millière (2007)
CclA	CclA-F (Forward GCATATGGTATCGCACAAGGTACAGC) CclA-R (Reverse GCTGTGAAGACACCTGATAAACCG)	65	124	Socholotuik (2012)

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 < 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).

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 ($P < 0.05$).

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 ($P < 0.10$).

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

3.3.1. Aerobic conditions

Under aerobic conditions, only presumptive *Pseudomonas* spp. ($P = 0.001$), presumptive LAB (on MRS and APT; $P = 0.001$) and *Listeria* spp. ($P = 0.01$) counts were significantly different amongst treatments during storage (control vs. Micocin® groups; Table 5). On day 0, the initial coliform, *Enterobacteriaceae* and presumptive *S. aureus* counts were below detection level (1.32 Log_{10} CFU/10 g) for both experimental groups; while presumptive *S. aureus* counts remained below 2 Log_{10} CFU/10 g for both as well, during the whole experiment. The presumptive *Pseudomonas* spp. counts varied from 1.05 to 7.50 CFU/g during the storage period and remained the prevailing microflora. Considering that end of shelf life is reached when cell count is at 7 Log_{10} CFU/g or higher, rabbit thighs reached that level after 8 days when stored under aerobic conditions. Interestingly, thighs from the Micocin® group had, on day 0, a presumptive *Pseudomonas* count of 1.17 Log above the control, but at the end of the storage period, it was 0.5 Log below (Fig. 1B). A similar pattern was also observed with TAM, but with a magnitude < 1 Log unit (Fig. 1A). Under such conditions, the various microbial counts performed were either similar or slightly above for the Micocin® group, but all below 1 Log unit difference.

3.3.2. Anaerobic conditions

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

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 (Figs. 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/10 g). 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

