Sustainable Food Technology

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d6fb00022c

Modulating spoilage microbiota and antimicrobial susceptibility in brown bear (*Ursus arctos*) shoulder meat (*musculus triceps brachii*) using monoterpene-enriched alginate coatings

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This study presents the first detailed characterization of microbial succession in brown bear (*Ursus arctos*) shoulder meat during refrigerated vacuum storage and the first evaluation of the modulating effects of pure monoterpenes applied *via* alginate coatings. Vacuum-packaged meat was treated with sodium alginate coatings containing monoterpenes (α-pinene, β-pinene, bornyl acetate, D-carvone, 3-carene, limonene, sabinene; 0.03% v/v) and stored at 4 °C for 7 days. Microbial dynamics were monitored by culture-based enumeration (log CFU g^{-1}) and species-level identification using MALDI-TOF MS. The initial microbiota, dominated by Enterobacteriaceae (50% on day 0, 71% on day 3), comprised the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia* and *Lelliottia*. Vacuum storage led to complete elimination of obligate aerobic *Pseudomonas* and *Acinetobacter* by day 3, followed by a pronounced shift towards lactic acid bacteria (LAB), which accounted for 35% of the microbiota by day 7. Monoterpene-enriched coatings tended to result in higher LAB counts (5.09–5.16 log CFU g^{-1}) compared to the control (4.92 log CFU g^{-1}), indicating suppression of Gram-negative spoilage bacteria rather than broad antimicrobial inhibition. All 36 bacterial species isolated from bear meat exhibited complete susceptibility to tested antibiotics (inhibition zones 20.33–54.33 mm), providing useful baseline data on wildlife-associated microbiota unexposed to anthropogenic antibiotic pressure. These findings suggest that monoterpenes may act as selective modulators of microbial succession, highlighting their potential for controlled preservation.

Received 18th January 2026
Accepted 1st April 2026

DOI: 10.1039/d6fb00022c

rsc.li/susfoodtech

Sustainability spotlight

This study contributes to sustainable food systems by developing natural preservation strategies for underexplored game meat. Brown bear meat represents a nutritionally valuable but insufficiently studied food resource, for which comprehensive data on microbial dynamics and preservation are lacking. The use of edible alginate coatings enriched with natural monoterpenes enables selective modulation of spoilage microbiota without synthetic preservatives, supporting food safety, reducing food waste, and promoting environmentally responsible processing of game meat derived from regulated wildlife management.

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1 Introduction

Brown bear meat (*Ursus arctos*) represents a nutritionally valuable yet underexplored game product characterized by a distinctive nutrient profile. Although bear meat consumption represents a niche market compared to conventional meats, it is traditionally consumed in several European and Asian regions where brown bear hunting is part of regulated wildlife management. In Slovakia, bear hunting is governed by the Game Management Act.[1] Despite its nutritional advantages, the processing and consumption of bear meat pose significant microbiological risks. In addition to bacterial contamination, the presence of parasites of the genus *Trichinella* represents a major safety concern, as these parasites can survive prolonged freezing and require thorough heat treatment to ensure consumer safety.[2–4] Kärssin *et al.*[3] reported a 14.7% prevalence of *Trichinella* spp. in brown bears in Estonia, identifying *T. britovi* and *T. nativa*, while Murakami *et al.*[4] detected *Trichinella* T9 in 2.5% of brown bears in Japan. Proper preservation strategies are therefore essential for both shelf-life extension and microbiological safety of this traditional game meat. In contrast to commercially produced meats, however, comprehensive data on the microbial quality and spoilage dynamics of bear meat remain extremely limited, and systematic microbiological characterizations of this raw material are scarce.

Meat represents an important component of the human diet due to its high content of proteins, essential amino acids, lipids, vitamins, and minerals. While most research has traditionally focused on commercially important meat types such as poultry, beef, and pork, there is a growing interest in less conventional sources of animal protein, including game meat, driven by changing consumer preferences and growing interest in alternative protein sources.[5] Game meat is often associated with lower environmental impact, natural feeding regimes, and reduced reliance on intensive farming systems. Kelava Ugarković *et al.*[6] reported that brown bear meat contains approximately 6% fat, increasing to about 9% in older individuals, while protein, dry matter, and ash contents are comparable to those of other game species. The fatty acid profile is dominated by monounsaturated fatty acids, with oleic acid (C18 : 1n-9) being the most abundant component. Younger animals exhibit a more favorable profile of essential polyunsaturated fatty acids. Due to its high energy and protein density, 100 g of cooked bear meat provides approximately 259 kcal and up to 32 g of protein, highlighting its potential as a nutrient-rich food resource.

At the same time, there is an increasing demand for natural and safe strategies to extend meat shelf life, as the excessive use of synthetic preservatives raises concerns regarding potential health risks and consumer acceptance. Edible coatings and films enriched with bioactive compounds have emerged as an effective and sustainable approach to slow spoilage processes in fresh meat during storage. Recent studies have demonstrated that sodium alginate is among the most widely used polysaccharide-based coating materials due to its excellent film-forming properties, non-toxicity, biodegradability, and ability to incorporate natural antimicrobial agents.[7,8] From a sustainability perspective, the application of edible coatings based on renewable biopolymers represents an attractive strategy to reduce food waste and improve the shelf life of perishable products without increasing the use of synthetic additives.

Previous research has shown that the combination of alginate with essential oils or their dominant monoterpenes can significantly inhibit microbial growth, reduce lipid oxidation, and extend the shelf life of poultry, beef, and pork. Mantzourani *et al.*[9] and Karam *et al.*[10] demonstrated that monoterpenes such as thymol and carvacrol exhibit strong antimicrobial activity against key spoilage microorganisms, including *Pseudomonas* spp., *Brochothrix thermosphacta*, and lactic acid bacteria. However, the antimicrobial efficacy of individual monoterpenes may strongly depend on the meat matrix, its intrinsic properties, and the composition of the associated microbial community. To date, no studies have investigated the application of alginate-based edible coatings enriched with monoterpenes in bear meat or evaluated their effects on microorganisms naturally colonizing this underexplored raw material.

Although growing interest exists in food preservation strategies, no comprehensive data are available on the use of alginate coatings combined with natural monoterpenes to preserve bear meat, nor on the characterization of microbial communities developing on this meat during refrigerated storage. Studies on other meat types identified *Pseudomonas lundensis* and *Brochothrix thermosphacta* as key spoilage microorganisms in aerobically stored beef and pork.[11,12] However, information regarding the dominant spoilage bacteria associated with bear meat, their susceptibility to pure monoterpenes, and their antibiotic resistance profiles remains lacking, representing a significant knowledge gap with implications for both food safety and sustainable preservation.

Based on the available evidence, it was hypothesized that alginate-based edible coatings enriched with selected monoterpenes were expected to reduce microbial growth in bear meat during refrigerated storage, and that dominant bacterial isolates would exhibit differential susceptibility to individual monoterpenes as well as to conventional antibiotics. Therefore, the aim of this study was to comprehensively evaluate the effect of an alginate-based edible film enriched with selected monoterpenes on the microbiological quality of bear meat stored at 4 °C. The specific objectives were to (i) monitor the dynamics of total microbial load and selected spoilage-related microbial groups during 7 days of refrigerated storage, (ii) identify dominant bacterial species using MALDI-TOF MS, (iii) select representative isolates with the highest identification scores and assess their susceptibility to pure monoterpenes using the disk diffusion method, and (iv) determine the antibiotic susceptibility profiles of these isolates in order to compare the effectiveness of natural monoterpenes with conventional antibiotics and evaluate their potential as alternative antimicrobial agents. This study provides the first systematic characterization of microbial succession in brown bear shoulder meat and the first evaluation of the effects of pure monoterpenes applied *via* alginate coatings, linking microbial dynamics, monoterpene activity, and antibiotic susceptibility within a food technology framework.

2 Material and methods

2.1 Bear meat

The meat used in this study was obtained from a brown bear (*Ursus arctos*) harvested during legally regulated hunting in the Východná–Liptovský Mikuláš area (Slovak Republic) on 21 June 2025. The animal was an adult individual with an estimated age of approximately 10 years and a body weight of 176 kg. Hunting was conducted by a licensed hunter in accordance with Slovak legislation governing wildlife management.

Immediately after evisceration and primary field dressing, the carcass was transported under refrigerated conditions (4 ± 1 °C) to the Agrobiotech Research Centre, Slovak University of Agriculture in Nitra. Upon arrival, the shoulder muscle (*musculus triceps brachii*), weighing approximately 10 kg, was excised for further analysis. The selected muscle was chosen due to its relevance for culinary use and its representative composition in bear meat products.

In the laboratory, the muscle was aseptically portioned into experimental samples according to the experimental design described below and subsequently subjected to coating application and microbiological analyses.

2.2 Monoterpene substances and other chemicals

Pure analytical-grade monoterpenes were used as bioactive components in the alginate-based coatings. The following compounds were applied: α-pinene (purity ≥98%), (−)-β-pinene (≥99%), sabinene (≥75%), D-carvone (≥96%), (−)-bornyl acetate (≥95%), (*S*)-(−)-limonene (≥96%) and 3-carene (≥98%). All monoterpene standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sodium alginate (sodium salt of alginic acid; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as the polysaccharide matrix for the preparation of edible coatings. Glycerol (≥99.0%, p.a.; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was employed as a plasticizing agent to improve film flexibility. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, p.a.; Centralchem, Bratislava, Slovak Republic) was used as a cross-linking agent to induce ionotropic gelation of the alginate coatings.

Polysorbate 80 (Tween 80; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added as an emulsifier to ensure homogeneous dispersion of the hydrophobic monoterpenes in the aqueous coating-forming solutions.

Sterile distilled water (conductivity <2 μS cm^{-1}) and sterile 0.85% (w/v) sodium chloride solution (Aloqeunce, Vráble, Slovak Republic) were used for the preparation of all solutions, emulsions, and microbial suspensions.

2.3 Preparation of the sodium alginate-based coating-forming solution

The sodium alginate-based coating-forming solution was prepared in a final volume of 2000 mL. Sterile distilled water (1600 mL) was transferred into a clean 2 L glass vessel. Sodium alginate (24.00 g; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was weighed using an analytical balance (Kern ABJ 220-4NM, Kern & Sohn GmbH, Balingen, Germany) and gradually dispersed into the water under continuous manual stirring with a glass rod to minimize the formation of lumps and undissolved agglomerates. After complete dispersion of the alginate powder, the solution volume was adjusted to 2000 mL with sterile distilled water.

The vessel was subsequently stored at 4 °C for 24 h to allow complete hydration and dissolution of the polymer, resulting in a homogeneous solution free of visible particles. Glycerol (7.20 g; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), corresponding to 30% (w/w) of the alginate mass, was then added to the hydrated alginate solution. The mixture was placed in a water bath at 30 °C and gently stirred manually with a glass rod until complete homogenization of the plasticizer within the solution was achieved. The final coating-forming solution contained 1.2% (w/v) sodium alginate and 0.36% (w/v) glycerol.

2.4 Preparation of coating-forming baths with monoterpenes

From the prepared alginate-based coating-forming solution, eight separate portions of 200 mL each were transferred into sterile glass vessels (Simax, Kavalier Glass a.s., Sázava, Czech Republic). One portion served as the control coating without monoterpenes (CA – sodium alginate coating), and no bioactive component was added.

For the remaining seven portions, individual monoterpene stock solutions were prepared. Each monoterpene (α-pinene, (−)-β-pinene, sabinene, D-carvone, (−)-bornyl acetate, (*S*)-(−)-limonene, and 3-carene) was handled using an automatic micropipette (Eppendorf Research plus, Eppendorf AG, Hamburg, Germany). Pure monoterpene (100 μL) was pipetted into a graduated test tube and diluted with a 0.5% (v/v) polysorbate 80 (Tween 80) solution prepared in sterile distilled water to a final volume of 5.00 mL. The stock solution was immediately vortexed (Biosan V-1 plus, Biosan, Riga, Latvia) for 30 s at 2500 rpm to ensure homogeneous dispersion of the monoterpene within the emulsion. Monoterpene stock solutions were prepared immediately before use (within 15 min) and used within the same working session to minimize losses of volatile components.

Subsequently, 3.00 mL of the corresponding monoterpene stock solution was added to each 200 mL coating-forming bath, resulting in a final monoterpene concentration of 0.03% (v/v). After addition, the baths were gently mixed by manual swirling and then sonicated in an ultrasonic bath (KRAINTEK 5, Kraintek s.r.o., Bratislava, Slovak Republic) for 5 min at 25 ± 2 °C to ensure uniform monoterpene distribution throughout the alginate solution. The control bath (CA) was prepared from the same alginate–glycerol solution without the addition of monoterpene stock solution or polysorbate 80.

2.5 Application of the alginate coating to meat samples

Prior to coating application, individual portions of bear meat (25.0 ± 1.0 g) were equilibrated to laboratory temperature (20 ± 2 °C) for approximately 10 min. The samples were then gently blotted dry with sterile absorbent paper towels to remove excess

surface moisture and to improve adhesion of the coating-forming solution.

Each meat portion was subsequently immersed into the corresponding coating-forming bath (CA or CA supplemented with a monoterpene) using sterile stainless-steel tweezers and maintained in the solution for 60 s. Gentle movement of the tweezers during immersion ensured complete surface coverage and facilitated the removal of air bubbles from the meat surface.

