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Effects of a *Carnobacterium maltaromaticum* strain at natural contamination levels on the microbiota of vacuum-packaged beef steaks during chilled storage

Peipei Zhang [a], Eric Ruan [b], Devin B. Holman [a], Xianqin Yang [a,*]

[a] Agriculture and Agri-Food Canada, 6000 C&E Trail, Lacombe, Alberta, T4L 1W1, Canada
[b] InnoTech Alberta, P O Bag 4000, Hwy 16A & 75 St, Vegreville, Alberta, T9C 1T4, Canada

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ABSTRACT

We investigated the impact of a *Carnobacterium maltaromaticum* strain at a natural contamination level on the dynamics of microbiota and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium on chilled vacuum-packaged (VP) beef. *C. maltaromaticum* A5 up to 2 log CFU/cm^2 was spiked onto beef steaks with or without *E. coli* O157:H7 and *S.* Typhimurium. The steaks were vacuum-packaged and stored at 2 °C for 12 weeks. The dynamics of the microbiota were determined using plating and 16S rRNA gene amplicon sequencing methods. The predominant bacterial species in the final microbiota were determined using whole metagenome and genome sequencing. *C. maltaromaticum* on inoculated meat peaked at week 2 (>80%) even though the initial relative abundance was as low as <1%. The relative abundance of indigenous *Latilactobacillus* was 0.1–0.5%, but gradually increased to 75.7–100% at week 12, displacing *Carnobacterium*. The predominant species in the final microbiota was identified as *Latilactobacillus sakei* subsp. *carnosus,* which harbored gene clusters encoding Sakacin G and a putative class IIc bacteriocin. Neither of the two pathogens was affected by *C. maltaromaticum* A5. Taken together, this study indicates that the initial microbial composition plays an important role in the fate of an introduced LAB strain.

1. Introduction

Both "good" and "bad" bacteria may establish persistence in the meat processing environment (Wang, He, & Yang, 2018). These bacteria are an important source of contamination on/in meat, some of which may affect the storage life and/or safety of meat in a positive or negative way (Stellato et al., 2016). The attainable storage life of vacuum-packaged (VP) beef and lamb often varies with processing facilities, which may have been colonized by different bacterial species/strains (Kaur, Bowman, Porteus, Dann, & Tamplin, 2017; Kaur, Williams, Bissett, Ross, & Bowman, 2021; M. K. Youssef, Gill, Tran, & Yang, 2014; M. K. Youssef, Gill, & Yang, 2014; M.K. Youssef, Tran, Zhang, Gill, & Yang, 2017). Lactic acid bacteria (LAB) are often a very small fraction (<10%) of the initial microbiota at the time meat is vacuum-packaged, but will gradually dominate the microbiota with extended storage at chiller temperatures (Chen et al., 2020; Small, Jenson, Kiermeier, & Sumner, 2012; Yang, Wang, Hrycauk, & Klassen, 2021; M. K. Youssef, Gill, Tran, & Yang, 2014). The impact of LAB on the shelf life of VP meat varies among bacterial species and even strains of the same species, depending on their sensory impacting metabolic products and their effect on the overall meat microbiota (Barcenilla, Ducic, López, Prieto, & Álvarez-Ordóñez, 2022).

Some Australian researchers have reported an extremely long shelf life (−0.5 °C, 30 weeks) for VP primal cuts, and *Carnobacterium maltaromaticum* was dominant in the meat microbiota (Kaur et al., 2017). We investigated the storage life of VP beef from several federally-inspected Canadian beef packing plants and noticed that products from one plant had an extremely long shelf life (−1.5 °C, >160 days) and this exceedingly long shelf life seemed to be associated with certain strains of *C. maltaromaticum* (M. K. Youssef, Gill, Tran, & Yang, 2014; M. K. Youssef, Gill, & Yang, 2014; M.K. Youssef et al., 2017). In addition, phylogenetic analysis showed that these *C. maltaromaticum* strains likely originated from the meat processing environment (Zhang, Badoni, Ganzle, & Yang, 2018). Phenotypic and genotypic analysis in our laboratory has shown that one of the *C. maltaromaticum* strains recovered from VP beef cuts with long storage life, A5, can inhibit in

* Corresponding author.
E-mail address: xianqin.yang@agr.gc.ca (X. Yang).

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dual cultures, a broad range of pathogenic and spoilage bacteria associated with meat, through production of bacteriocins and/or organic acids (Zhang, Ganzle, & Yang, 2019). *C. maltaromaticum* has been explored as a protective culture in various foods including salmon, beef, ham, and cheese (Aymerich, Rodriguez, Garriga, & Bover-Cid, 2019; Danielski et al., 2020; dos Reis et al., 2011; Hu, Balay, Hu, McMullen, & Ganzle, 2019; Nilsson et al., 2004; Spanu et al., 2018). However, a high inoculation level ($\geq$4 $\log_{10}$ CFU per gram/cm^2) of *C. maltaromaticum* was often used in these studies and information on the impact of low concentrations of carnobacteria on meat microbiota is limited.

In the present study, *C. maltaromaticum* strain A5 was inoculated onto VP beef steaks at 0.5–2 log CFU/cm^2, to simulate levels that would be expected for naturally contaminated meat during production (Chen et al., 2020; Kiermeier et al., 2013; Yang et al., 2021; M. K. Youssef, Gill, Tran, & Yang, 2014; M. K. Youssef, Gill, & Yang, 2014). The potential effect of the introduced *Carnobacterium* strain on the native meat microbiota and on the growth of two main meat-borne pathogens (*Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium) was investigated via both conventional plate counting and high-throughput sequencing methods. Also, we investigated the potential effect of *C. maltaromaticum* on meat sensory quality by comparing the odors and volatile compounds of the inoculated and un-inoculated groups.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Carnobacterium maltaromaticum A5 was recovered from VP beef in our previous study and was found to have antibacterial activity against a large number of bacteria *in vitro* (M. K. Youssef, Gill, Tran, & Yang, 2014; Zhang et al., 2019). *S.* Typhimurium ATCC 14028 was obtained from the American Type Culture Collection (Manassas, VA, USA) and *E. coli* O157:H7 1934 was kindly provided by Dr. Alexander O. Gill (Health Canada, Ottawa, ON, Canada). The three bacterial strains were streaked onto tryptic soy agar (TSA; Oxoid, Mississauga, ON, Canada) and incubated at 25 °C for 48 (*C. maltaromaticum*) or 24 h (*S.* Typhimurium and *E. coli* O157:H7), and then sub-cultured in half-strength brain heart infusion broth (BHI; Oxoid) at 25 °C for 24 h. The overnight cultures were ten-fold serially diluted in 0.1% (w/v) peptone water (Fisher Scientific, Edmonton, AB, Canada). Cell density of the suspensions was determined by plating on TSA. Appropriate dilutions were used as inoculum for beef steaks.