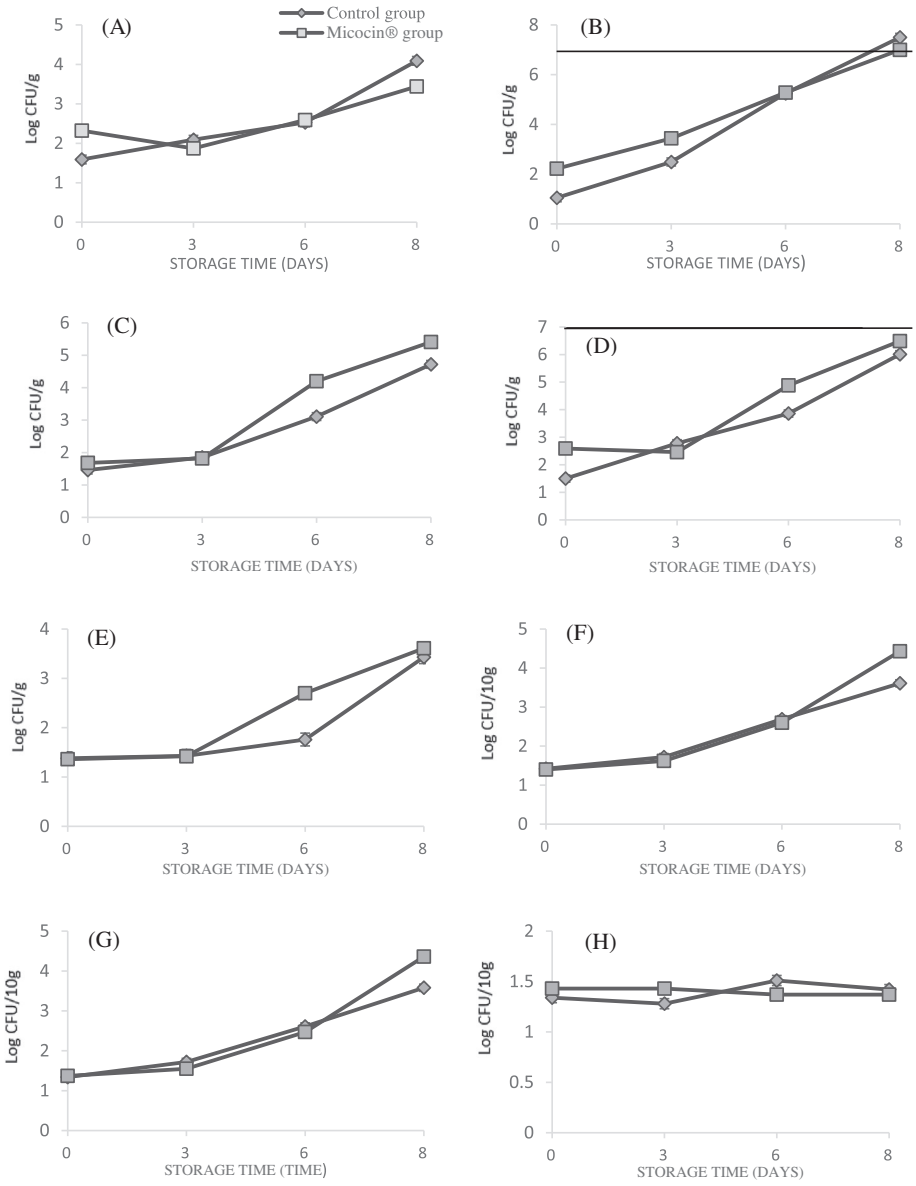

Fig. 1. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D) and *Listeria* spp. (E) counts in Log_{10} CFU/g, and *Enterobacteriaceae* (F), coliform (G) and presumptive *Staphylococcus aureus* (H) counts in Log_{10} CFU/10 g on rabbit thighs 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.

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 and 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 > 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 (Figs. 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 temperatures and under aerobic as well as anaerobic conditions. The effect of treatment under anaerobiosis was significant ($P = 0.025$) for ground meat stored at 4 and 10 °C on day 15. But greatest control of *L. monocytogenes* was observed for ground meat from the Micocin® supplemented group stored at 4 °C under anaerobic conditions reaching a 2.1 Log unit difference compared to the control (Fig. 5). The effect of temperature and treatment on *L. monocytogenes* growth in ground meat was also revealed by its growth rate (Table 10). A