After immersion, the samples were removed from the coating bath and placed on a sterile stainless-steel grid (mesh size 5 × 5 mm) positioned above a collection vessel. The samples were allowed to drain for 2 min at laboratory temperature to remove excess alginate solution and to form a uniform pre-gel coating layer on the meat surface.

2.6 Cross-linking of the alginate coating

Following the draining step, the coated meat samples were immediately immersed in a 1.5% (w/v) calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) solution for 3 min at laboratory temperature (20 ± 2 °C) to induce gelation of the alginate coating through ionic cross-linking with Ca^{2+} ions. The cross-linking solution was freshly prepared each day by dissolving the appropriate amount of $CaCl_2 \cdot 2H_2O$ in sterile distilled water.

After cross-linking, the samples were removed using sterile stainless-steel tweezers and placed on a sterile stainless-steel grid positioned above a collection vessel for 2 min to allow removal of excess $CaCl_2$ solution and the formation of a stable and uniform coating film on the meat surface.

2.7 Vacuum-packaging and storage

After the draining step, all samples, including the uncoated control, were vacuum-packaged in three-layer PA/PE barrier vacuum bags (150 × 200 mm, thickness 90 μm, oxygen permeability <50 $cm^3\ m^{-2}$ per 24 h; Chosen, Czech Republic) using a benchtop vacuum-packaging machine (Lavezzini ECO-VAC ECO-STAR, Lavezzini S.r.l., Fiorenzuola d'Arda, Italy). The vacuum level was set to 99.5%, corresponding to a residual pressure below 5 mbar.

Each package was labelled using a water-resistant permanent marker with a code indicating the treatment type (C – uncoated control, CAC – alginate coating without monoterpene, CA + α, CA + β, CA + BA, CA + D-car, CA + 3-car, CA + L, and CA + S for alginate coatings containing individual monoterpenes), the packaging date, and the planned sampling day.

Immediately after labelling, the packaged samples were transferred to a refrigerated storage chamber and stored at 4 ± 1 °C for the entire duration of the experiment until microbiological analyses were performed. Storage temperature was continuously monitored using a digital thermometer with an accuracy of ± 0.1 °C.

2.8 Experimental design

Bear meat portions were divided using a sterile knife into individual samples weighing 25.0 ± 1.0 g, with efforts made to achieve maximum uniformity in sample size and shape. Individual portions were randomly assigned, using a random number generator, to nine experimental groups according to surface treatment:

(1) Group C (uncoated control) – no coating applied.

(2) Group CAC (sodium alginate coating) – alginate coating without monoterpene.

(3) Group CA + α – alginate coating containing α-pinene.

(4) Group CA + β – alginate coating containing (−)-β-pinene.

(5) Group CA + BA – alginate coating containing (−)-bornyl acetate.

(6) Group CA + D-car – alginate coating containing D-carvone.

(7). Group CA + 3-car – alginate coating containing 3-carene.

(8) Group CA + L – alginate coating containing (*S*)-(−)-limonene.

(9) Group CA + S – alginate coating containing sabinene.

All monoterpene-containing coatings were prepared at the same final monoterpene concentration of 0.03% (v/v) in the coating-forming solution.

For each experimental group and sampling day, three independent biological replicates ($n = 3$) were prepared, with each replicate corresponding to a separately vacuum-packaged meat portion. The total number of samples analysed in the experiment was 108 (9 treatment groups × 4 sampling time points × 3 biological replicates).

Microbiological analyses were performed on days 0 (immediately after coating application and vacuum-packaging), 3, 5, and 7 of refrigerated storage at 4 ± 1 °C. These sampling points were selected to represent the typical storage duration of fresh game meat under retail and domestic refrigeration conditions. On each sampling day, three independent packages per experimental group were opened for analysis and were not returned to storage to avoid repeated handling and potential cross-contamination.

2.9 Microbiological analyses

On days 0, 3, 5, and 7 of storage at 4 ± 1 °C, samples designated for microbiological analysis were removed from refrigerated storage and equilibrated to laboratory temperature (21 ± 1 °C) for approximately 15 min. From each vacuum-packaged sample, 25.0 ± 0.1 g of meat was aseptically transferred into a sterile stomacher bag (BagPage®, Interscience, Saint-Nom-la-Bretèche, France) containing 225 mL of sterile 0.85% (w/v) NaCl solution. Samples were homogenized using a laboratory stomacher (BagMixer® 400, Interscience, France) at 230 rpm for 60 s, yielding a primary suspension corresponding to a 10^{-1} dilution.

Decimal dilutions (10^{-2}–10^{-6}) were subsequently prepared in sterile 0.85% (w/v) NaCl solution. Appropriate dilutions were plated onto selective and non-selective culture media as described below. After incubation, plates containing 30–300 typical colonies were selected for enumeration, and microbial counts were expressed as log CFU g^{-1}.

2.9.1 Total viable count (TVC). Total viable counts were determined by spreading 0.1 mL of the appropriate dilution onto the surface of Plate Count Agar (PCA; Oxoid, Basingstoke, United Kingdom) using a sterile L-shaped spreader. Plates were incubated at 30 ± 1 °C for 24–48 h in an incubator (Memmert

INE 400, Memmert GmbH, Schwabach, Germany). After incubation, all visible colonies were enumerated and results were expressed as log CFU g^{-1}.

2.9.2 Coliform bacteria. Coliform bacteria were enumerated on Violet Red Bile Lactose Agar (VRBL; Oxoid) using the pour-plate method. Briefly, 1.0 mL of the appropriate dilution was pipetted into sterile Petri dishes and overlaid with approximately 15 mL of molten and tempered (45 ± 2 °C) VRBL agar prepared according to the manufacturer's instructions. After gentle mixing and solidification, plates were incubated at 37 ± 1 °C for 24 h. Typical lactose-positive colonies (dark pink to purple, with a zone of bile salt precipitation) were enumerated and results were expressed as log CFU g^{-1}.

2.9.3 *Pseudomonas* spp. Bacteria of the genus *Pseudomonas* were quantified on *Pseudomonas* Agar Base (Oxoid) supplemented with CFC selective supplement (cetrimide, fucidin, cephalosporin; Oxoid), according to the manufacturer's recommendations. Aliquots of 0.1 mL of the appropriate dilution were spread on the agar surface using a sterile Drigalski spatula. Plates were incubated at 25 ± 1 °C for 48 h. After incubation, typical colonies exhibiting greenish to yellowish pigmentation and yellow-green fluorescence under UV light (365 nm) were enumerated and results were expressed as log CFU g^{-1}.

2.9.4 Lactic acid bacteria (LAB). Lactic acid bacteria were enumerated by spreading 0.1 mL of the appropriate dilution onto de Man Rogosa-Sharpe agar (MRS; Merck, Darmstadt, Germany). Plates were incubated at 37 ± 1 °C for 48–72 h under microaerophilic conditions in a CO_2 incubator (Memmert ICO150med, Memmert GmbH, Schwabach, Germany) set to 5% CO_2. After incubation, typical LAB colonies (small, cream to whitish, smooth surface) were enumerated and results were expressed as log CFU g^{-1}.

2.10 Identification of bacterial isolates using MALDI-TOF MS

2.10.1 Preparation of bacterial isolates. Representative bacterial isolates were selected from Plate Count Agar, Violet Red Bile Lactose Agar, *Pseudomonas* Agar, and de Man–Rogosa–Sharpe agar plates at different storage time points for species-level identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Typical colonies differing in morphology were selected to capture the diversity of the microbial community present on bear meat during refrigerated storage.

Individual colonies were aseptically transferred using a sterile inoculating loop onto fresh Plate Count Agar plates and incubated under conditions appropriate for the respective bacterial group, corresponding to the incubation temperature and duration used for the original isolation medium. After incubation for 18–24 h, pure cultures were obtained, and a small amount of fresh bacterial biomass was collected using a sterile wooden applicator for MALDI-TOF MS analysis. It should be noted that the results reflect only the cultivable fraction of the microbiota.

2.10.2 Preparation of the MALDI target. A thin layer of bacterial biomass was applied directly onto the surface of a stainless-steel MALDI target plate (MTP 384 target plate ground steel; Bruker Daltonics, Bremen, Germany) at the designated position using a sterile wooden applicator. The applied biomass was allowed to air-dry completely at laboratory temperature for approximately 2–3 min.

Subsequently, 1.0 μL of matrix solution containing α-cyano-4-hydroxycinnamic acid (α-CHCA; Bruker Daltonics), prepared according to the manufacturer's protocol as a saturated solution in acetonitrile/water/trifluoroacetic acid (50 : 47.5 : 2.5, v/v/v), was applied to each dried bacterial spot. The matrix was allowed to air-dry completely at laboratory temperature for approximately 5 min, resulting in the formation of matrix crystals embedding bacterial proteins and rendering the samples ready for mass spectrometric analysis.

2.10.3 Calibration and measurement. Prior to each analytical series, the MALDI-TOF MS instrument (Microflex LT, Bruker Daltonics, Bremen, Germany) was calibrated using the Bacterial Test Standard (Bruker Daltonics) in accordance with the manufacturer's recommendations. Mass spectra were acquired in positive linear mode over a mass-to-charge (m/z) range of 2000–20 000.

For each sample, spectra were generated as the sum of 240 laser shots collected from multiple positions on the same spot to ensure representative and reproducible spectral profiles.

2.10.4 Identification and evaluation of isolates. Acquired mass spectra were automatically processed and analysed using MALDI Biotyper software (version 3.1; Bruker Daltonics) and compared against the Bruker BDAL reference library (version 10.0), containing more than 11 000 reference spectra. Identification reliability was evaluated based on the log(score) values generated by the software.

Log(score) values of 2.300 or higher were considered indicative of reliable species-level identification. Values between 2.000 and 2.299 were interpreted as probable species-level identification or reliable genus-level identification, while scores between 1.700 and 1.999 indicated probable genus-level identification. Scores below 1.700 were regarded as unreliable and were excluded from further consideration.

Only isolates achieving log(score) values of at least 2.000 were considered sufficiently reliable for inclusion in subsequent experiments.

2.10.5 Selection of bacterial isolates for further testing. Following MALDI-TOF MS identification, isolates with log(score) values ≥2.000 were selected for further analyses. From these reliably identified isolates, bacterial species that occurred most frequently during refrigerated storage and represented dominant members of the microbial community on individual selective media were chosen.

For each selected species, multiple independent strains (at least three to five per species) originating from different experimental groups (C, CAC, and CA with individual monoterpenes) and from different storage time points were included. This approach was adopted to account for inter-strain

variability and potential adaptive responses associated with storage conditions and surface treatments.

The selected bacterial isolates were subsequently used to evaluate the antimicrobial activity of pure monoterpenes using the disk diffusion method and to determine antibiotic susceptibility profiles.

2.11 Antimicrobial effect of monoterpenes determined by the disk diffusion method

The antimicrobial activity of individual monoterpenes against selected bacterial isolates was evaluated using a standardized disk diffusion assay. Fresh bacterial cultures (18–24 h) grown on Mueller–Hinton agar (MHA; Oxoid, Basingstoke, United Kingdom) under conditions corresponding to their optimal growth temperatures were used for inoculum preparation. Total viable count isolates were cultivated at 30 °C, *Pseudomonas* spp. at 25 °C, and coliform bacteria together with lactic acid bacteria (LAB) at 37 °C (LAB in microaerophilic conditions). Bacterial suspensions were prepared in sterile 0.85% (w/v) NaCl solution and adjusted to a turbidity equivalent to 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU mL^{-1}, using a densitometer (DEN-1, Biosan, Riga, Latvia).

An aliquot of 100 μL of the standardized bacterial suspension was evenly spread over the entire surface of MHA plates (Ø 90 mm) using a sterile L-shaped spreader. Spreading was performed successively in three directions (0°, 60°, and 120°) to ensure uniform distribution of the inoculum. The inoculated plates were allowed to stand at laboratory temperature for 5–10 min to facilitate absorption of the inoculum into the agar surface.

Sterile paper disks with a diameter of 6 mm (Oxoid) were impregnated immediately prior to application with 10 μL of pure monoterpene using an automatic micropipette. The tested monoterpenes included α-pinene, (−)-β-pinene, sabinene, D-carvone, (−)-bornyl acetate, (*S*)-(−)-limonene, and 3-carene, all applied in their pure liquid form as supplied by the manufacturer (Sigma-Aldrich). Following impregnation, disks were air-dried for 2–3 min under a laminar flow cabinet to minimize uncontrolled spreading of the volatile compounds on the agar surface.

The prepared disks were placed onto the surface of the inoculated MHA plates using sterile tweezers. A maximum of four disks was positioned on each plate, ensuring a minimum distance of 24 mm between disk centers and at least 15 mm from the plate edge to prevent overlapping inhibition zones. For quality control, each plate also included a negative control disk impregnated with 10 μL of sterile distilled water and a disk impregnated with 10 μL of 0.5% (v/v) polysorbate 80 solution to verify that neither the paper disk nor the emulsifier affected bacterial growth.