2.2. Inoculation of beef steaks

Frozen VP eye of round primal cuts were obtained from the Lacombe Research and Development Centre abattoir and were made into steaks using a cutting machine. The steaks were ~1.5 cm thick and each had a surface area of ~200 cm^2. Two days prior to the experiment, the beef steaks were placed in a 2 °C incubator to thaw. The beef steaks were divided into eight groups of 21 steaks with 1–4 and 5–8 as spoilage and pathogen groups, respectively (Table 1). Each steak was put into a plastic pouch (Winpak Ltd., Winnipeg, MB, Canada) and inoculated with 2 ml of 0.1% peptone water (group 1 steaks) or bacterial suspensions of various combinations (groups 2–8) with 1 ml on each side. *C. maltaromaticum* A5 was inoculated at 0 (group 1 and 5), 0.5 (group 2 and 6), 1 (group 3 and 7) and 2 (group 4 and 8) log CFU/cm^2, respectively. Pathogen group beef steaks were also inoculated with 2 log CFU/cm^2 of *S.* Typhimurium and *E. coli* O157:H7. The inoculated steaks were vacuum-packaged using a Multivac vacuum sealer A300/16 (Sepp Haggenmuller KG, Wolfertschwenden, Germany) and stored in a 2 °C incubator with even distribution on all five shelves. Five data loggers (Temprecord, Auckland, New Zealand) were placed in the middle of each shelf to monitor the temperature.

2.3. Meat sampling and processing

Three beef steaks selected at random were withdrawn from each group on the next day (week 0) after packaging and at bi-weekly intervals thereafter until week 12. Each beef steak was assigned a name which included sampling week, group and replicate ID. For example, W00_1A represented beef steak replicate A withdrawn at week 0 from group 1. Each pack was opened aseptically and placed on ice. Odor assessment was performed for spoilage group beef steaks within 30 min of opening. The steaks of all groups were then massaged from outside the plastic bags for 1 min. Two milliliters of purge (meat exudate) were withdrawn from each pack and stored at −20 °C for potential volatile compounds analysis using headspace solid phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). Ten milliliters of 0.1% peptone water was added to each pack and massaged for 1 min, and then the rinsates were withdrawn. Aliquots of 500 μl of the rinsates from each steak in the spoilage group were stored at −20 °C for microbial profiling using sequencing methods. A 700 μl portion of rinsates from each steak was mixed with 300 μl of glycerol solution (50%, v/v) and stored at −20 °C for potential isolation of bacterial strains.

2.4. pH measurement and odor assessment

The potential effect of *C. maltaromaticum* A5 on meat odor was investigated by assessing the off-odor intensity of spoilage group beef steaks. The assessment was performed by an experienced five-member panel as described by Gill and Badoni (2002) with slight changes. Each beef steak was assigned a score of 1 (no off-odor), 2 (slight off-odor), 3 (moderate off-odor) or 4 (strong off-odor). Four types of off-odors were listed in the survey form, which included stale, acid, dairy and putrid. If one person sensed a type of off-odor for a beef steak, the steak was assigned with a "+" for this type of odor or a "-" otherwise. The "+" ratio of an odor among all data points (from all people in the

Table 1
Inoculation levels of the three bacterial strains used in this study.

		Spoilage group				Pathogen group			
		1	2	3	4	5	6	7	8
Aimed inoculation level ($\log_{10}$ CFU/cm^2)	*C. maltaromaticum*	0	0.5	1	2	0	0.5	1	2
	E. coli O157:H7	[b]	\	\	\	2	2	2	2
	S. Typhimurium	\	\	\	\	2	2	2	2
Estimated inoculation level[a] ($\log_{10}$ CFU/cm^2)	*C. maltaromaticum*	0	0.7	1.2	2.2	0	0.7	1.2	2.2
	E. coli O157:H7	\	\	\	\	2.2	2.2	2.2	2.2
	S. Typhimurium	\	\	\	\	2.3	2.3	2.3	2.3
Recovered level ($\log_{10}$ CFU/cm^2)	*C. maltaromaticum*	0	0.1 ± 0.1	0.4 ± 0.3	1.6 ± 0.1	0	0.2 ± 0.2	0.2 ± 0.3	1.3 ± 0.1
	E. coli O157:H7	\	\	\	\	1.5 ± 0.1			
	S. Typhimurium	\	\	\	\	1.4 ± 0.02			

[a] The estimated inoculation level was calculated based on the population density of the overnight cultures of three strains used for inoculation.
[b] No inoculation.

panel) assigned to an individual beef steak was defined as a ratio of positive (RP) in this study. The pH was also measured for each steak of spoilage groups using a Fisherbrand Accumet AP115 Portable pH Meter (Fisher Scientific, Edmonton, AB, Canada).

2.5. Microbiological analysis by plating

The concentrations of total aerobic bacteria (all groups), carnobacteria (all groups), *S.* Typhimurium (pathogen groups) and *E. coli* O157:H7 (pathogen groups) were determined using conventional plating methods. Rinsates from each beef steak were 10-fold diluted and appropriate dilutions were spread-plated onto Petrifilm Aerobic Count Plates (3M, St. Paul, MN, USA), Cresol Red Thallium Acetate Sucrose Inulin Agar (CTSI) (Wasney, Holley, & Jayas, 2001), Xylose Lysine Deoxycholate Agar (XLD; Oxoid) and Sorbitol MacConkey Agar supplemented with Cefixime-Tellurite (CT-SMAC; Oxoid) to enumerate total aerobic counts (TAC), carnobacteria, *S.* Typhimurium and *E. coli* O157:H7, respectively. The Petrifilm plates and CTSI were incubated at 25 °C for 72 h, and XLD and CT-SMAC plates were incubated at 35 °C for 24 h. Petrifilms bearing 20–200 colonies and the agar plates bearing 30–300 colonies were enumerated. Most of the group 1 steaks at all sampling weeks and two beef steaks of group 2 at week 0 did not have presumptive *Carnobacterium* on CTSI plates at the detection limit of −0.3 log CFU/cm^2, and a value of −0.5 log CFU/cm^2 was arbitrarily assigned to these steaks for statistical analysis.