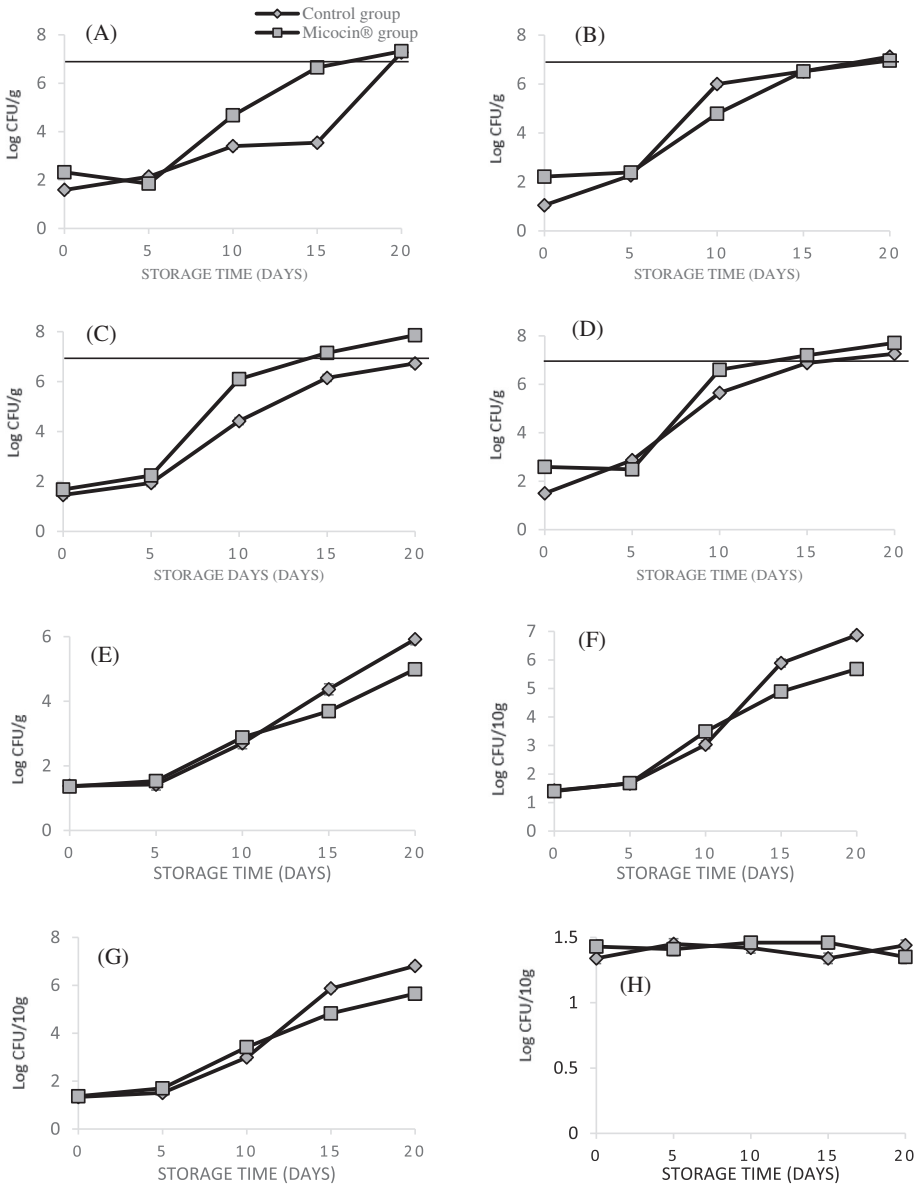

Fig. 2. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D) and *Listeria* spp. (E) counts in Log_{10} CFU/g, and *Enterobacteriaceae* (F), coliform (G) and presumptive *Staphylococcus aureus* (H) counts in Log_{10} CFU/10 g on rabbit thighs 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.

Table 5
Different *P* values of microbial counts on thigh samples stored at 4 °C 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 °C 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 ($P < 0.05$).

temperature of 10 °C favours growth of *L. monocytogenes* under both aerobic and anaerobic conditions, whereas the effect of supplementing the ration with Micocin® led to a better control of this bacterium under anaerobic storage ($P < 0.0001$; Fig. 5B). The effect of treatment in aerobiosis was significant only on day 15 ($P = 0.03$; Fig. 5A) where the Micocin® supplemented group was 1.05 to 1.43 Log below the control group at 4 and 10 °C, respectively. But under anaerobic conditions, *C. maltaromaticum* reduced significantly