For each bacterial strain and each tested monoterpene, three technical replicates were prepared using independent agar plates. Immediately after disk placement, plates were incubated in an inverted position at temperatures corresponding to the optimal growth conditions of the tested bacteria, namely 30 °C for mesophilic bacteria, 25 °C for *Pseudomonas* spp., and 37 °C for coliform bacteria and lactic acid bacteria. Incubation time was 24 h for fast-growing strains and extended to 48 h for slow-growing isolates when required.

After incubation, inhibition zones were evaluated by measuring the diameter of the clear zone from the edge of the disk, using a digital caliper with an accuracy of ±0.1 mm. Measurements were performed in two perpendicular directions and the mean value was recorded. A completely clear zone without visible bacterial growth was considered indicative of antimicrobial activity. In cases where no distinct inhibition zone was observed and the measured diameter corresponded to the disk diameter only, the isolate was classified as resistant or tolerant to the respective monoterpene under the applied experimental conditions.

2.12 Determination of antibiotic susceptibility of bacterial isolates

Antibiotic susceptibility testing was performed in parallel on the same panel of bacterial isolates used for evaluation of monoterpene antimicrobial activity. The disk diffusion method was applied in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, version 2025). Preparation of bacterial inocula followed the same procedure as described for monoterpene testing, with suspensions adjusted to a turbidity equivalent to 0.5 McFarland standard and evenly spread over the entire surface of Mueller-Hinton agar plates (Ø 90 mm; Oxoid) using a sterile cotton swab in three directions.

After allowing the inoculated plates to rest for 5–10 min, commercial antibiotic disks (Oxoid, Basingstoke, United Kingdom) containing defined concentrations of antibiotics were applied to the agar surface using sterile tweezers or an automatic disk dispenser. The selection of antibiotics was based on EUCAST 2025 guidelines and tailored to the taxonomic group of the tested isolates. For Enterobacterales (including *Enterobacter*, *Escherichia*, *Klebsiella*, *Citrobacter*, *Hafnia*, *Kluyvera*, *Lelliottia*, *Pantoea*, *Rahnella*, *Raoultella*, *Serratia*, *Erwinia*, and *Ewingella*), the tested antibiotics were piperacillin (ATB 1–30 μg), ceftazidime (ATB 2–10 μg), imipenem (ATB 3–10 μg), and tobramycin (ATB 4–10 μg). For *Pseudomonas* spp. (*Pseudomonas fluorescens*, *P. fragi*, *P. lundensis*, *P. synxantha*, *P. taetrolens*, and *P. trivialis*), piperacillin (ATB 1–30 μg), ceftazidime (ATB 2–10 μg), imipenem (ATB 3–10 μg), and amikacin (ATB 4–30 μg) were applied. *Acinetobacter* species (*A. calcoaceticus*, *A. dispersus*, and *A. guillouiae*) were tested against imipenem (ATB 1–10 μg), ciprofloxacin (ATB 2–5 μg), amikacin (ATB 3–30 μg), and tobramycin (ATB 4–10 μg). For lactic acid bacteria and related Gram-positive genera (*Lactococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Lactobacillus*, and *Kurthia*), susceptibility testing was performed using piperacillin (ATB 1–30 μg), ceftazidime (ATB 2–10 μg), imipenem (ATB 3–10 μg), and tobramycin (ATB 4–10 μg).

A maximum of six antibiotic disks was placed on each agar plate in accordance with EUCAST recommendations regarding minimum distances between disks. Plates were incubated in an inverted position at 35 ± 1 °C for 18–24 h under standard

EUCAST conditions. For each bacterial strain, three technical replicates were prepared.

Following incubation, inhibition zone diameters were measured in mm using a digital caliper, with measurements taken in two perpendicular directions and averaged. The resulting values were interpreted according to current EUCAST 2025 breakpoint criteria for the respective antibiotic and bacterial group. Isolates were classified as susceptible (*S*), intermediate (*I*), or resistant (*R*). For bacterial species for which EUCAST breakpoints were not available, including lactic acid bacteria and certain environmental isolates, interpretative criteria from the Clinical and Laboratory Standards Institute (CLSI M100, 33rd edition, 2023) or published peer-reviewed literature were applied. The specific interpretative standards used for each species are indicated in the Results section.

2.13 Statistical analyses

All microbiological data were subjected to logarithmic transformation prior to statistical analysis. Results are reported as mean ± standard deviation (SD), and all experiments were performed in triplicate ($n = 3$). Statistical analyses were conducted using JMP Student Edition 18.2 (SAS Institute, Cary, NC, USA) and CoStat software version 6.451 (CoHort Software, Pacific Grove, CA, USA). Before inferential analyses, the assumptions underlying ANOVA were assessed: residual normality was evaluated using the Shapiro–Wilk test, and variance homogeneity was examined using Bartlett's test.

Microbiological data were analyzed by a two-way analysis of variance (ANOVA), with treatment and storage day included as fixed factors, and their interaction was also considered. When significant main effects were detected, post hoc comparisons were performed using Dunnett's test, comparing each treatment with the uncoated control within the same storage day. *P*-values were adjusted according to the Holm correction, and differences were considered statistically significant at $p < 0.05$.

The effects of monoterpenes and antibiotics on the 36 bacterial strains tested were evaluated using a one-way analysis of variance (ANOVA), followed by mean comparisons using Tukey's honestly significant difference (HSD) post hoc test, with significance set at $p < 0.05$.

3 Results and discussion

3.1 Microbiological counts

Total viable count (TVC) increased significantly during refrigerated storage in all experimental groups ($p < 0.001$). Two-way ANOVA revealed a highly significant effect of both treatment ($F = 9.05$, $p < 0.001$) and storage day ($F = 727.05$, $p < 0.001$), as well as a significant treatment × day interaction ($F = 2.72$, $p < 0.001$) (Table 1). The significant interaction indicates that the effect of the different treatments on TVC was not constant over time but varied across storage days. This suggests that the antimicrobial efficacy of the coatings depended on storage duration, with some formulations being more effective at specific time points. Overall, samples treated with alginate-based coatings, particularly those enriched with active compounds, generally exhibited lower TVC values compared to the uncoated control, although the magnitude of this effect varied during storage.

Coliform bacteria count also increased significantly over storage time in all experimental groups ($p < 0.001$). Two-way ANOVA showed a significant main effect of treatment ($F = 11.26$, $p < 0.001$) and storage day ($F = 481.02$, $p < 0.001$), while no significant treatment × day interaction was detected ($F = 1.07$, $p = 0.39$) (Table 1). The absence of a significant interaction indicates that the effect of storage time on coliform growth was consistent across treatments. Nevertheless, the significant treatment effect highlights the ability of the applied formulations to modulate coliform levels throughout storage. In general, samples treated with alginate coatings containing active compounds displayed lower coliform counts than the uncoated control, indicating a sustained inhibitory effect without altering the overall growth trend.

Lactic acid bacteria (LAB) counts were strongly influenced by storage day ($F = 1033.13$, $p < 0.001$), while a weaker but statistically significant main effect of treatment was also observed ($F = 2.12$, $p = 0.045$). In addition, a significant treatment × day

Table 1 Two-way ANOVA results (*F* values and significance levels) for the effects of treatment, storage day, and their interaction on microbial counts (log CFU g^{-1}) during refrigerated storage[a]

Parameters	Factors	*F*	*P*-Value	Significance
Total viable count (log CFU g^{-1})	Treatment	9.05	<0.001	***
	Day	727.05	<0.001	***
	Treatment × Day	2.72	<0.001	***
Coliform bacteria count (log CFU g^{-1})	Treatment	11.26	<0.001	***
	Days	481.02	<0.001	***
	Treatment × Day	1.07	0.39	ns
Lactic acid bacteria count (log CFU g^{-1})	Treatment	2.12	0.045	*
	Days	1033.13	<0.001	***
	Treatment × Day	1.90	0.019	*
Pseudomonas bacteria count (log CFU g^{-1})	Treatment	0.83	0.58	ns
	Days	2.05	0.115	ns
	Treatment × Day	1.49	0.098	ns

[a] Treatment refers to the different experimental formulations applied to the samples, while Day refers to storage time.

interaction was detected ($F = 1.90$, $p = 0.019$) (Table 1). This interaction suggests that the impact of the different treatments on LAB populations varied over storage time, indicating a time-dependent response rather than a uniform effect across all days. Such behavior may reflect differences in the ability of LAB to adapt to the coated environments and to the presence of active compounds, as well as their intrinsic tolerance to antimicrobial agents.

For *Pseudomonas* spp., two-way ANOVA revealed no significant effects of treatment ($F = 0.83$, $p = 0.58$), storage day ($F = 2.05$, $p = 0.115$), or their interaction ($F = 1.49$, $p = 0.098$) (Table 1). These results indicate that *Pseudomonas* counts were not significantly affected by storage time or treatment under the experimental conditions. The lack of significant differences among treatments suggests a limited sensitivity of *Pseudomonas* spp. to the tested coatings and active compounds, which is consistent with the well-documented resilience of this microbial group to various antimicrobial strategies.

Pseudomonas spp. were sporadically detected during storage, with the majority of samples showing counts below the detection limit throughout the experimental period. Occasional positive counts were observed in isolated samples at days 3 and 7; however, no consistent growth pattern was evident across treatments or storage time. Due to the predominance of zero values and the sporadic nature of detection, no statistically meaningful differences among treatments could be identified and therefore were not discussed.

3.1.1 Coliform bacteria. Coliform bacteria are widely recognized as key indicators of the hygienic quality of meat and meat products, as their presence and proliferation may reflect fecal contamination, environmental exposure, or inadequate hygiene during processing and storage.[13] Coliform counts increased progressively during refrigerated storage in all samples ($p < 0.001$) (Fig. 1). On day 0, most coated treatments showed lower coliform counts compared to the uncoated control. Significant reductions were already observed for CA + A, CA + B, CA + BA, CA + D-car, CA + 3-car, CA + L and CA + S ($p < 0.05$), with the strongest effect recorded for CA + 3-car ($p < 0.01$), whereas CAC did not differ significantly from the control.

At day 3, a moderate antimicrobial effect was evident for nearly all coatings. CA + A, CA + B, CA + D-car and CA + L significantly reduced coliform counts compared to the control ($p < 0.05$ to $p < 0.01$), while CA + BA and CA + 3-car exhibited the strongest reductions ($p < 0.001$). No significant differences were observed for CAC.

At day 5, several treatments still showed significantly lower coliform levels than the control, including CA + A, CA + B, CA + BA, CA + D-car, CA + 3-car and CA + S ($p < 0.05$ to $p < 0.001$). In particular, CA + D-car and CA + BA resulted in the most marked reductions. Conversely, CAC and CA + L did not differ significantly from the control at this time point.

At day 7, fewer coatings maintained significant differences relative to the control. CA + A, CA + D-car and CA + 3-car showed modest but significant reductions ($p < 0.05$), CA + B exhibited

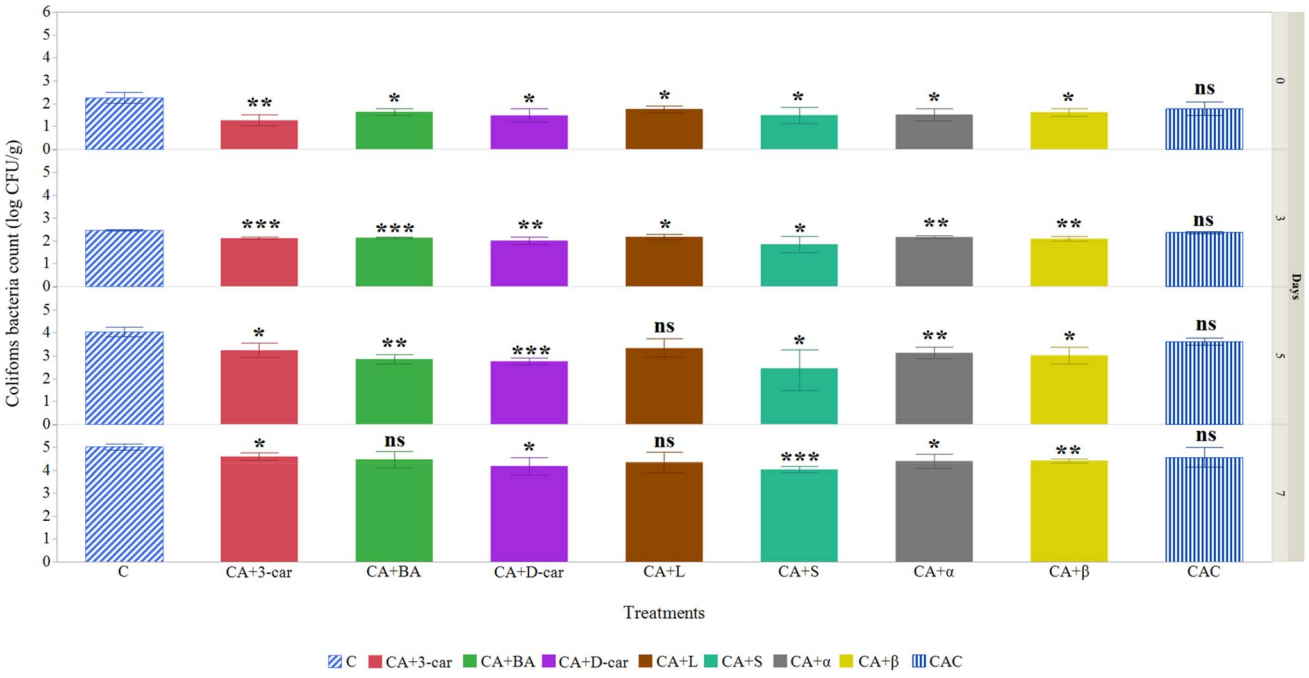

Fig. 1 Coliform bacteria count (log CFU g^{-1}) in bear meat samples treated with sodium alginate coating (CA) with different monoterpenes addition, measured during refrigerated storage on the 0, 3, 5 and 7 days. Values are expressed as mean (bars indicate ± standard deviation) ($n = 3$). Data were analyzed by two-way ANOVA with treatment and storage day as fixed factors. Asterisks indicate significant differences compared to uncoated control (C) according to Dunnett's test (* = $p < 0.05$, ** = $p < 0.01$, ***$p < 0.001$, ns = not significant, $p \geq 0.05$). C = uncoated control; CAC = sodium alginate coating without monoterpene; CA + α = alginate coating containing α-pinene; CA + β = alginate coating containing (−)-β-pinene; CA + BA = alginate coating containing (−)-bornyl acetate; CA + D-car = alginate coating containing D-carvone; CA + 3-car = alginate coating containing 3-carene; CA + L = alginate coating containing (*S*)-(−)-limonene; CA + S = alginate coating containing sabinene.

a stronger effect ($p < 0.01$), and CA + S remained highly effective ($p < 0.001$). No significant differences were detected for CAC, CA + BA or CA + L. It is worth noting that, at this stage, some statistically significant differences were observed despite mean coliform counts being close to those of the control, reflecting increased variability and the sensitivity of Holm-corrected Dunnett's pairwise comparisons.