2.6. Microbial profiling by 16S rRNA gene amplicon analysis

Meat rinsates of spoilage groups were subjected to DNA extraction using a QIAGEN DNeasy Blood & Tissue kit (QIAGEN, Toronto, ON, Canada) per manufacturer's instructions for Gram-positive bacteria. The V4 region of the 16S rRNA gene was amplified using primers 515-F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806-R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). The amplicon preparation, library construction, and sequencing were carried out by Genome Quebec (Montreal, QC, Canada). The sequencing was carried out on an Illumina MiSeq instrument (Illumina, Inc., San Diego, CA, USA) using the Illumina MiSeq Reagent Kit v2 (500 cycles). The primers in the raw sequencing reads were removed using Cutadapt v2.9 (Martin, 2011). Dada2 v1.16.0 in R v4.0.2 was used to remove potential contamination from PhiX and reads with low quality, merge paired-end reads, and assign taxonomy (Callahan et al., 2016). The forward and reverse reads were truncated to 210 and 200 bases, respectively. The RDP naïve Bayesian classifier and the SILVA 16S rRNA gene database (release 138.1) were used to assign taxonomy to the amplicon sequence variants (ASVs) (Quast et al., 2013). Package decontam v1.6.0 was used to remove likely contaminants among the ASVs (Davis, Proctor, Holmes, Relman, & Callahan, 2018). The alpha diversity of the sequenced samples was summarized using Phyloseq v1.30.0 with the Shannon and inverse Simpson diversity indices and the number of observed ASVs (McMurdie & Holmes, 2013). To account for unequal sequencing depth, variance stabilizing transformation was performed for each sample using DESeq2 package v1.26.0 (Anders & Huber, 2010; McMurdie & Holmes, 2014). A principal coordinate analysis (PCoA) was performed for the Euclidean distance for each sampling week.

2.7. SPME-GC-MS analysis of volatile compounds

The SPME-GC-MS for volatile compounds analysis was performed by the Mass Spectrometry Lab in the Department of Chemistry at the University of Alberta. A Bruker Scion 456 TQ GC-MS instrument (Billerica, MA, USA) equipped with a commercial CTC CombiPAL Autosampler (Leap Technologies, San Diego, USA) and a Phenomenex Zebron ZB-5 fused silica capillary column (30 m × 0.25 mm id × 0.25 μm film thickness) was used. A SPME fiber, DVB/CAR/PDMS-50/30μm (needle size 23-ga) (Supelco, Bellefonte, PA, USA) was used for the absorption of volatile compounds. An aliquot of 1 ml purge sample was added to a 20 ml headspace vial (Sigma, Canada) with 2 ml of 25% (w/v) NaCl and 10 μl of 6.7 ng/μl of 3, 3-dimethyl-2-butanol as an internal standard (Jaaskelainen et al., 2013). The mixture of each sample was homogenized by vortexing and then stored at −20 °C until analysis. Before injection to GC-MS, the samples were pre-incubated for SPME at 40 °C for 30 min with agitation at 250 rpm, and the injector was kept at 250 °C with split mode (5:1). The oven temperature was programmed as follows: started at 40 °C, held for 5 min; increased to 150 °C with a heating rate of 4 °C/min; increased to 250 °C with a heating rate of 30 °C/min; held for 5 min. The carrier gas was Helium at flow rate of 1 ml/min in GC and detector was mass spectrometry in full scan mode with a range of *m/z* 29–350. Bruker MS workstation (v8.2) was used for data analysis. VOCs identification in the samples was done using used NIST 2011 as database profiling and also compared with previous studies on vacuum-packaged beef (Casaburi et al., 2011; Dainty, Edwards, & Hibbard, 1989; Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; Ferrocino et al., 2013; Hernandez-Macedo et al., 2012; Yang, Balamurugan, & Gill, 2009).

2.8. Identification of predominant bacterial strains in the final microbiota

Bacterial species that were dominant in the microbiota on beef steaks W12_1A-W12_1C and W12_4A-W12_4C were identified using both whole metagenome sequencing (WMS) and whole genome sequencing (WGS). To recover predominant bacterial strains, the glycerol stock of rinsates obtained from the six beef steaks were thawed and serially diluted using 0.1% peptone water. An aliquot of 100 μl of the 10^6, 10^7, 10^8-fold dilutions was each spread-plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid) and incubated at 25 °C for 48h. The reason for using MRS was that the predominant genera were lactic acid bacteria as determined by 16S rRNA gene amplicon analysis. One colony was picked from each agar plate bearing well-separated colonies at the highest dilution. Six colonies in total were picked from six rinsate samples and sub-cultured in BHI at 25 °C for 24h. The DNA of these isolates and from six rinsates was extracted as described before and subjected to WMS and WGS by Genome Quebec. The sequencing libraries were constructed using a NEBNext Ultra™ II DNA Library Prep Kit and were sequenced for 300 cycles on a SP flow cell of an Illumina NovaSeq 6000 instrument. The target sequencing depth for WGS was >100X coverage per genome, while the target number of reads for WMS was >1 Gb/sample. FastQC v0.11.9 and Bowtie2 v2.4.2 were used to examine the quality of sequencing reads and remove contamination from PhiX and the host genome (*Bos taurus* DNA; reference: GCF_002263795.1 in the Assembly database of the National Center for Biotechnology Information (NCBI)), respectively (Andrews, 2010; Langmead & Salzberg, 2012). Trimmomatic v0.39 was used to remove adapter sequences, reads with a quality score of <20 over a 4-bp sliding window and reads shorter than 50 bp (Bolger, Lohse, & Usadel, 2014). The metagenomes and genomes were assembled using SPAdes v3.14.1 with k-mers set at 21, 33, 55, 77 and 99 bases with (WMS) or without (WGS) using the option "-meta" (Bankevich et al., 2012).