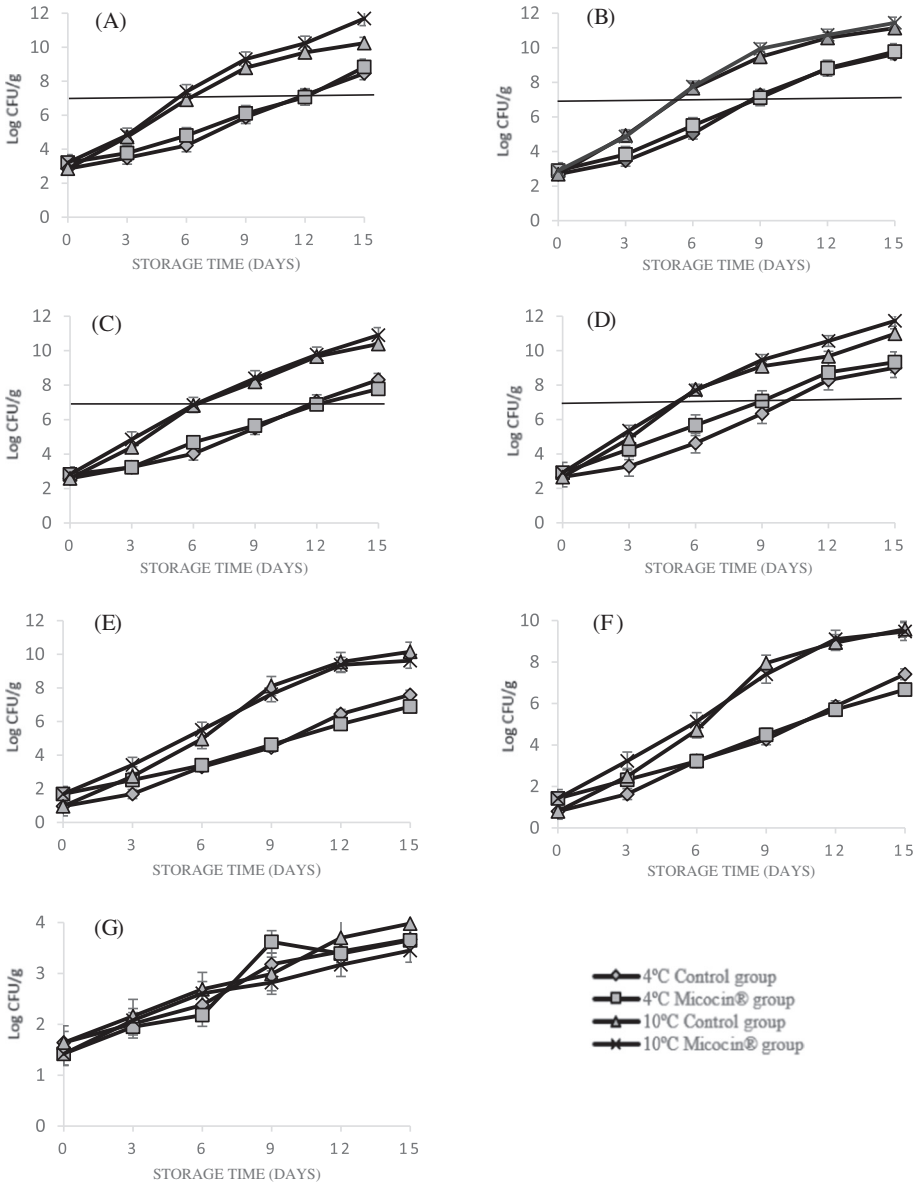

Fig. 3. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D), *Enterobacteriaceae* (E), coliform (F), and presumptive *Staphylococcus aureus* (G) counts in Log_{10} CFU/g on ground meat uninoculated rabbit between 0 and 15 days stored at 4 and 10 °C 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.

L. monocytogenes stored at 4 and 10 °C ($P = 0.0001$) with a reduction of > 1.5 Log reaching 2.1 Log on day 15.

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

Faeces microbial analysis during the feeding period is presented in Table 11. The female rabbit had just been weaned before their arrival (< 2 d). During the experiment, the difference between the two experimental groups was below 1 Log unit. After one week of feeding, all cell counts were fairly high (> 7.85 CFU/g). But, in weeks 2 and 3, *Enterobacteriaceae*, coliform and *E. coli* counts were below 4.70 CFU/g, whereas TAM and presumptive LAB on MRS and APT were above 5.62 CFU/g demonstrating a shift in the fecal microflora towards a more desirable profile. Using PCR analysis of three specific sequences, namely 16S-cpg, ISR, and CclA, the presence in the faeces of *C. maltaromaticum* producing carnocyclin A was followed. Its presence was revealed during the whole duration of the feeding period for the Micocin® supplemented group, but only for the first week for the control (Table 12).