In the present study, which represents the first comprehensive evaluation of coliform dynamics in refrigerated brown bear meat, coliform counts on day 0 (immediately after coating application and vacuum-packaging) ranged from 1.27 ± 0.24 log CFU g^{-1} in samples coated with alginate and 3-carene (CA + 3-car) to 2.27 ± 0.24 log CFU g^{-1} in the uncoated control (C). These relatively low initial counts indicate that carcass handling, transport, and laboratory processing were performed under appropriate hygienic conditions, despite the inherently higher microbiological risk associated with wild game meat. During refrigerated storage at 4 °C, coliform populations increased in all experimental groups. In the uncoated control samples (C), counts rose from 2.27 ± 0.24 log CFU g^{-1} on day 0 to 5.03 ± 0.12 log CFU g^{-1} on day 7, corresponding to an increase of approximately 2.8 log CFU g^{-1}. This growth pattern is consistent with previous observations in wild game meat and reflects the ability of psychrotrophic members of Enterobacterales to proliferate at refrigeration temperatures.[5,14,15]

Application of the alginate coating without bioactive compounds (CAC) resulted in only a limited inhibitory effect, with coliform counts reaching 4.58 ± 0.44 log CFU g^{-1} on day 7, corresponding to a reduction of approximately 0.5 log CFU g^{-1} compared to the uncoated control. These data clearly demonstrate that the alginate matrix alone acts primarily as a physical barrier, providing only marginal protection against coliform proliferation. Similar limited effects of pure alginate coatings have been reported for chicken and pork meat, where antimicrobial efficacy was achieved only after incorporation of active compounds.[7,8]

In contrast, enrichment of the alginate coating with monoterpenes enhanced antimicrobial efficacy, although the magnitude of inhibition differed among individual compounds. Among the tested coatings, the strongest inhibitory effect against coliform bacteria was observed for D-carvone (CA + D-car), with counts of 4.19 ± 0.38 log CFU g^{-1} on day 7, representing a reduction of approximately 0.8 log CFU g^{-1} relative to the uncoated control. Comparable inhibitory effects were also recorded for limonene (CA + L: 4.35 ± 0.44 log CFU g^{-1}), α-pinene (CA + α: 4.40 ± 0.30 log CFU g^{-1}), and β-pinene (CA + β: 4.43 ± 0.09 log CFU g^{-1}). These results are consistent with previous reports demonstrating moderate but reproducible antimicrobial activity of monoterpenes against enterobacteria in meat systems,[9,10] although the extent of inhibition observed here was lower than that reported for leaner meat matrices.

The observed differences among monoterpenes are likely driven by variations in their chemical structure and modes of action against Gram-negative bacteria. Cyclic monoterpenes such as pinenes and limonene have been shown to compromise the integrity of the outer membrane, induce oxidative stress, and disrupt essential cellular processes.[16] However, in high-fat matrices such as bear meat, partial partitioning of lipophilic monoterpenes into the lipid phase may reduce their effective concentration at the meat surface, where bacterial growth predominantly occurs, thereby limiting their antimicrobial impact.

Interestingly, sabinene (CA + S: 4.04 ± 0.12 log CFU g^{-1}) exhibited inhibitory activity comparable to other monoterpenes, despite being reported as less potent against Gram-negative bacteria in simplified *in vitro* systems. This observation underscores the importance of evaluating antimicrobial efficacy directly in complex food matrices, where interactions between the compound, the food structure, and the indigenous microbiota may substantially influence the final outcome.

Despite the observed antimicrobial effects, coliform counts in all treatments exceeded 4 log CFU g^{-1} by day 7. These results suggest that alginate coatings enriched with monoterpenes at the tested concentration (0.03% v/v) can delay but not fully suppress coliform growth, and that higher concentrations or additional hurdle technologies may be required for more effective preservation.

3.1.2 Total viable count (TVC). Total viable count (TVC) is a fundamental indicator of the microbiological quality of meat and reflects the overall microbial load associated with the product. Total viable counts increased significantly during refrigerated storage in all samples ($p < 0.001$) (Fig. 2). On day 0, several coated treatments already showed significantly lower TVC values compared to the uncoated control. Specifically, CA + B, CA + 3-car, CA + BA, CA + S and CA + A resulted in significantly reduced counts after Holm correction ($p < 0.05$), whereas no significant differences were observed for CA + D-car, CA + L or CAC.

At day 3, no significant differences in TVC were detected between any coated treatment and the control ($P > 0.05$), even before adjustment for multiple comparisons, indicating a comparable microbial load among all samples at this early storage stage.

At day 5, a clear antimicrobial effect emerged for selected coatings. CA + L showed the strongest reduction in TVC relative to the control ($p < 0.001$), while CA + 3-car, CA + D-car and CA + S also exhibited significantly lower counts after Holm correction ($p < 0.05$). In contrast, CAC, CA + A, CA + B and CA + BA did not differ significantly from the control at this time point.

At day 7, a pronounced and highly robust effect was observed. All coated treatments resulted in significantly lower TVC values compared to the uncoated control, even after correction for multiple comparisons. The strongest reductions were recorded for CA + L, CA + D-car, CA + 3-car and CA + S ($p < 0.001$), followed by CA + B, CA + BA and CA + A ($p < 0.01$), while CAC also showed a modest but significant reduction ($p < 0.05$).

On day 0 (immediately after coating application and vacuum-packaging), the mean TVC values across all experimental groups were low, ranging from 2.26 ± 0.06 log CFU g^{-1} in samples coated with alginate enriched with 3-carene, sabinene, and bornyl acetate (CA + 3-car, CA + S, CA + BA) to 2.81 ± 0.05 log CFU g^{-1} in the uncoated control (C). These low initial counts indicate that the bear meat used in this study was fresh and that

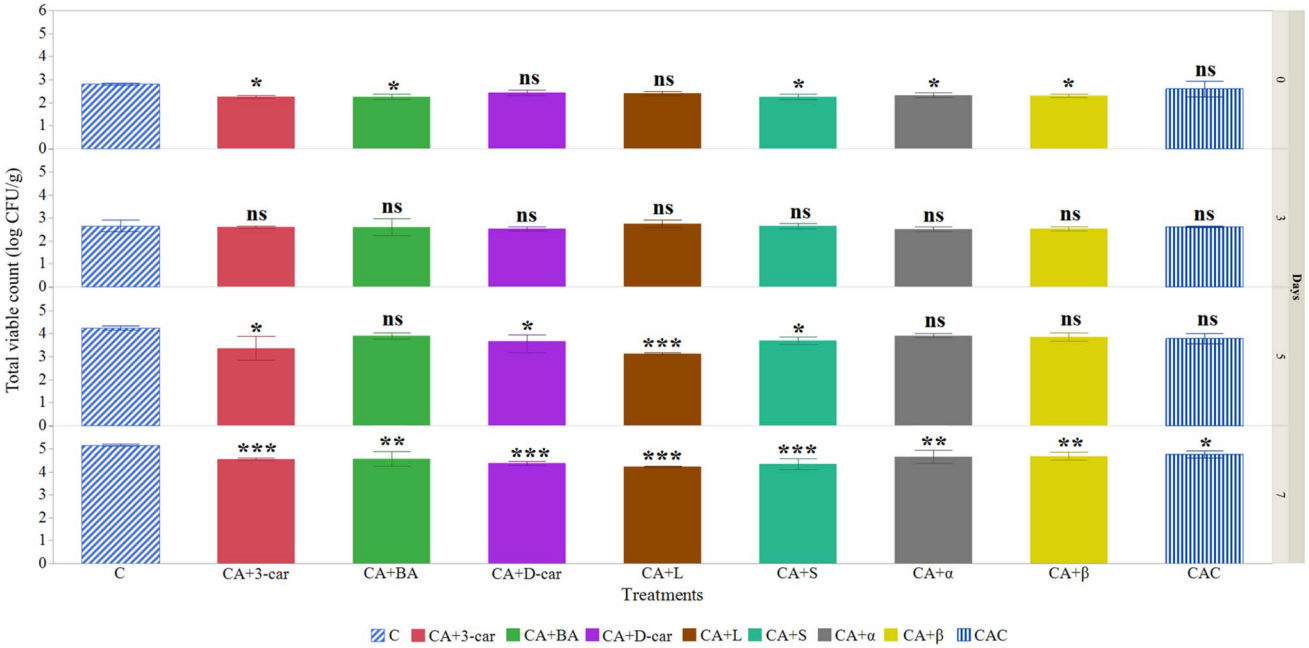

Fig. 2 Total viable count (TVC) (log CFU g^{-1}) in bear meat samples treated with sodium alginate coating (CA) with different monoterpenes addition, measured during refrigerated storage on the 0, 3, 5 and 7 days. Values are expressed as mean (bars indicate ± standard deviation) ($n = 3$). Data were analyzed by two-way ANOVA with treatment and storage day as fixed factors. Asterisks indicate significant differences compared to uncoated control (C) according to Dunnett's test (* = $p < 0.05$, ** = $p < 0.01$, ***$p < 0.001$, ns = not significant, $p \geq 0.05$). C = uncoated control; CAC = sodium alginate coating without monoterpene; CA + α = alginate coating containing α-pinene; CA + β = alginate coating containing (−)-β-pinene; CA + BA = alginate coating containing (−)-bornyl acetate; CA + D-car = alginate coating containing D-carvone; CA + 3-car = alginate coating containing 3-carene; CA + L = alginate coating containing (*S*)-(−)-limonene; CA + S = alginate coating containing sabinene.

slaughtering, handling, and sample preparation were performed under appropriate hygienic conditions.

During refrigerated storage at 4 °C, TVC increased progressively in all experimental groups. In the uncoated control group (C), TVC increased from 2.81 ± 0.05 log CFU g^{-1} on day 0 to 5.17 ± 0.05 log CFU g^{-1} on day 7, corresponding to an increase of approximately 2.4 log CFU g^{-1}. Such microbial growth is typical for fresh meat stored under refrigerated conditions and is mainly driven by the proliferation of psychrotrophic microorganisms, particularly Gram-negative bacteria such as *Pseudomonas* spp., *Acinetobacter* spp., and other spoilage-associated taxa that dominate meat microbiota at low temperatures.[17] These bacteria are known for their ability to grow at refrigeration temperatures and for producing proteolytic and lipolytic enzymes that contribute to the degradation of meat proteins and lipids, ultimately leading to sensory deterioration.

Application of the alginate coating without monoterpenes (CAC) resulted in a moderate reduction of microbial growth compared to the uncoated control. On day 7, TVC in the CAC group reached 4.78 ± 0.15 log CFU g^{-1}, representing a reduction of approximately 0.4 log CFU g^{-1} relative to the control. This limited inhibitory effect can be attributed to the barrier properties of the alginate film, which restricts oxygen diffusion and moisture exchange at the meat surface, thereby slowing the growth of aerobic spoilage microorganisms. However, the alginate matrix alone was insufficient to provide substantial antimicrobial protection, which is consistent with previous findings reported for other meat types.

Incorporation of monoterpenes into the alginate coating moderately enhanced antimicrobial efficacy against the total microbial population. Among the tested treatments, the most notable inhibitory effect was observed for the limonene-enriched coating (CA + L), where TVC reached only 4.24 ± 0.03 log CFU g^{-1} on day 7, corresponding to a reduction of approximately 0.9 log CFU g^{-1} compared to the uncoated control. Similar reductions were observed for coatings containing sabinene (CA + S: 4.36 ± 0.23 log CFU g^{-1}) and D-carvone (CA + D-car: 4.40 ± 0.10 log CFU g^{-1}), both achieving reductions of approximately 0.8 log CFU g^{-1}.