For WMS, contigs shorter than 500 bp were removed using a python script (Douglass et al., 2019). Bowtie2 was used to map sequencing reads to contigs and MetaBAT2 v2.12.1 was used to bin contigs into metagenome-assembled genomes (MAGs) (Kang et al., 2019). The taxonomy of each MAG was assigned using GTDB-Tk v1.7.0 with the GTDB database v2022-04-08 (Chaumeil, Mussig, Hugenholtz, & Parks, 2020). The completeness and contamination of each MAG was assessed using CheckM v1.1.3 (Parks, Imelfort, Skennerton, Hugenholtz, & Tyson, 2015). The MAGs with completeness <95% and contamination >5% were removed. The presence of bacteriocin-encoding gene clusters in the remaining MAGs was determined using Bagel4 (van Heel et al., 2018). The relative abundance of bacterial species in the metagenomes was calculated using MetaPhlan v3.0.13 with the trimmed sequencing reads as input (Beghini et al., 2021).

For WGS, contigs with less than 10X coverage or shorter than 500 bp were removed. The species identity of each isolate was determined using GTDB-Tk. Each genome was ordered using Mauve v2015-02-13 with *L. sakei* DSM 20017 (Accession no. GCF_002370355.1 in the Assembly database of NCBI) as a reference genome (Darling, Mau, Blattner, & Perna, 2004). To identify the subspecies identity of each isolate, the average nucleotide identities (ANI) between the six genomes and *L. sakei* genomes with known subspecies identity in NCBI were calculated using fastANI v1.32 (Jain, Rodriguez-R, Phillippy, Konstantinidis, & Aluru, 2018). The presence of bacteriocin-encoding gene clusters in the six genomes was detected using Bagel4.

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed to compare bacterial population density, alpha diversity, pH, odor density, and concentration of volatile compounds among groups and sampling weeks. Sidak method was used for post hoc pairwise comparison. A significance level of 0.05 was used for all these analyses.

For the 16S rRNA gene amplicons, permutational multivariate analysis of variance (PERMANOVA) was carried out on the Euclidean distances using the function adonis2() of Vegan v2.5.6 to test the overall difference among groups (Jari Oksanen et al., 2020). Post hoc pairwise comparisons were performed using PairwiseAdonis v0.4 (https://github.com/pmartinezarbizu/pairwiseAdonis). The difference with $P < 0.05$ was regarded as significant. Figures were plotted using ggplot2 v3.3.5 (Wickham, 2016).

3. Results

3.1. The bacterial population density on VP beef steaks

The mean storage temperature during the 12 weeks was 1.9 ± 0.1 °C, with slight fluctuations during sampling at bi-weekly intervals (Fig. S1). Compared to the estimated inoculation levels, lower numbers of the three bacterial strains were recovered from inoculated beef steaks, with *Carnobacterium* at 0.1 ± 0.1, 0.4 ± 0.3, and 1.6 ± 0.1 log CFU/cm^2 for groups 2–4 steaks and at 0.2 ± 0.2, 0.2 ± 0.3, and 1.3 ± 0.1 log CFU/cm^2 for groups 6–8 steaks, and *E. coli* O157:H7 and *S.* Typhimurium at 1.5 ± 0.1 log CFU/cm^2 and 1.4 ± 0.02 log CFU/cm^2 for groups 5–8 (Table 1).

Carnobacterium spp. were not recovered from most (40/42) un-inoculated steaks (groups 1 and 5) (Figs. 1A and 2A), indicating that the *Carnobacterium* recovered from groups 2–4 and 6–8 steaks were mainly from inoculation rather than the native microbiota. Despite the difference in the initial numbers, *Carnobacterium* on all inoculated groups reached stationary phase at around week 6 with a similar maximum population density of approximately 7 log CFU/cm^2. Compared to their respective un-inoculated control (groups 1 and 5), group 2–4 and 6–8 steaks showed higher aerobic counts at weeks 2, 4, and 8 (group 2–4) or 6 and 8 (group 6–8), but there was no significant difference for other weeks (Figs. 1B and 2B). The final aerobic counts for all groups at week 12 were 8.1–8.7 log CFU/cm^2. The proportions of carnobacteria in total aerobic counts for groups 2–4 steaks were 1.9, 38.8, and 71.0% initially, increased to 90.5, 83.9 and 90.0% at week 2 and gradually decreased to 5.4, 19.3 and 1.7% by week 12 (Fig. 3A).

The group 6–8 steaks were also inoculated with both *E. coli* O157:H7 and *S.* Typhimurium to investigate whether *C. maltaromaticum* A5 could inhibit the growth of these two pathogens. The numbers for both pathogens decreased during storage, but no inhibition by *C. maltaromaticum* A5 was observed (Fig. 2C and D).

3.2. 16S rRNA gene amplicon analysis

The impact of *C. maltaromaticum* A5 on the dynamics of the meat microbiota was also assessed using 16S rRNA gene amplicon sequencing. On average, 31,024 sequences/sample were obtained from the 84 samples. The initial microbiota on all four groups of beef steaks at week 0 mainly had higher alpha diversity than at other weeks in terms of ASV richness (number of observed ASVs) and diversity (Shannon and inverse Simpson) ($P < 0.05$; Fig. S2). The alpha diversity among groups differed significantly for all three indices at week 2; however, no differences were observed for any of the other weeks. There was an overall significant difference among groups at most sampling weeks except for weeks 0 and 8 as determined by PERMANOVA and group 1 tended to be separated from other groups in the PCoA (Fig. S3). However, the more conservative pairwise comparison did not find significant between-group differences at any of the sampling weeks. For week 0 steaks, 1.6–68.5% of the 16S rRNA gene sequence amplicons were not assigned to a bacterial genus (Fig. S4). The 10 most relatively abundant genera included *Staphylococcus, Clostridium, Escherichia, Carnobacterium, Rhodococcus, Veillonella, Rhodanobacter, Pseudomonas, Jeotgalicoccus* and *Dyella* (Fig. S4). From week 4 for group 1 or week 2 for groups 2–4 onwards, the microbiota was mainly dominated by *Latilactobacillus* and/or *Carnobacterium* on most beef steaks, with a considerable proportion of *Leuconostoc* and *Pseudomonas* on five (W04_1B, W08_2A, W08_3A, W10_1A, and W12_1C) and two (W10_1B and W10_1C) beef steaks, respectively (Fig. 4). The relative abundance of *Carnobacterium* for group 2–4 beef steaks inferred from 16S rRNA gene sequencing data showed a similar trend to the relative abundance determined by the plating method, i.e., the proportion of *Carnobacterium* increased from week 0 to week 2 and decreased there onwards (Fig. 3). Even so, the fraction of *Carnobacterium* in the initial total population determined (0, 1.9, 38.8, and 71.0% for groups 1–4) by plating was much higher than by sequencing (0.0, 0.0, 1.0, and 14.8%, respectively). This difference diminished with storage time. Nevertheless, *C. maltaromaticum* was able to establish dominance during early storage of VP meat, even when its initial relative abundance was below 1% of the total population (groups