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

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

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

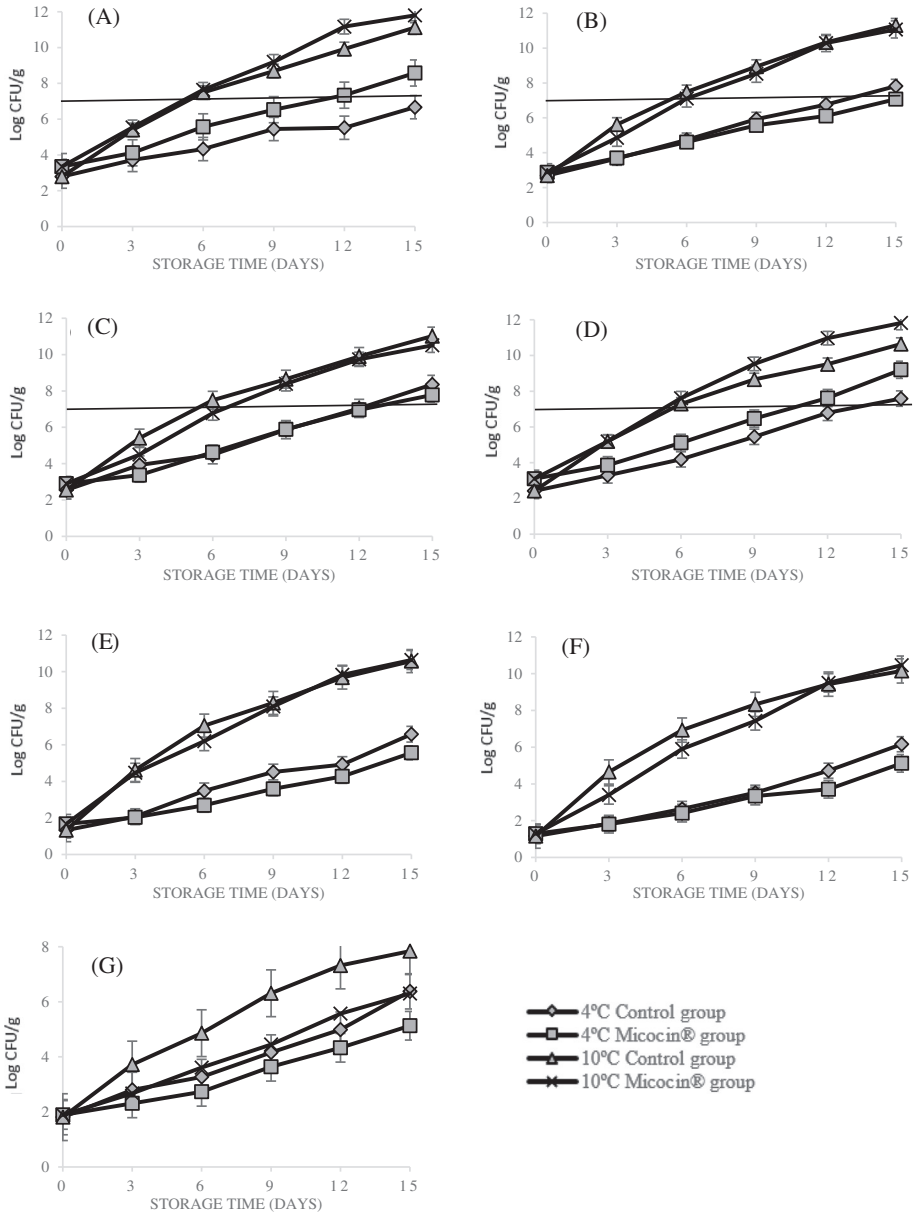

Fig. 4. Total aerobic mesophilic (A), presumptive *Pseudomonas* (B), presumptive LAB on MRS (C), presumptive LAB on APT (D), *Enterobacteriaceae* (E), coliform (F), and presumptive *Staphylococcus aureus* (G) counts in Log_{10} CFU/g on ground meat uninoculated rabbit between 0 and 15 days stored at 4 and 10 °C 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.

Table 7
Different *P* values of microbial counts on uninoculated ground meat samples stored at 4 and 10 °C 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 °C 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 ($P < 0.10$).