Interestingly, the antimicrobial efficacy of individual monoterpenes differed depending on the target microbial group. While limonene and sabinene exhibited the strongest inhibitory effect against total viable counts, their activity against coliform bacteria was slightly less pronounced compared to some other monoterpenes. In contrast, α-pinene and β-pinene, which showed relatively strong activity against coliforms, exhibited a weaker overall effect on TVC, with values of 4.67 ± 0.29 and 4.70 ± 0.17 log CFU g^{-1} on day 7, respectively. This differential behavior suggests that individual monoterpenes exhibit selective antimicrobial activity toward specific microbial groups, likely reflecting differences in their chemical structure, lipophilicity, and mechanisms of action.[18]

Limonene, a monocyclic monoterpene, has been shown to disrupt bacterial cell membranes through multiple mechanisms, including increased membrane permeability, induction of oxidative stress, and interference with energy metabolism, resulting in broad-spectrum antimicrobial activity.[19,20] Han *et al.*[19] demonstrated that limonene causes severe structural damage to bacterial cell envelopes, inhibits respiratory enzymes and ATPase activity, and induces leakage of intracellular components. Gupta *et al.*[20] further confirmed that limonene promotes the formation of reactive oxygen species, leading to DNA damage, protein damage, and membrane disruption in *Escherichia coli*. In contrast, bicyclic monoterpenes such as α- and β-pinene may interact differently with bacterial membranes, potentially explaining their narrower antimicrobial spectrum and preferential activity against Gram-negative enterobacteria.

Despite the observed inhibitory effects, TVC values in all experimental groups exceeded 4 log CFU g^{-1} by day 7, remaining however well below the commonly cited acceptability limit of 7 log CFU g^{-1} for fresh meat. These findings indicate that the tested monoterpene concentration (0.03% v/v) effectively delayed but did not fully suppress microbial proliferation, suggesting that higher concentrations or complementary preservation strategies may be needed for substantial shelf-life extension.

3.1.3 Lactic acid bacteria (LAB). Lactic acid bacteria (LAB) constitute an important component of the natural microbiota of fresh meat and play a key role in the spoilage of vacuum-packaged products, where the growth of oxygen-dependent psychrotrophic bacteria is restricted.[21]

LAB counts increased significantly during refrigerated storage in all treatments ($p < 0.001$) (Fig. 3). On day 0, no statistically significant differences between coated treatments and the uncoated control were detected after correction for multiple comparisons, although some treatments showed lower LAB counts than the control before adjustment.

At day 3, several alginate-based coatings resulted in significantly lower LAB counts compared to the control even after Holm correction, including CA + L, CA + A, CA + S, CA + 3-car, CA + BA and CA + D-car ($p < 0.05$). In contrast, CAC and CA + B did not differ significantly from the control. These results indicate a transient inhibitory effect of specific formulations on LAB growth at this storage time.

At day 5, no significant differences in LAB counts were observed among treatments, either before or after correction for multiple comparisons, suggesting a convergence of LAB populations across all samples.

Similarly, at day 7, although some treatments showed nominal differences relative to the control without correction, none of these differences remained significant after Holm adjustment. Overall, these findings suggest that the effect of the coatings on LAB populations was time-dependent and limited to the early storage phase, with no persistent impact at later stages.

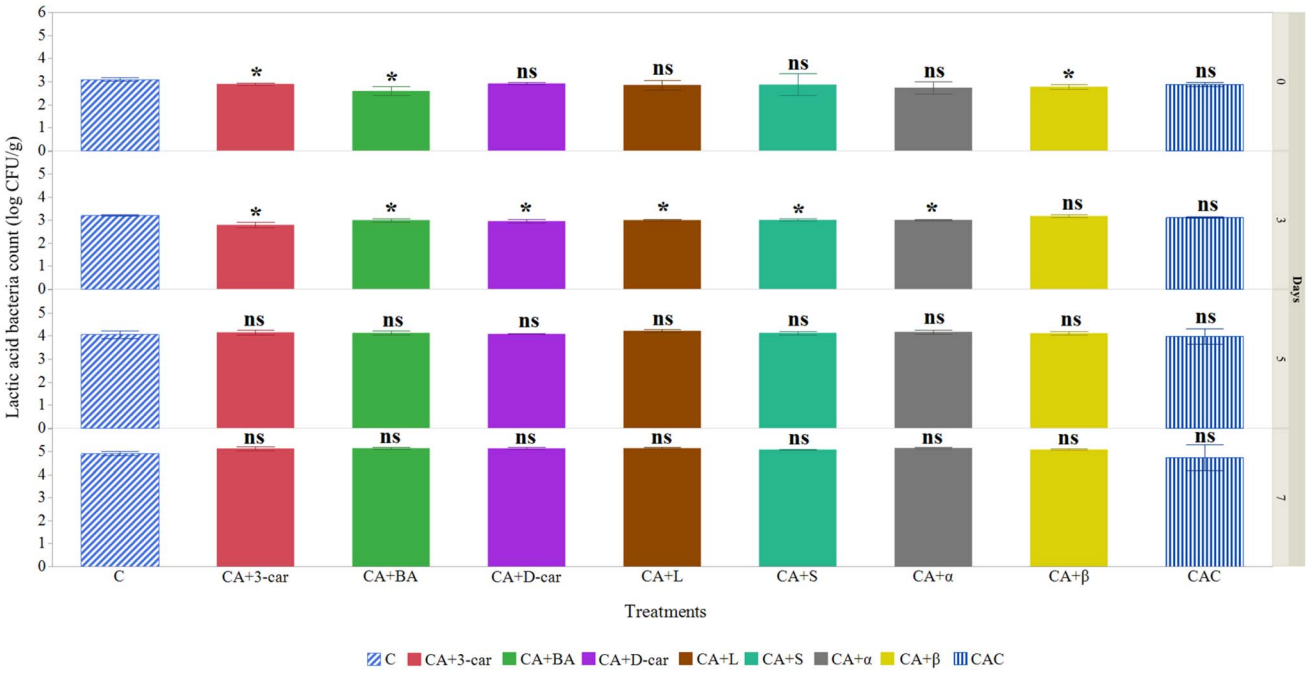

Fig. 3 Lactic acid bacteria (LAB) count (log CFU g^{-1}) in bear meat samples treated with sodium alginate coating (CA) with different monoterpenes addition, measured during refrigerated storage on the 0, 3, 5 and 7 days. Values are expressed as mean (bars indicate ± standard deviation) ($n = 3$). Data were analyzed by two-way ANOVA with treatment and storage day as fixed factors. Asterisks indicate significant differences compared to uncoated control (C) according to Dunnett's test (* = $p < 0.05$, ns = not significant, $p \geq 0.05$). C = uncoated control; CAC = sodium alginate coating without monoterpene; CA + α = alginate coating containing α-pinene; CA + β = alginate coating containing (−)-β-pinene; CA + BA = alginate coating containing (−)-bornyl acetate; CA + D-car = alginate coating containing D-carvone; CA + 3-car = alginate coating containing 3-carene; CA + L = alginate coating containing (*S*)-(−)-limonene; CA + S = alginate coating containing sabinene.

On day 0, LAB counts were relatively low across all experimental groups, ranging from 2.60 ± 0.21 log CFU g^{-1} in samples coated with alginate enriched with bornyl acetate (CA + BA) to 3.10 ± 0.07 log CFU g^{-1} in the uncoated control (C). These values were consistently lower than the corresponding total viable counts (TVC), indicating that LAB did not represent the dominant fraction of the initial microbiota of bear meat.

During refrigerated storage at 4 °C, LAB populations increased markedly in all experimental groups. In the uncoated control group (C), LAB counts increased from 3.10 ± 0.07 log CFU g^{-1} on day 0 to 4.92 ± 0.10 log CFU g^{-1} on day 7, corresponding to an increase of approximately 1.8 log CFU g^{-1}. This trend is characteristic of vacuum-packaged meat, where facultatively anaerobic and anaerobic LAB progressively dominate the microbial ecosystem in the absence of oxygen. Psychrotrophic LAB, particularly species belonging to the genera *Leuconostoc*, *Lactococcus*, and *Lactobacillus*, are widely recognized as major spoilage organisms in chilled vacuum-packed meat products.[22]

MALDI-TOF MS analysis confirmed this typical succession pattern, revealing *Lactococcus lactis* as the dominant LAB species during the later stages of storage, accompanied by the presence of *Lactobacillus sakei*, *Leuconostoc mesenteroides*, and *Carnobacterium divergens*. A comparable microbial shift was reported by Rodriguez-Caturla *et al.*[23] in vacuum-packaged beef, where post-storage reduction in microbial diversity was associated with the dominance of LAB, regardless of meat pH (5.4–6.1) and storage temperature.

Application of the alginate coating without monoterpenes (CAC) exerted only a marginal inhibitory effect on LAB growth. On day 7, LAB counts in the CAC group reached 4.74 ± 0.57 log CFU g^{-1}, corresponding to a reduction of approximately 0.2 log CFU g^{-1} compared to the uncoated control. This observation confirms that alginate films without incorporated antimicrobial agents do not possess intrinsic inhibitory activity against LAB and primarily function as physical barriers with limited impact on facultatively anaerobic microorganisms.

In contrast to their effects on coliform bacteria and total viable counts, enrichment of alginate coatings with monoterpenes did not suppress LAB growth. On the contrary, LAB proliferation was slightly enhanced in most monoterpene-treated groups. By day 7, LAB counts in monoterpene-containing coatings ranged between 5.09 and 5.16 log CFU g^{-1}, exceeding those observed in the control group (4.92 log CFU g^{-1}). The highest LAB counts were recorded in samples coated with α-pinene (CA + α: 5.16 ± 0.03 log CFU g^{-1}), bornyl acetate (CA + BA: 5.15 ± 0.05 log CFU g^{-1}), and D-carvone (CA + D-car: 5.15 ± 0.06 log CFU g^{-1}).

This apparently contrasting effect, whereby monoterpenes promoted rather than inhibited LAB growth, can be explained by several complementary mechanisms. First, monoterpenes generally exhibit stronger antimicrobial activity against Gram-negative bacteria than against Gram-positive LAB, whose thick peptidoglycan layer provides enhanced protection against membrane-disrupting compounds.[24] Chen *et al.*[25] demonstrated that the minimum inhibitory concentration of cinnamaldehyde against *Lactobacillus sakei* was approximately 50-fold higher than that required to inhibit Gram-negative bacteria, highlighting the intrinsic tolerance of LAB to essential oil components.

Second, selective inhibition of competing microbial groups, particularly *Pseudomonas* spp. and Enterobacterales, may have resulted in competitive release, allowing LAB to exploit available nutrients more efficiently under reduced interspecific competition. Such ecological shifts are well documented in meat microbiota. Andreevskaya *et al.*[22] reported that during interspecific competition, fast-growing LAB species such as *Leuconostoc gelidum* enhance carbohydrate catabolism and nutrient scavenging, thereby accelerating their proliferation when competing bacteria are suppressed. A similar mechanism likely occurred in the present study, where monoterpene-mediated inhibition of Gram-negative bacteria indirectly favored LAB growth.

Third, certain LAB species may exhibit inherent tolerance to or even metabolic interactions with monoterpenes, conferring a selective advantage in environments enriched with these compounds. Bukvicki *et al.*[26] highlighted the complex and species-specific interactions between LAB and essential oil components, emphasizing that tolerance to monoterpenes varies substantially among LAB strains.

Although monoterpenes did not exert a direct inhibitory effect on LAB, the observed increase in LAB populations under vacuum-packaging may have both positive and negative effects on meat quality. On one hand, LAB produce lactic acid, leading to pH reduction that may suppress the growth of pathogenic bacteria and contribute to microbiological safety. On the other hand, excessive LAB growth is associated with sensory deterioration, including sour off-flavors, slime formation, and unpleasant odors, ultimately limiting the acceptable shelf life of vacuum-packaged meat. Therefore, while alginate coatings enriched with monoterpenes effectively modulated the microbial community structure of bear meat, their application should be carefully optimized to balance antimicrobial efficacy against spoilage-associated LAB.

3.2 Bacterial microbiota identification using MALDI-TOF MS

The bacterial diversity of bear meat samples was analyzed using MALDI-TOF MS during 7-days storage under vacuum-packaging at 4 °C. This approach enabled rapid and reliable identification of bacterial isolates at the species level based on ribosomal protein mass spectral profiles.

3.2.1 Initial microbiota (day 0). On day 0, prior to refrigerated storage, the bacterial microbiota of bear shoulder muscle exhibited high taxonomic diversity (Fig. 4). A total of 23 bacterial species belonging to eight families were identified. The dominant bacterial family was Enterobacteriaceae, accounting for approximately 50% of the total microbiota, and comprising nine genera, including *Escherichia* (7%), *Lelliottia* (7%), *Enterobacter* (12% combined from three species), *Klebsiella* (5%), *Serratia* (6%), *Raoultella* (4%), and *Kluyvera* (2%). The most abundant individual species within this family were *Escherichia coli* (7%) and *Lelliottia amnigena* (7%).

Fig. 4 Bacterial microbiota composition of brown bear shoulder meat (*musculus triceps brachii*) during vacuum storage at 4 °C on day 0, determined by MALDI-TOF MS. The sunburst diagram shows the relative abundance (%) of bacterial families, genera, and species.