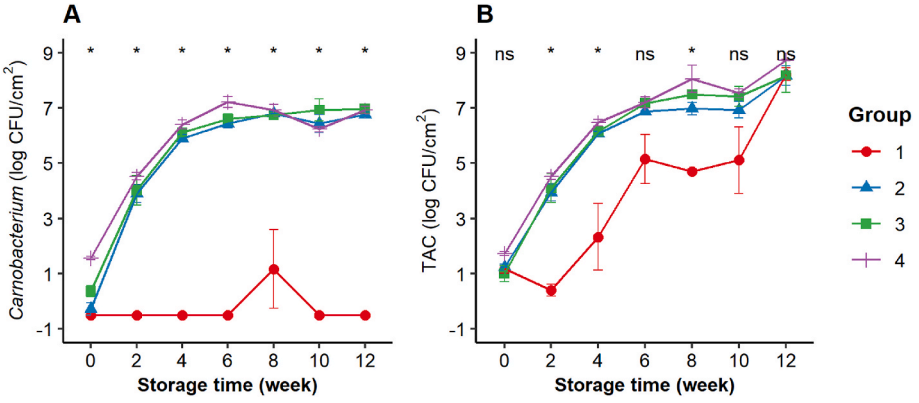

Fig. 1. The cell counts of *Carnobacterium* spp. (A) and total aerobes (TAC; B) on vacuum-packaged beef steaks of groups 1–4 during storage at 2 °C. The steaks were inoculated with 0, 0.7, 1.2 or 2.2 log CFU/cm^2 of *Carnobacterium maltaromaticum* A5. Error bars represent the standard error of the mean of three replicates. ANOVA was performed to compare the groups at each sampling week, and a "*" is shown if there is a significant between-group difference ($P < 0.05$) or "ns" is shown otherwise.

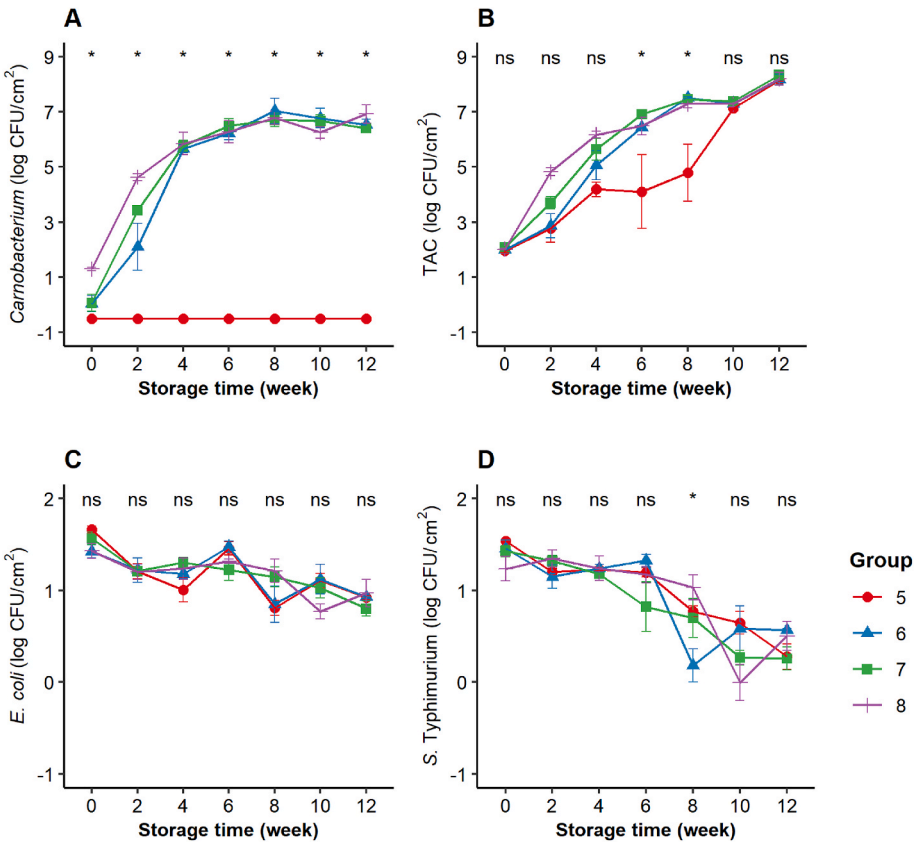

Fig. 2. The cell counts of *Carnobacterium* spp. (A), total aerobes (TAC; B), *Escherichia coli* O157:H7 (C), and *Salmonella* Typhimurium (D) on groups 5–8 vacuum-packaged beef steaks during storage at 2 °C. The steaks were inoculated with 0, 0.7, 1.2 or 2.2 log CFU/cm^2 of *Carnobacterium maltaromaticum* A5. All steaks were also inoculated with 2.2 log CFU/cm^2 of *E. coli* O157:H7 and 2.3 log CFU/cm^2 of *S.* Typhimurium. Error bars represent the standard error of the mean of three replicates. ANOVA was performed to compare the groups at each sampling week, and a "*" is shown if there is a significant between-group difference ($P < 0.05$) or "ns" is shown otherwise.