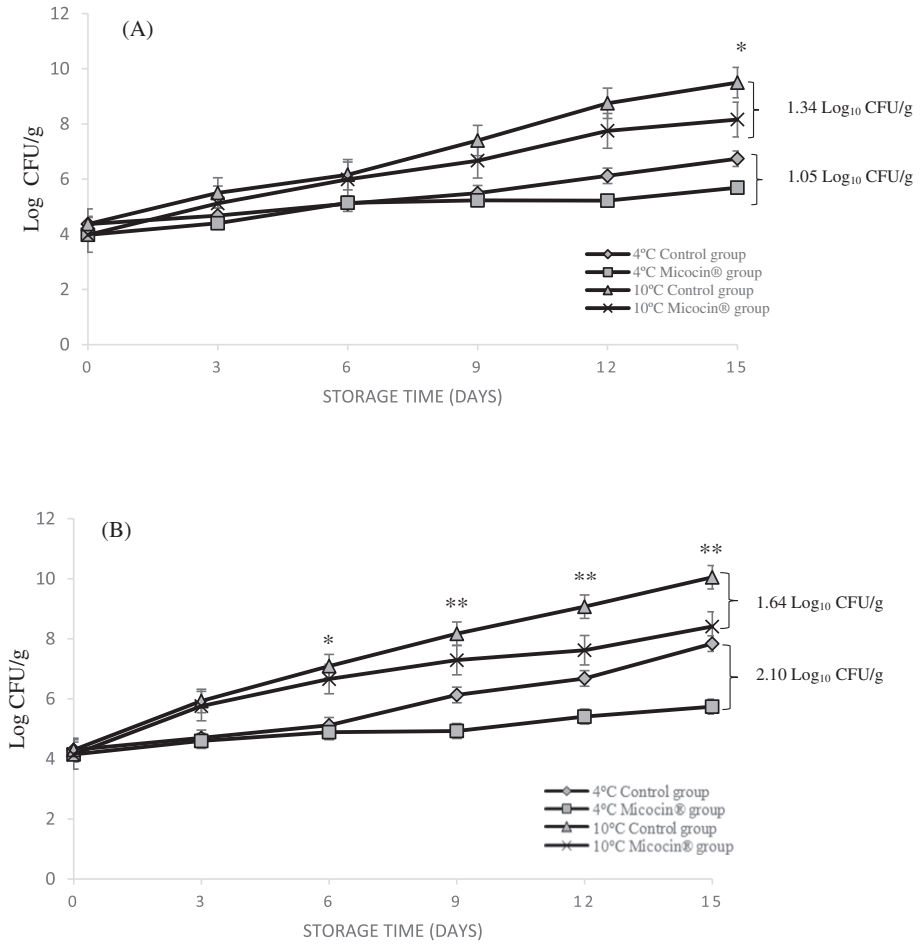

Fig. 5. Growth of a cocktail of five *Listeria monocytogenes* strains inoculated at 4 Log_{10} CFU/g on ground rabbit meat from animals fed a control diet or a diet supplemented with Micocin® containing *Carnobacterium maltaromaticum* CB1 at a level of 8 Log_{10} CFU/kg of feed. Meat was stored under 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).

C. maltaromaticum CB1 (Micocin®), we were able to modulate its presence in the faeces, on the thighs and in ground meat.

4. Discussion

4.1. Growth performance and meat quality

As expected, the effect on growth performance was limited when Micocin® was added to the feed and, on average, both experimental groups reached the minimal slaughter weight of 2.2 kg (Table 3). In order to follow the rabbit slaughter schedule at the abattoir and to avoid cross contamination between the two experimental groups, rabbits from the Micocin® supplemented group had to be slaughtered a week later. So, lighter rabbits were therefore placed in the Micocin® group and remained as such for the whole duration of the experiment except when slaughter weight was compared ($P = 0.0003$). However, study with balanced groups with respect to weight will have to be performed to confirm the beneficial effect on growth performance from the supplementation. Amber, Yakout, and Hamed (2004) showed improved daily weight gain and performance index with rabbits fed diet containing dried *Lactobacillus acidophilus* (probiotics). Oso et al. (2013) reported a limited impact on the growth rate, but other studies report positive effects with Bioplus 2B and *Bacillus cereus* var *toyoi* on rabbits (Kritas et al., 2008; Trocino, Xiccato, Carraro, & Jimenez, 2005). Health status of the animals was followed on a daily basis, and no detrimental effect was associated with the supplementation whatsoever. Although the pHu after slaughter was lower in the BF, but not in the LL muscle from the control group ($P = 0.004$, Table 4), a variation of < 0.2 pH unit is of little biological significance (Blasco & Piles, 1990). Similarly to pHu, colour, only for the BF, was affected by the supplementation with Micocin®; indeed, meat was darker, less red and less yellow than the control meat ($P < 0.05$, Table 4). According to Neffe-Skocińska, Jaworska, Kołożyn-Krajewska, Dolatowski, and Jachacz-Jówko (2015), a decrease in the value of the yellow colour parameter b^* may be a result of the lactic acid bacteria growth during meat products ripening. Colour is generally accepted as one of the major attributes upon which consumers make purchasing decisions (Font-i-Furnols & Guerrero, 2014). Furthermore, the colour parameters of meat are related to pHu, which influences the oxidation of the heme pigments (Hulot & Ouhayoun, 1999). According to Fraysse and Darre (1989), low

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 °C 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 °C in aerobic and anaerobic conditions.

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

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

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

4.2. Modulation of the microflora

Micocin® is a protective culture (*C. maltaromaticum* CB1) autorized in Canada, in the US and many other countries for applications in ready-to-eat meat products (Health Canada, 2010). It was used as a feed additive in this study, since it is easy to track with a set of three genes including the one for carnocyclin A. It was isolated originally from pork and has not been genetically modified according to the manufacturer's official information (FDA, 2009). Hence, it is most likely widely distributed in the meat production/processing environment (Health Canada, 2010). In addition, it may contribute, at least in part, to the sporadic detection of *C. maltaromaticum* producing carnocyclin A in the control group along with possible cross contamination despite strict biosecurity measures. Its absence on rabbit thighs stored at 4 °C under aerobic conditions for 8 days and under anaerobic conditions for 15 and 20 days may reflect a better ability of other indigenous microbes to prevail in such conditions. Furthermore, detection was done on single colonies isolated from the APT agar plate with the thighs where it was done on the whole cell pellet from the meat homogenate for ground meat in order to improve detection. *C. maltaromaticum*, a facultative anaerobe, is expected to exert a competitive exclusion effect that will vary according to the different strains constituting the indigenous microflora and this may explain the various differences observed on the thighs compared to ground meat. During storage, all microbial counts increased more rapidly at 10 than at 4 °C and the extent vary with the ability of microbial groups tested to grow at such temperature.