The second most abundant family was Streptococcaceae (12%), represented exclusively by *Lactococcus lactis*, which was the sole lactic acid bacterium detected in the initial microbiota. Moraxellaceae accounted for 12% of the isolates and was represented by the genus *Acinetobacter*, including *A. calcoaceticus* (5%), *A. guillouiae* (5%), and *A. dispersus* (2%). Pseudomonadaceae constituted 10% of the microbiota and included *Pseudomonas fragi* (5%) and *P. lundensis* (5%). Additional families detected at lower relative abundances included Yersiniaceae (9%), Erwiniaceae (7%), and Hafniaceae (5%).

The observed composition is consistent with previous studies on fresh meat microbiota. Yang *et al.*[27] reported *Pseudomonas* (19.5%) and *Acinetobacter* (10%) as dominant genera in meat processing environments, with direct transfer from equipment to meat surfaces. Similarly, Stellato *et al.*[28] demonstrated that members of Enterobacteriaceae and *Pseudomonas* dominate the microbiota of retail meat products. Our findings corroborate these observations, with Enterobacteriaceae and *Pseudomonas* constituting a substantial fraction of the initial microbiota of bear meat.

The presence of *E. coli* and other enterobacteria (*Serratia*, *Hafnia*, *Klebsiella*) indicates contamination originating from the gastrointestinal tract during slaughter and dressing. Wang *et al.*[29] identified *Pseudomonas*, *Acinetobacter*, *Pantoea*, and *Raoultella* as major spoilage-associated genera in chilled pork, aligning closely with the taxa detected in bear meat in the present study.

Notably, lactic acid bacteria were poorly represented in the initial microbiota, with *Lactococcus lactis* accounting for only 12% of all isolates. Similar trends were reported by Roch *et al.*,[30] who observed that LAB constituted a minor component of the initial microbiota of vacuum-packaged beef but became dominant during prolonged refrigerated storage. This pattern suggests that LAB are not primary contaminants during slaughter but rather proliferate during storage under anaerobic conditions. The absence of other LAB genera (*Lactobacillus*, *Leuconostoc*, *Carnobacterium*) on day 0 may therefore reflect

Fig. 5 Bacterial microbiota composition of brown bear shoulder meat (*musculus triceps brachii*) during vacuum storage at 4 °C on day 3, determined by MALDI-TOF MS. The sunburst diagram shows the relative abundance (%) of bacterial families, genera, and species.

their ecological adaptation to vacuum storage rather than initial contamination.

3.2.2 Microbiota on storage day 3. By day 3 of vacuum storage at 4 °C, pronounced shifts in the bacterial community structure were observed (Fig. 5). The total number of identified species decreased from 23 to 12, indicating strong selective pressure exerted by reduced oxygen availability combined with refrigeration. This reduction in diversity reflects early-stage microbial succession typical for vacuum-packaged fresh meat.

Enterobacteriaceae remained the dominant bacterial family, with its relative abundance increasing markedly from 50% on day 0 to approximately 71% on day 3. Within this family, *Escherichia coli* showed a pronounced increase from 7% to 20%, while *Klebsiella oxytoca* increased from 5% to 12%. In addition, *Citrobacter braakii*, which was not detected in the initial microbiota, emerged as a major component, accounting for 13% of the total isolates. This expansion of Enterobacteriaceae is characteristic of the early storage phase under vacuum conditions, before lactic acid bacteria become dominant.

Lactic acid bacteria increased substantially, with Streptococcaceae and Lactobacillaceae together rising from 12% to 26% of the total microbiota. *Lactococcus lactis* increased slightly from 12% to 14%, and three additional LAB species appeared for the first time: *Lactococcus garvieae* (5%), *Leuconostoc mesenteroides* (5%), and *Weissella viridescens* (2%). The emergence of these psychrotrophic LAB indicates the beginning of microbial adaptation to anaerobic and refrigerated storage conditions.

Hafniaceae remained a stable component of the microbiota, represented exclusively by *Hafnia alvei* (8%), suggesting good adaptation of this species to vacuum-packaging and low temperature.

A notable finding was the complete disappearance of obligate aerobic psychrotrophic bacteria. Members of Moraxellaceae (*Acinetobacter* spp.), which accounted for 12% of the microbiota on day 0, and Pseudomonadaceae (*Pseudomonas* spp.), representing 10% initially, were not detected on day 3. This confirms the strong inhibitory effect of vacuum-packaging on strictly aerobic spoilage bacteria. Similar rapid declines of *Pseudomonas* and *Acinetobacter* during the early storage phase of vacuum-packaged meat have been reported by Rovira *et al.*[31]

The dominance of Enterobacteriaceae and the appearance of *Citrobacter braakii* are typical for the transitional phase of microbial succession in vacuum stored meat. Rodriguez-Caturla *et al.*[23] reported a comparable pattern in vacuum-packaged beef, where Enterobacteriaceae dominated during the first 3–7 days of storage before being progressively outcompeted by LAB. The concurrent emergence of *Leuconostoc* and *Weissella* suggests the beginning of a shift toward LAB-dominated spoilage microbiota, which is characteristic of longer storage periods under anaerobic conditions.

3.2.3 Microbiota on storage day 5. At the intermediate storage point of 4 °C vacuum-packaging, a pronounced restructuring of the bacterial community was observed on day 5, accompanied by a marked increase in diversity from 12 to 22 identified species (Fig. 6). This shift indicates a transitional phase in microbial succession, characterized by the coexistence of anaerobic- and aerobic-tolerant bacterial populations.

The most striking finding was the re-emergence of aerobic psychrotrophic bacteria, particularly members of the family Pseudomonadaceae, which accounted for approximately 17% of the total microbiota despite vacuum-packaging. Five *Pseudomonas* species were identified, namely *Pseudomonas fragi* (4%), *P. lundensis* (4%), *P. synxantha* (4%), *P. taetrolens* (3%), and *P. fluorescens* (2%). In addition, Moraxellaceae reappeared in the form of *Acinetobacter calcoaceticus* (4%), which had been completely absent on storage day 3.

In contrast, the relative abundance of Enterobacteriaceae declined substantially from 71% on day 3 to 49% on day 5, accompanied by a profound change in species composition. Notably, *Escherichia coli* and *Citrobacter braakii*, which dominated the microbiota on day 3 (20% and 13%, respectively), were no longer detected. Instead, the prevalence of *Serratia* spp. increased from 8% to 12%, with *Serratia liquefaciens* (8%), *S. plymuthica* (2%), and *S. marcescens* (2%) identified. Furthermore, *Rahnella aquatilis* reached 7% abundance, while *Enterobacter* spp. collectively accounted for 9% of the microbiota.

Lactic acid bacteria (LAB) showed a slight decline in relative abundance from 26% on day 3 to 22% on day 5. Within this group, *Leuconostoc mesenteroides* increased from 5% to 8%, whereas *Lactococcus lactis* decreased moderately from 14% to 12%, suggesting ongoing competition and niche differentiation among LAB during storage.

The reappearance of *Pseudomonas* spp. under vacuum conditions represents a notable and somewhat unexpected observation. While the exact mechanism cannot be determined from the present study, this observation may be consistent with emerging evidence suggesting that certain *Pseudomonas* species could be capable of growth under reduced oxygen conditions. Watson *et al.*[32] demonstrated that certain *Pseudomonas* species isolated from vacuum-packaged meat are capable of anaerobic growth *via* arginine fermentation pathways. Similarly, Yang *et al.*[33] reported the persistence of *P. fluorescens* and *P. lundensis* in vacuum-packaged beef toward the end of storage. The detection of five distinct *Pseudomonas* species in the present study highlights the heterogeneous adaptive capacity within this genus and suggests that vacuum-packaging alone may not completely suppress all psychrotrophic aerobic bacteria during intermediate storage phases.

The pronounced turnover within Enterobacteriaceae, characterized by the disappearance of fast-growing species (*E. coli*, *Citrobacter*) and the emergence of more stress-tolerant taxa (*Serratia*, *Rahnella*), reflects competitive dynamics under oxygen-limited and refrigerated conditions. Shao *et al.*[34] described similar patterns of enterobacterial succession in meat systems, where early colonizers are gradually replaced by species better adapted to environmental stressors.

Overall, the high microbial diversity observed on storage day 5 likely represents a transitional ecological stage preceding microbiota stabilization. This phase appears to be critical for determining the subsequent dominance of LAB during prolonged vacuum storage and underscores the dynamic nature of microbial interactions in game meat, which remains largely unexplored. The present findings therefore provide novel insight into the temporal succession of bacterial communities

Fig. 6 Bacterial microbiota composition of brown bear shoulder meat (*musculus triceps brachii*) during vacuum storage at 4 °C on day 5, determined by MALDI-TOF MS. The sunburst diagram shows the relative abundance (%) of bacterial families, genera, and species.

in vacuum-packaged bear meat and contribute to a deeper understanding of spoilage ecology in this unconventional meat matrix.

3.2.4 Microbiota on storage day 7 final spoilage community. On storage day 7, a pronounced reduction in bacterial diversity was observed, with the number of identified species decreasing from 22 to 13, indicating stabilization of the spoilage-associated microbial community (Fig. 7). This stage represents the final phase of microbial succession under vacuum storage at 4 °C, characterized by dominance of bacteria adapted to anaerobic and refrigerated conditions.

Lactic acid bacteria (LAB) became the predominant microbial group, accounting for 35% of the total microbiota, representing a substantial increase from 22% on day 5. *Lactococcus lactis* reached 22% and emerged as the single most abundant species within the community. Additional LAB species contributing to the spoilage consortium included *Lactobacillus sakei* (8%) and *Leuconostoc mesenteroides* (5%), confirming their

Fig. 7 Bacterial microbiota composition of brown bear shoulder meat (*musculus triceps brachii*) during vacuum storage at 4 °C on day 7, determined by MALDI-TOF MS. The sunburst diagram shows the relative abundance (%) of bacterial families, genera, and species.

key role in late-stage spoilage of vacuum-packaged meat. Two LAB species typical of advanced storage phases, *Carnobacterium divergens* (2%) and *Kurthia zopfii* (2%), appeared exclusively on day 7.

The relative abundance of Enterobacteriaceae further declined to 35%, accompanied by continued shifts in species composition. *Ewingella americana* increased markedly to 13% and became the dominant representative of this family, followed by *Kluyvera intermedia* (10%), *Lelliottia amnigena* (9%), *Rahnella aquatilis* (9%), and *Enterobacter ludwigii* (8%). This pattern reflects a transition from early-stage, fast-growing enterobacteria to more stress-tolerant species capable of persistence under refrigerated and oxygen-limited conditions.

Pseudomonadaceae decreased from 17% on day 5 to 10% on day 7. Within this family, *Pseudomonas lundensis* remained the dominant species (8%), accompanied by *Pseudomonas trivialis* (2%). The persistence of *P. lundensis* despite vacuum-packaging supports emerging evidence that certain *Pseudomonas* species exhibit partial adaptation to anaerobic environments.

The dominance of *Lactococcus lactis* and the appearance of *Lactobacillus sakei* are consistent with numerous studies on vacuum-packaged meat. Samelis and Kakouri[35] reported that *Latilactobacillus sakei* dominated 65.8% of isolates from spoiled

vacuum-packaged beef, followed by *Leuconostoc carnosum* and *Carnobacterium divergens*. Similarly, Fontana *et al.*[36] demonstrated that *L. sakei* and *L. curvatus* persist throughout refrigerated storage of vacuum-packaged beef, reaching maximal populations of approximately 7–8 log CFU cm^{-2}. Lauritsen *et al.*[37] further identified *Carnobacterium*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* as key spoilage genera in refrigerated packaged meat, corroborating the LAB-dominated spoilage profile observed in the present study.

The detection of *Carnobacterium divergens* is particularly notable, as this species is considered a hallmark spoilage organism of vacuum-packaged meat. Ercolini *et al.*[38] observed *C. divergens* as a dominant species in vacuum stored beef after four days of storage, while Casaburi *et al.*[39] demonstrated its spoilage potential through the production of volatile organic compounds under both aerobic and anaerobic conditions. The presence of *Kurthia zopfii*, although at low abundance, further supports the classification of day 7 as an advanced spoilage stage, as this species has been repeatedly associated with late storage phases of vacuum-packaged meat.

Despite the overall decline of Enterobacteriaceae from their peak abundance on day 3 (71%) to 35% on day 7, this family remained a substantial component of the spoilage microbiota. The predominance of *Ewingella americana*, *Rahnella aquatilis*, and *Kluyvera intermedia* reflects a well-documented succession pattern, in which early colonizers such as *Escherichia coli* and *Citrobacter* spp. are gradually replaced by more resilient taxa adapted to low temperatures and anaerobic environments. This ecological transition, culminating in LAB dominance, represents the characteristic spoilage trajectory of vacuum-packaged meat and confirms that bear meat follows a broadly comparable, yet previously undocumented, microbial succession pathway.

3.2.5 Overall microbial succession during 7-days storage. Analysis of microbial succession during 7-days storage of bear meat under vacuum-packaging at 4 °C revealed pronounced and dynamic changes in the composition, diversity, and dominance structure of the bacterial community (Fig. 7). The initially diverse microbiota, comprising 23 bacterial species on day 0, underwent strong selective pressure imposed by vacuum-packaging and refrigeration, resulting in a reduction to 12 species on day 3. This was followed by a transient increase in diversity to 22 species on day 5 and subsequent stabilization at 13 species on day 7, indicating establishment of a mature spoilage-associated community.