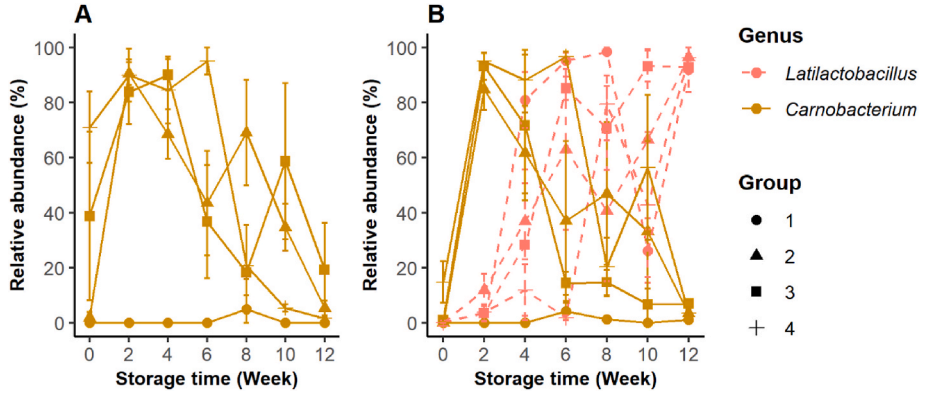

Fig. 3. The relative abundance of *Carnobacterium* and *Latilactobacillus* on group 1–4 beef steaks as determined by plate counting (A) or 16S rRNA gene amplicon sequencing (B).

2 and 3). Interestingly, the relative abundance of *Latilactobacillus* in the initial microbiota of all four groups was 0.1–0.5%, but it eventually increased to 75.7–100% at week 12, displacing *Carnobacterium* (Figs. 3B and 4).

3.3. Predominant bacterial species in the final microbiota

To better understand the predominate bacterial species in the final microbiota, WMS was performed for week 12 beef steaks from groups 1 and 4 (n = 6). A total of 113,542,136–422,436,328 bp were obtained from each sample after removing host DNA (cattle) and reads with low quality. The microbial composition determined by WMS and 16S rRNA gene amplicon sequencing was similar (Figs. 4 and 5A). From WMS analysis, *L. sakei* accounted for 67.2–100% of the microbiota on these steaks (Fig. 5A). Beef steak W12_1C had a considerable proportion of *Leuconostoc carnosum* (26.9%) and *Carnobacterium divergens* (5.9%). Binning of contigs failed for one sample from group 1 steaks (W12_1A), which was likely attributable to low coverage of the bacterial genome caused by the presence of a high quantity of host DNA. All MAGs with completeness >95% and contamination <5% were assigned to bacterial species. Interestingly, all these MAGs had bacteriocin-encoding genes (Fig. 5B). Presumptive *Latilactobacillus* spp., based on 16S rRNA gene amplicon sequencing analysis, were recovered from week 12 beef steaks. The six isolates sequenced by WGS and the MAGs assigned to *L. sakei* had between-genome ANI values of >99.99% (Table S1). These isolates/MAGs were further identified as *L. sakei* subsp. *carnosus* based on the ANI between their genomes and *L. sakei* genomes with known sub-species identity in NCBI (Table S1). Both genomes and MAGs of *L. sakei* harbored genes synthesizing a putative class IIc bacteriocin. A sakacin G encoding gene cluster was also found in the genomes, but not in the