C. maltaromaticum producing carnocyclin A was detected in the faeces collected from the Micocin® supplemented group (Table 12) suggesting that the organism survived the GI passage. It is not known to be particularly resistant to low stomach pH, but being imbedded within the pellet, the feed matrix may have provided a protective effect.

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 °C	0	−	−	−	−	−	−
		3	−	−	−	+	+	+
		6	−	−	−	−	−	−
		9	+	−	−	−	−	−
		12	+	−	−	−	−	−
		15	+	+	−	+	+	−
	10 °C	3	−	−	−	−	−	−
		6	+	−	−	+	−	−
		9	+	−	−	+	+	−
		12	+	+	−	+	−	−
		15	+	+	−	+	−	−
Total positive			7	3	0	6	3	1
Micocin®	4 °C	0	+	+	+	+	−	−
		3	+	+	+	+	+	+
		6	+	+	+	+	+	+
		9	+	+	+	+	+	+
		12	+	+	−	+	+	+
		15	+	+	−	+	+	+
	10 °C	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).

However, because the faeces were collected in the pan underneath the cages, part of the contamination may have come from the feed falling onto them as well. Incidence of *C. maltaromaticum* producing carnocyclin A was definitely higher on thighs and in ground meat from the Micocin® supplemented group more so in anaerobic conditions (Saucier et al., 2016) confirming that microorganisms in the feed can end up on the meat either by contamination from the environment or the faeces (Huffman, 2002).

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

4.3. Meat safety

The most convincing evidence that the feeding strategy described here is a valuable and promising approach to better control microbial contamination and growth on meat comes from the 2.1 Log difference obtained in ground meat stored under anaerobic conditions à 4 °C and experimentally inoculated with a five strain cocktail of *L. monocytogenes* (Fig. 5, Table 9). The inhibition effect observed in ground meat from the Micocin® supplemented group directly supports our hypothesis that feeding desirable microorganisms to farm animals can lead to safer products, including meat. According to Ammor and Mayo (2007), LAB are generally added to food in order to meet safety, shelf life, technological effectiveness and economic feasibility criteria. Many LAB associated with meat, including *C. maltaromaticum*, are known for their bactericidal or bacteriostatic activity against other strains, species or genera of bacteria (Imazaki, Jacques-Houssa, Kergourlay, Daube, & Clinquart, 2015). Bacteriocins alone are usually ineffective against gram-negative bacteria because of the outer membrane that acts as a barrier to these inhibitory peptides (Gänzle, Hertel, & Hammes, 1999; Vaara, 1992). According to Martin-Visscher et al. (2008; Martin-Visscher, Yoganathan, Sit, Lohans, and Vederas, 2011), even if carnobacteriocin BM1 and piscicolin 126 have a potent activity against *L. monocytogenes*, the antimicrobial effect is primarily due to carnocyclin A. These conclusions were also supported by those of Liu, Basu, Miller, and McMullen (2014) who confirmed that carnocyclin A is the active compound in Micocin® with strong anti-listerial activity. However, Jack et al. (1996) has demonstrated that piscicolin 126 is effective against *L. monocytogenes* in a commercial ham for up to 14 days of storage at 10 °C. Although the CclA gene was used in this study to track the presence of *C. maltaromaticum* CB1 on meat, it also most probably, at least in part, contributes to the microbial inhibition and the competitive exclusion observed, along with the two other bacteriocins produced. Nevertheless, these antimicrobial peptides are ideal candidates for strategic use against *L. monocytogenes* and further research is necessary to find microorganisms with a broader and stronger antimicrobial activity, especially for meat stored under aerobic conditions where LAB do not prevail readily.

5. Conclusion

This study demonstrates that it is possible to positively modulate carcass and meat contamination by the introduction of a desirable microflora, here *C. maltaromaticum* CB1, into the feed of weaned rabbits until they reached slaughter weight. The results show that dietary

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

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