The most prominent feature of microbial succession was the gradual shift from an *Enterobacteriaceae*-dominated microbiota to a lactic acid bacteria (LAB)-dominated community. Enterobacteriaceae accounted for 50% of the microbiota on day 0, increased sharply to 71% on day 3, then declined to 49% on day 5 and further to 35% on day 7. In contrast, LAB increased from 12% on day 0 to 26% on day 3, slightly decreased to 22% on day 5, and ultimately became the dominant group on day 7, representing 35% of the total microbiota.

Within Enterobacteriaceae, marked species-level succession was observed. Fast-growing species such as *Escherichia coli* dominated the early storage phase, increasing from 7% on day 0 to 20% on day 3, but were completely absent on days 5 and 7. Similarly, *Citrobacter braakii*, which emerged on day 3 at 13%, disappeared in later storage stages. These early colonizers were progressively replaced by more stress-tolerant species adapted to low temperature and anaerobic conditions, including *Ewingella americana* (increasing from 5% on day 0 to 13% on day 7), *Rahnella aquatilis* (from 4% to 9%), and *Kluyvera intermedia* (from 2% to 10%).

LAB succession followed a characteristic pattern associated with vacuum-packaged meat. *Lactococcus lactis*, present from the beginning at 12%, increased steadily throughout storage and became the dominant species on day 7 (22%). *Leuconostoc mesenteroides* appeared on day 3 (5%), increased to 8% on day 5, and slightly declined to 5% on day 7. Typical late-stage spoilage species, including *Lactobacillus sakei* (8%) and *Carnobacterium divergens* (2%), appeared exclusively on day 7, highlighting their role in advanced spoilage phases.

Of particular interest was the dynamic behavior of aerobic psychrotrophic bacteria. Moraxellaceae, represented by *Acinetobacter* spp. (12% on day 0), and Pseudomonadaceae, represented by *Pseudomonas* spp. (10% on day 0), were completely absent on day 3, confirming the strong inhibitory effect of vacuum-packaging on obligate aerobes. However, on day 5, both groups re-emerged, with *Acinetobacter* reaching 4% and *Pseudomonas* increasing to 17%, represented by five distinct species. The mechanism underlying this reappearance remains unclear from the present data and would require further investigation, although adaptive responses to reduced oxygen conditions cannot be excluded. On day 7, the proportion of *Pseudomonas* declined to 10%, yet *Pseudomonas lundensis* (8%) remained a significant member of the spoilage community, underscoring heterogeneity in the anaerobic tolerance of this genus.

Overall, the observed microbial succession follows the three-phase spoilage model described for vacuum-packaged meat. The early phase (days 0–3) was characterized by dominance of Enterobacteriaceae and disappearance of aerobic psychrotrophic bacteria. The transitional phase (days 3–5) exhibited maximal diversity, partial return of *Pseudomonas* spp., and increasing LAB abundance. The late spoilage phase (days 5–7) was defined by LAB dominance and stabilization of the microbial community. A comparable succession pattern was reported by Rodriguez-Caturla *et al.*[23] for vacuum-packaged beef with pH values between 5.4 and 6.1, where LAB dominance became evident only after 7 days of storage. Furthermore, Andreevskaya *et al.*[22] described competitive interactions among LAB species during meat storage, with faster-growing taxa such as *Leuconostoc* spp. facilitating metabolic shifts that ultimately favor *Lactobacillus* and *Carnobacterium*. This mechanism likely explains the gradual transition from *Lactococcus* dominance to the emergence of typical late-stage spoilage LAB observed in the present study.

3.3 Antimicrobial activity of monoterpenes

3.3.1 Overall antimicrobial activity. The antimicrobial activity of seven pure monoterpenes was evaluated against 36 bacterial species isolated from bear meat using the disk

diffusion method. All tested compounds showed detectable inhibitory effects, with inhibition zone diameters ranging from 3 to 8 mm, demonstrating a broad antimicrobial activity against the indigenous microbiota associated with bear meat.

Among the tested compounds, D-carvone exhibited the strongest overall antimicrobial activity. The largest inhibition zone was observed against *Lactobacillus sakei* (6.67 mm), while the weakest effect was recorded against *Enterobacter cloacae* (3.33 mm). Sabinene displayed the widest activity range, with inhibition zones ranging from 7.33 mm against *Serratia marcescens* to 3.67 mm against *Pseudomonas taetrolens*. Limonene showed pronounced activity against *Serratia plymuthica* (6.67 mm) but comparatively weaker inhibition of *Pseudescherichia vulneris* (4.33 mm) (Table 2).

The pronounced efficacy of D-carvone is consistent with numerous reports highlighting the superior antimicrobial properties of oxygenated monoterpenes. Ghannay *et al.*[40] demonstrated strong antibacterial activity of caraway essential oil rich in carvone and limonene, while Yahia Balla[41] reported broad-spectrum antimicrobial effects of *Mentha viridis* oil dominated by D-carvone. Oxygenated monoterpenes are recognized as potent antimicrobial agents due to their ability to disrupt bacterial membranes, interfere with energy metabolism, and induce leakage of intracellular components.[42]

Among bicyclic monoterpene hydrocarbons, α-pinene exhibited the weakest antimicrobial activity, with inhibition zones ranging from 5.33 mm against *Acinetobacter guillouiae* to 3.33 mm against *Pseudomonas taetrolens*. β-Pinene demonstrated moderate activity, reaching a maximum inhibition of 5.67 mm against *Klebsiella oxytoca* and a minimum of 3.67 mm against *Weissella viridescens*. Bornyl acetate showed its strongest inhibitory effect against *Escherichia coli* (5.67 mm) and the weakest against *Enterobacter cloacae* (3.33 mm) (Table 2). These findings support previous observations that bicyclic monoterpene hydrocarbons generally exhibit lower antimicrobial potency compared to oxygenated or monocyclic monoterpenes. Aggarwal *et al.*[43] reported that carvone consistently outperformed limonene and pinene derivatives against a broad spectrum of bacterial species.

3-Carene demonstrated intermediate antimicrobial activity, with the strongest inhibition observed against *Ewingella americana* (5.67 mm) and the weakest against *Acinetobacter calcoaceticus* (3.33 mm). The substantial interspecies variability observed for all monoterpenes, particularly sabinene, indicates pronounced differences in bacterial susceptibility, reflecting variability in cell envelope structure, membrane composition, and intrinsic defense mechanisms. Kim *et al.*[44] demonstrated that structural modifications of monoterpenes, such as oxidation of limonene to carveol, significantly enhance antimicrobial activity, underscoring the importance of molecular structure in determining antibacterial efficacy.

3.3.2 Species-specific susceptibility patterns. Marked differences in susceptibility were observed among individual bacterial species. *Serratia marcescens* exhibited exceptional sensitivity to sabinene, with an inhibition zone of 7.33 mm, representing the highest value recorded in this study. This species also showed strong susceptibility to D-carvone (6.67 mm) (Table 2). *Serratia plymuthica* displayed high sensitivity to both limonene and 3-carene (6.67 mm each). The pronounced susceptibility of *Serratia* spp. is notable, as members of this genus are frequently characterized by intrinsic or acquired resistance to multiple conventional antibiotics. This suggests that monoterpenes may bypass classical resistance mechanisms, likely through non-specific membrane-targeting effects rather than interaction with defined intracellular targets.

Among lactic acid bacteria, *Lactobacillus sakei* (Table 2) demonstrated the highest susceptibility to D-carvone (6.67 mm), whereas *Weissella viridescens* showed the weakest response to β-pinene (3.67 mm). These findings indicate heterogeneity in susceptibility even among phylogenetically related LAB species, potentially reflecting differences in cell wall thickness, membrane lipid composition, or stress response systems.

Members of the genus *Pseudomonas* exhibited generally lower susceptibility to monoterpenes. *Pseudomonas taetrolens* showed minimal inhibition by both α-pinene (3.33 mm) and sabinene (3.67 mm). This reduced susceptibility aligns with the well-documented intrinsic resistance mechanisms of *Pseudomonas*, including low outer membrane permeability, efficient efflux pump systems, and enzymatic detoxification pathways (Table 2).

Enterobacteriaceae displayed heterogeneous susceptibility profiles. *Escherichia coli* responded strongly to bornyl acetate (5.67 mm), whereas *Enterobacter cloacae* exhibited minimal susceptibility to both D-carvone and bornyl acetate (3.33 mm). *Klebsiella oxytoca* showed peak inhibition by β-pinene (5.67 mm), while *Ewingella americana* exhibited the highest sensitivity to 3-carene (5.67 mm) (Table 2). These findings highlight substantial interspecies variability within Enterobacteriaceae, reflecting diverse physiological and structural traits influencing monoterpene susceptibility.

3.3.3 Implications for food preservation. The pronounced *in vitro* susceptibility to oxygenated monoterpenes contrasts with *in situ* observations where LAB was not inhibited and in some cases exhibited enhanced growth. This discrepancy reflects well-recognized limitations of disk diffusion assays for volatile and hydrophobic compounds, including uneven diffusion and rapid volatilization.[42,45] The results should therefore be interpreted as preliminary comparative susceptibility data only and not as definitive indicators of antimicrobial performance in food systems. Nevertheless, they confirm the superior potency of oxygenated monoterpenes and reveal species-specific variability relevant for rational selection of bioactive compounds for food preservation.

3.4 Antibiotic susceptibility

Antibiotic susceptibility of all 36 bacterial species isolated from bear meat was evaluated using the disk diffusion method in accordance with EUCAST guidelines. Isolates were grouped into four taxonomic categories and tested against antibiotic panels relevant to each group. All tested bacterial species were classified as susceptible to all antibiotics, with inhibition zone diameters ranging from 20 to 55 mm (Table 3), indicating the absence of acquired antibiotic resistance among the isolates.

Table 2 Inhibition zone diameters (mm) obtained by the disk diffusion method against 36 different bacterial strains using 7 different monoterpenes treatments