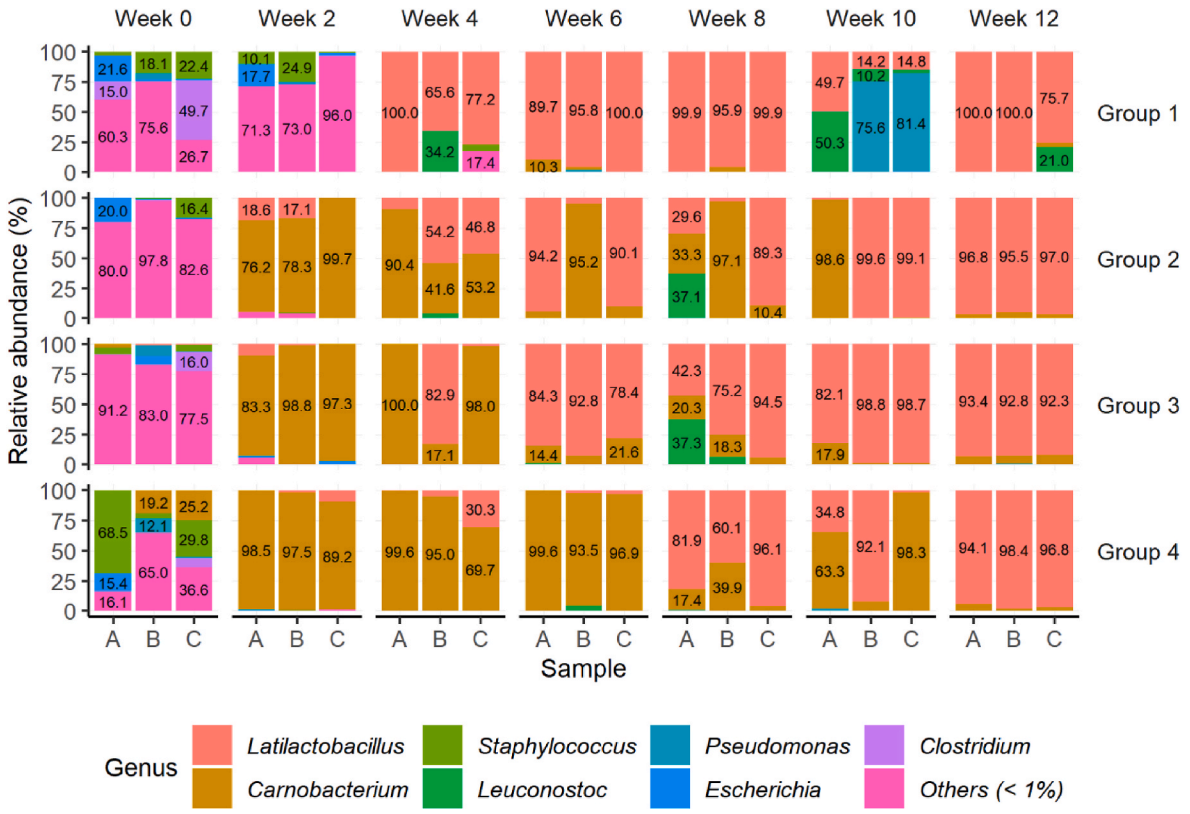

Fig. 4. The relative abundance of bacterial genera on beef steaks of groups 1–4. The relative abundance is shown for all three replicates ("A", "B" and "C") of each group from each sampling time. The bars in the same row and column are for steaks from the same group and sampling week, respectively. Genera with a the relative abundance of less than 1% in all samples were categorized into "Others" in the figure. The percentage value is labeled for each genus with a relative abundance >10%. The group 1–4 steaks were, respectively, inoculated with 0, 0.7, 1.2 and 2.2 log CFU/cm^2 of *Carnobacterium maltaromaticum* A5, vacuum-packaged and stored at 2 °C.

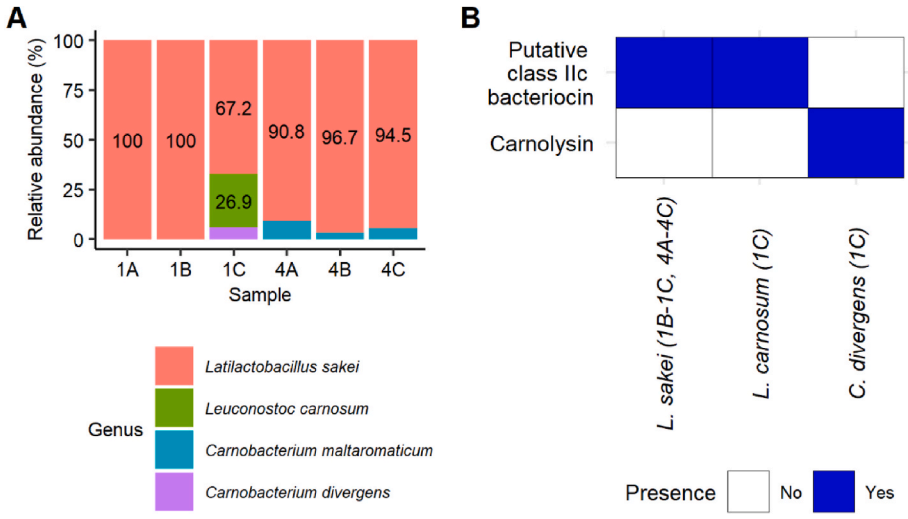

Fig. 5. The relative abundance of bacterial species on (Panel A), and the bacteriocins predicted in metagenome-assembled genomes from (Panel B) group 1 and 4 vacuum-packaged beef steaks stored at 2 °C for 12 weeks. In panel A, the relative abundance was calculated using trimmed whole metagenome sequencing reads and is shown for all three replicates ("A", "B" and "C") of each group. The percentage value is labeled for the species with >10% of relative abundance. In panel B, the group and replicate of beef steaks containing a corresponding bacterial species are labeled in parenthesis after each species name.

MAGs.

3.4. Odor assessment and SPME-GC-MS analysis

The pH of all spoilage group beef steaks was 5.6 ± 0.1 and no significant differences among the four groups or storage times were observed (Fig. S5A). The scores for odor intensity increased during storage; however, no significant differences among the groups in odor intensity were observed (Fig. S5B). The ratio of positive of each odor type did not differ ($P > 0.05$) between groups, but it increased from week 0 (mean, 0.02) to week 12 (0.09) with more putrid odor (mean, 0.09) identified followed by stale (0.08), acid (0.04) and dairy (0.03) (Fig. S6).

To investigate whether volatile compounds differed among beef steaks inoculated or not with *C. maltaromaticum* A5, SPME-GC-MS analysis was performed for the purge samples from group 1 and 4 beef steaks throughout storage. A total of 28 compounds had been identified in GC-MS analysis (Fig. S7). A number (n = 11) of compounds showed

relatively lower concentration in group 4 than group 1 beef steaks even at the commencement of storage for unclear reason. Throughout the storage, there was no significant change of the difference between two groups (panels with red frame line in Fig. S7). For other compounds, group 4 steaks mostly had similar concentrations compared to group 1.

4. Discussion

The present study investigated the impact of *C. maltaromaticum* A5 spiked at 0.5–2 log CFU/cm^2, natural contamination levels that would be expected on beef at production, on the dynamics of the microbiota on beef steaks and the survival of *E. coli* O157 and *S.* Typhimurium during chilled (2 °C) storage for up to 12 weeks. This *C. maltaromaticum* strain can inhibit the growth of spoilage-associated bacteria and foodborne pathogens *in vitro* (15 °C, anaerobic), resulting from the production of bacteriocins (carnobacteriocin B1, B2 and/or carnolysin) and organic acids (formic acid and/or acetic acid) (Zhang et al., 2019).

Both conventional plating and 16S rRNA gene amplicon sequencing methods were used to determine the dynamic change of microbiota on VP beef steaks. The former method showed a much higher proportion of *Carnobacterium* in the initial bacterial population than the latter one, suggesting that a large fraction of the initial microbiota may not be cultivable under the conditions routinely used for recovering bacteria from meat. The bias of 16S rRNA gene PCR primers for certain bacterial species may be another factor contributing to the relatively lower percentage detected by 16S rRNA gene sequencing (Jovel et al., 2016). Even so, both methods demonstrated that *C. maltaromaticum* A5 became dominant in the early storage in a manner independent of its concentration in the initial microbiota, indicating that the VP chilled beef environment favors the growth of this species. Interestingly, the predominant status of *C. maltaromaticum* was gradually superseded by *Latilactobacillus* spp. Published relevant studies have mainly characterized the dynamic change of the microbial community on chilled VP beef to the genus instead of species level. In these studies, *Carnobacterium* spp. gradually became dominant and either maintained its predominant status until the end of storage or were displaced by *Latilactobacillus* or other LAB species (Chen et al., 2019; Chen et al., 2020; Mandeep Kaur, Bowman, Porteus, Dann, & Tamplin, 2017; Yang et al., 2021; M. K. Youssef, Gill, Tran, & Yang, 2014; M. K. Youssef, Gill, & Yang, 2014). These findings suggest that the initial microbiota on VP beef played an important role in the fate of introduced LAB strains and vice versa as the outcome of predominance is dictated by the inherent properties of indigenous LAB strains and the incoming strain(s).

The population density of both *S.* Typhimurium and *E. coli* O157:H7 decreased during storage. Nevertheless, no significant differences were observed between groups with or without inoculated *C. maltaromaticum*. The low storage temperature (2 °C) is likely one of the important factors leading to the decrease in concentration of both pathogens. The reduction of *Salmonella* (in Trypticase Soy Broth with 0.6% Yeast Extract, TSBYE) and *E. coli* O157:H7 (in BHI and chicken slurry) at 4 °C has been reported in previous studies (Conner & Kotrola, 1995; Morey & Singh, 2012). It is also possible that the effect of *C. maltaromaticum* in the present study on the two pathogens has been masked by the potential inhibitory activity of the indigenous *L. sakei*, as *L. sakei* gradually became dominant irrespective of *C. maltaromaticum* inoculation. It has been reported that *L. sakei* showed an inhibition effect on the growth of *E. coli* and *S.* Typhimurium in ground beef under chilled vacuum or modified atmosphere packaging (S. Chaillou et al., 2014). Hu et al. (2019) investigated the effect of chitosan and *C. maltaromaticum* on the growth of *E. coli* on refrigerated VP lean beef and observed a decrease in *E. coli*. A higher level of inoculation may be necessary for *C. maltaromaticum* A5 to show inhibitory activity or to compete with other bacterial species in the microbial community. Nilsson et al. (2004) found that a higher initial level of *C. maltaromaticum* (10^3 vs. 10^6 CFU/ml) resulted in a larger log reduction (3 vs. 5) in the maximum population density of *Listeria monocytogenes* in a cold-smoked salmon model system. Similarly, the inoculated beef steaks did not show any significant differences in either odor intensity or volatile compounds profile. These findings were in agreement with a study by Casaburi et al. (2011), which found that the contribution to the spoilage odor of beef stored in air or under VP by 45 *C. maltaromaticum* strains at initial level of 10^3–10^4 CFU/g was negligible.

The predominant *Latilactobacillus* strain was identified to be *L. sakei* subsp. *carnosus*. The displacement of *C. maltaromaticum* A5 by *L. sakei* could be attributable to one or a combination of the following factors: better acid tolerance, faster growth rate and bacteriocin production potential of *L. sakei*. *L. sakei* is a species often found on VP meat (pH is often <5.7) or fermented meat products and the metabolism of this species is believed to be well adapted to what meat can provide (Stéphane Chaillou et al., 2005; Torriani et al., 1996; Zheng et al., 2020). *L. sakei* has been found to be dominant in the microbiota of chilled VP pork (4 °C) or beef (6 °C) at the end of storage (Jääskeläinen, Hultman, Parshintsev, Riekkola, & Björkroth, 2016; Jiang et al., 2010). In a study by Leisner, Greer, Dilts, and Stiles (1995), a *L. sakei* strain had a much higher growth rate than two *C. maltaromaticum* strains inoculated on sterile VP beef slices stored at 2 °C. The *L. sakei* subsp. *carnosus* strain recovered in the present study harbors two gene clusters for bacteriocin production including sakacin G and other putative bacteriocins. Sakacin G is a class IIa bacteriocin showing anti-listerial activities (Todorov et al., 2011). We investigated the mutual inhibitory activity of the recovered *L. sakei* subsp. *carnosus* strain and *C. maltaromaticum* A5 under VP beef relevant conditions using a spot-lawn assay (BHI agar, pH 5.5 ± 0.2, anaerobic, 15 °C, Fig. S8). The *L. sakei* subsp. *carnosus* strain inhibited *C. maltaromaticum* A5 but the latter strain did not show inhibition of the former. Numerous studies have explored using *L. sakei* to control the growth of pathogenic bacteria such as *L. monocytogenes* and *Salmonella* (Barcenilla et al., 2022), however, there are limited studies on the contribution of *L. sakei* to meat spoilage. The predominance status of *L. sakei* indicates it may play a major role in shaping the microbiota of VP beef steaks during chilled storage. In addition, bacteriocin-encoding genes were found in all the MAGs with completeness >95% (from the beef steaks at the end of storage), indicating the important role played by bacteriocins in the competition of host bacterial strains against closely related LAB species on VP meat.

5. Conclusion

The findings in this study demonstrate *C. maltaromaticum* A5 at natural contamination levels on VP beef was outcompeted by an indigenous *L. sakei* strain during chilled storage under vacuum. It did not affect meat spoilage odor or the growth of *E. coli* O157:H7 or *S.* Typhimurium. The *L. sakei* strain played a more important role than *C. maltaromaticum* in shaping the meat microbiota. Caution should be given when discussing or comparing the succession of LAB on VP meat, or rather in general, as differences in the initial microbiota resulting from different processing facilities, incoming animals, downstream manipulations etc. may also have significant effects, in addition to the conditions that VP meat is subjected to immediately before packaging.

Chemical compounds studied in this article

Sakacin G (PubChem CID: unknown); Acetoin (PubChem CID: 179); 1-Octen-3-ol (PubChem CID: 18827); 2,7-Dimethyloctane (PubChem CID: 14070); 2-pentyl-Furan (PubChem CID: 19602).

CRediT authorship contribution statement

Peipei Zhang: Methodology, Formal analysis, Writing – original draft. **Eric Ruan:** Methodology, Writing – review & editing. **Devin B. Holman:** Methodology, Writing – review & editing. **Xianqin Yang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

None.

Data availability

The sequencing data obtained in this study have been deposited under the BioProject PRJNA786256 in NCBI. The accession numbers for 16S rRNA gene amplicon and WMS sequencing reads, MAGs are SRR17223157-SRR17223242 and SRR17227705-SRR17227710, and JAMQER000000000-JAMQEX000000000, respectively. The accession numbers for sequencing reads and genomes for six recovered *L. sakei* isolates are SRR17133724-SRR17133729 and JAJSCQ000000000-JAJSCV000000000, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113944.

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