Group	Microorganism	*p*-Value[a]	α-Pinene	β-Pinene	Bornyl acetate	D-Carene	3-Carene	Limonene	Sabinene
Acinetobacter spp.	*Acinetobacter calcoaceticus*	***	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}	5.67 ± 0.58^{a}	3.33 ± 0.58^{b}	5.33 ± 0.58^{a}	6.33 ± 0.58^{a}
Acinetobacter spp.	*Acinetobacter dispersus*	**	4.67 ± 0.58^{bc}	3.67 ± 0.58^{c}	4.33 ± 0.58^{bc}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{bc}	5.67 ± 0.58^{ab}
Acinetobacter spp.	*Acinetobacter guillouiae*	*	5.33 ± 0.58^{a}	4.33 ± 0.58^{ab}	4.67 ± 0.58^{ab}	5.33 ± 0.58^{a}	4.67 ± 0.58^{ab}	5.67 ± 0.58^{a}	3.67 ± 0.58^{b}
Enterobacterales	*Citrobacter braakii*	*	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}
Enterobacterales	*Enterobacter bugandensis*	**	3.67 ± 0.58^{b}	4.67 ± 0.58^{ab}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Enterobacter cloacae*	***	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	3.33 ± 0.58^{b}	3.33 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Enterobacter ludwigii*	**	4.33 ± 0.58^{b}	4.67 ± 0.58^{b}	4.67 ± 0.58^{b}	5.67 ± 0.58^{ab}	4.33 ± 0.58^{b}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}
Enterobacterales	*Erwinia persicina*	***	4.67 ± 0.58^{bc}	3.67 ± 0.58^{c}	4.33 ± 0.58^{bc}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	3.67 ± 0.58^{c}
Enterobacterales	*Escherichia coli*	***	3.67 ± 0.58^{c}	5.33 ± 0.58^{ab}	5.67 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{bc}	3.67 ± 0.58^{c}
Enterobacterales	*Ewingella americana*	***	3.33 ± 0.58^{c}	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.67 ± 0.58^{ab}	4.67 ± 0.58^{bc}	3.67 ± 0.58^{c}
Enterobacterales	*Hafnia alvei*	**	3.67 ± 0.58^{b}	4.67 ± 0.58^{ab}	4.33 ± 0.58^{ab}	5.67 ± 0.58^{a}	5.33 ± 0.58 ^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Klebsiella oxytoca*	ns	4.33 ± 0.58^{a}	5.67 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Kluyvera intermedia*	**	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Lelliottia amnigena*	**	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}
Enterobacterales	*Pantoea agglomerans*	**	4.33 ± 0.58 ^{b}	4.67 ± 0.58^{b}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}
Enterobacterales	*Pantoea vagans*	**	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	3.67 ± 0.58 ^{b}	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Pseudescherichia vulneris*	*	3.67 ± 0.58^{b}	4.67 ± 0.58^{ab}	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	4.33 ± 0.58^{ab}	3.67 ± 0.58^{b}
Enterobacterales	*Rahnella aquatilis*	**	3.67 ± 0.58^{b}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	4.67 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Raoultella planticola*	ns	4.67 ± 0.58^{a}	4.67 ± 0.58^{a}	4.67 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}	5.67 ± 0.58^{a}	5.67 ± 0.58^{a}
Enterobacterales	*Raoultella terrigena*	**	4.33 ± 0.58^{b}	5.33 ± 0.58^{ab}	5.67 ± 0.58^{ab}	6.67 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.33 ± 0.58 ^{ab}	4.67 ± 0.58^{b}
Enterobacterales	*Serratia liquefaciens*	**	4.33 ± 0.58^{b}	4.67 ± 0.58^{b}	5.67 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.67 ± 0.58^{ab}	6.67 ± 0.58^{a}
Enterobacterales	*Serratia marcescens*	***	5.33 ± 0.58^{bc}	5.33 ± 0.58^{bc}	3.67 ± 0.58^{d}	6.67 ± 0.58^{ab}	4.67 ± 0.58^{cd}	5.67 ± 0.58^{bc}	7.33 ± 0.58^{a}
Enterobacterales	*Serratia plymuthica*	***	3.67 ± 0.58^{c}	4.33 ± 0.58^{bc}	5.33 ± 0.58^{ab}	6.67 ± 0.58^{a}	5.67 ± 0.58^{ab}	6.67 ± 0.58^{a}	6.67 ± 0.58^{a}
Lactic acid bacteria	*Carnobacterium divergens*	**	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Lactic acid bacteria	*Kurthia zopfii*	*	4.33 ± 0.58^{b}	5.33 ± 0.58^{ab}	5.67 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{b}	5.33 ± 0.58^{ab}
Lactic acid bacteria	*Lactobacillus sakei*	***	3.67 ± 0.58^{c}	3.67 ± 0.58^{c}	5.33 ± 0.58^{ab}	6.67 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{bc}
Lactic acid bacteria	*Lactococcus garvieae*	*	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{b}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{b}	6.33 ± 0.58^{a}
Lactic acid bacteria	*Lactococcus lactis*	**	4.67 ± 0.58^{ab}	3.67 ± 0.58^{b}	5.67 ± 0.58^{a}	4.67 ± 0.58^{ab}	5.67 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Lactic acid bacteria	*Leuconostoc mesenteroides*	***	3.67 ± 0.58^{c}	4.67 ± 0.58^{bc}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{bc}	6.33 ± 0.58^{a}
Lactic acid bacteria	*Weissella viridescens*	***	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	6.67 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	6.67 ± 0.58^{a}
Pseudomonas spp.	*Pseudomonas fluorescens*	***	3.33 ± 0.58^{b}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	6.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}
Pseudomonas spp.	*Pseudomonas fragi*	*	4.33 ± 0.58^{b}	5.33 ± 0.58^{ab}	5.33 ± 0.58^{ab}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	5.67 ± 0.58^{ab}	6.33 ± 0.58^{a}
Pseudomonas spp.	*Pseudomonas lundensis*	**	3.33 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	4.33 ± 0.58^{ab}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}
Pseudomonas spp.	*Pseudomonas synxantha*	**	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	3.67 ± 0.58^{b}
Pseudomonas spp.	*Pseudomonas taetrolens*	***	3.33 ± 0.58^{b}	3.67 ± 0.58^{b}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.33 ± 0.58^{a}	5.67 ± 0.58^{a}	3.67 ± 0.58^{b}
Pseudomonas spp.	*Pseudomonas trivialis*	*	4.67 ± 0.58^{b}	5.67 ± 0.58^{ab}	4.67 ± 0.58^{b}	6.33 ± 0.58^{a}	5.33 ± 0.58^{ab}	4.67 ± 0.58^{b}	5.67 ± 0.58^{ab}

[a] Values are expressed as mean ± standard deviation ($n = 3$). Different lowercase letters within the same row indicate significant differences among treatments ($p < 0.05$), according to one-way ANOVA followed by Tukey's HSD. Significance levels: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = not significant ($p \geq 0.05$).

Table 3 Inhibition zone diameters (mm) obtained by the disk diffusion method against different bacterial strains using antibiotics (ATB 1–4) treatments

Group	Microorganism	*p*-Value[a]	ATB 1	ATB 2	ATB 3	ATB 4
Acinetobacter spp.	*Acinetobacter calcoaceticus*	***	29.67 ± 0.58^{c}	52.67 ± 0.58^{a}	29.67 ± 0.58^{c}	33.67 ± 0.58^{b}
Acinetobacter spp.	*Acinetobacter dispersus*	***	25.67 ± 0.58^{b}	50.67 ± 0.58^{a}	22.33 ± 0.58^{c}	24.33 ± 0.58^{b}
Acinetobacter spp.	*Acinetobacter guillouiae*	***	27.33 ± 0.58^{b}	53.33 ± 0.58^{a}	22.67 ± 0.58^{d}	24.67 ± 0.58^{c}
Enterobacterales	*Citrobacter braakii*	**	24.33 ± 0.58^{a}	22.33 ± 0.58^{b}	24.33 ± 0.58^{a}	22.67 ± 0.58^{b}
Enterobacterales	*Enterobacter bugandensis*	***	26.33 ± 0.58^{a}	25.33 ± 0.58^{ab}	22.33 ± 0.58^{c}	24.67 ± 0.58^{b}
Enterobacterales	*Enterobacter cloacae*	**	23.67 ± 0.58^{ab}	24.67 ± 0.58^{a}	23.33 ± 0.58^{ab}	22.33 ± 0.58^{b}
Enterobacterales	*Enterobacter ludwigii*	***	24.33 ± 0.58^{c}	26.33 ± 0.58^{b}	25.67 ± 0.58^{bc}	29.67 ± 0.58^{a}
Enterobacterales	*Erwinia persicina*	*	26.67 ± 0.58^{a}	25.67 ± 0.58^{ab}	26.33 ± 0.58^{a}	24.67 ± 0.58^{b}
Enterobacterales	*Escherichia coli*	**	24.33 ± 0.58^{a}	25.33 ± 0.58^{a}	24.67 ± 0.58^{a}	22.67 ± 0.58^{b}
Enterobacterales	*Ewingella americana*	**	25.33 ± 0.58^{b}	27.33 ± 0.58^{a}	24.33 ± 0.58^{b}	25.67 ± 0.58^{b}
Enterobacterales	*Hafnia alvei*	***	24.33 ± 0.58^{b}	26.33 ± 0.58^{a}	22.67 ± 0.58^{c}	24.33 ± 0.58^{b}
Enterobacterales	*Klebsiella oxytoca*	***	25.33 ± 0.58^{c}	23.33 ± 0.58^{d}	27.33 ± 0.58^{b}	29.33 ± 0.58^{a}
Enterobacterales	*Kluyvera intermedia*	ns	25.67 ± 0.58^{a}	26.67 ± 0.58^{a}	25.33 ± 0.58^{a}	26.33 ± 0.58^{a}
Enterobacterales	*Lelliottia amnigena*	***	26.67 ± 0.58^{a}	26.67 ± 0.58^{a}	23.67 ± 0.58^{b}	26.33 ± 0.58^{a}
Enterobacterales	*Pantoea agglomerans*	***	26.33 ± 0.58^{a}	23.33 ± 0.58^{b}	27.33 ± 0.58^{a}	27.33 ± 0.58^{a}
Enterobacterales	*Pantoea vagans*	***	25.67 ± 0.58^{b}	25.67 ± 0.58^{b}	29.33 ± 0.58^{a}	23.33 ± 0.58^{c}
Enterobacterales	*Pseudescherichia vulneris*	***	27.67 ± 0.58^{b}	24.33 ± 0.58^{c}	30.67 ± 0.58^{a}	27.33 ± 0.58^{b}
Enterobacterales	*Rahnella aquatilis*	**	25.33 ± 0.58^{b}	27.33 ± 0.58^{a}	24.33 ± 0.58^{b}	25.67 ± 0.58^{b}
Enterobacterales	*Raoultella planticola*	***	24.33 ± 0.58^{b}	26.33 ± 0.58^{a}	22.67 ± 0.58^{c}	24.33 ± 0.58^{b}
Enterobacterales	*Raoultella terrigena*	***	25.33 ± 0.58^{c}	23.33 ± 0.58^{d}	27.33 ± 0.58^{b}	29.33 ± 0.58^{a}
Enterobacterales	*Serratia liquefaciens*	*	25.33 ± 0.58^{b}	26.67 ± 0.58^{a}	25.33 ± 0.58^{b}	26.33 ± 0.58^{ab}
Enterobacterales	*Serratia marcescens*	***	25.00 ± 1.00^{b}	27.67 ± 0.58^{a}	24.33 ± 0.58^{b}	27.33 ± 0.58^{a}
Enterobacterales	*Serratia plymuthica*	***	27.33 ± 0.58^{a}	24.67 ± 0.58^{b}	27.67 ± 0.58^{a}	25.33 ± 0.58^{b}
Lactic acid bacteria	*Carnobacterium divergens*	*	25.67 ± 0.58^{b}	26.67 ± 0.58^{ab}	25.67 ± 0.58^{b}	27.33 ± 0.58^{a}
Lactic acid bacteria	*Kurthia zopfii*	***	28.33 ± 0.58^{b}	24.67 ± 0.58^{c}	31.33 ± 0.58^{a}	27.67 ± 0.58^{b}
Lactic acid bacteria	*Lactobacillus sakei*	***	27.67 ± 0.58^{b}	24.67 ± 0.58^{c}	29.33 ± 0.58^{a}	27.67 ± 0.58^{b}
Lactic acid bacteria	*Lactococcus garvieae*	***	27.33 ± 0.58^{b}	25.67 ± 0.58^{c}	31.33 ± 0.58^{a}	26.33 ± 0.58^{bc}
Lactic acid bacteria	*Lactococcus lactis*	***	27.33 ± 0.58^{b}	24.67 ± 0.58^{c}	30.67 ± 0.58^{a}	27.67 ± 0.58^{b}
Lactic acid bacteria	*Leuconostoc mesenteroides*	***	27.67 ± 0.58^{b}	24.33 ± 0.58^{c}	31.33 ± 0.58^{a}	27.67 ± 0.58^{b}
Lactic acid bacteria	*Weissella viridescens*	***	27.67 ± 0.58^{b}	24.33 ± 0.58^{c}	30.67 ± 0.58^{a}	27.33 ± 0.58^{b}
Pseudomonas spp.	*Pseudomonas fluorescens*	***	53.67 ± 0.58^{a}	52.67 ± 0.58^{ab}	51.67 ± 0.58^{b}	20.33 ± 0.58^{c}
Pseudomonas spp.	*Pseudomonas fragi*	***	49.67 ± 0.58^{b}	53.67 ± 0.58^{a}	54.33 ± 0.58^{a}	23.33 ± 0.58^{c}
Pseudomonas spp.	*Pseudomonas lundensis*	***	50.67 ± 0.58^{b}	51.67 ± 0.58^{b}	53.33 ± 0.58^{a}	24.33 ± 0.58^{c}
Pseudomonas spp.	*Pseudomonas synxantha*	***	52.67 ± 0.58^{b}	54.33 ± 0.58^{a}	52.67 ± 0.58^{b}	24.67 ± 0.58^{c}
Pseudomonas spp.	*Pseudomonas taetrolens*	***	53.67 ± 0.58^{a}	50.33 ± 0.58^{b}	53.67 ± 0.58^{a}	23.67 ± 0.58^{c}
Pseudomonas spp.	*Pseudomonas trivialis*	***	50.33 ± 0.58^{b}	53.33 ± 0.58^{a}	51.67 ± 0.58^{b}	21.67 ± 0.58^{c}

[a] Values are expressed as mean ± standard deviation ($n = 3$). Different lowercase letters within the same row indicate significant differences among treatments ($p < 0.05$), according to one-way ANOVA followed by Tukey's HSD. Significance levels: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = not significant ($p \geq 0.05$).

3.4.1 Enterobacterales. Twenty Enterobacterales species were tested against piperacillin, ceftazidime, imipenem, and tobramycin. Susceptibility to piperacillin ranged from 23.67 mm in *Enterobacter cloacae* to 27.67 mm in *Pseudescherichia vulneris*. Ceftazidime exhibited the strongest inhibition against *Serratia marcescens* (27.67 mm) and the weakest against *Citrobacter braakii* (22.33 mm). Imipenem demonstrated the widest range of activity, with inhibition zones from 22.33 mm in *Enterobacter bugandensis* to 30.67 mm in *Pseudescherichia vulneris*. Tobramycin showed maximal activity against *Enterobacter ludwigii* (29.67 mm) and minimal activity against *Enterobacter cloacae* (22.33 mm) (Table 3).

The complete susceptibility of all Enterobacterales isolates contrasts with the global emergence of extended-spectrum β-lactamase-producing and carbapenem-resistant strains in food-production environments,[28] supporting the concept that wildlife-associated bacteria unexposed to anthropogenic antibiotic pressure retain natural susceptibility profiles.

3.4.2 *Pseudomonas* species. Six *Pseudomonas* species were evaluated against piperacillin, ceftazidime, imipenem, and amikacin. All isolates exhibited exceptionally high susceptibility, with inhibition zones substantially exceeding EUCAST clinical breakpoints. Piperacillin inhibition ranged from 49.67 mm in *Pseudomonas fragi* to 53.67 mm in *Pseudomonas fluorescens*. Ceftazidime showed the strongest activity against *Pseudomonas synxantha* (54.33 mm) and the weakest against *Pseudomonas taetrolens* (50.33 mm). Imipenem inhibition ranged from 51.67 mm in *Pseudomonas trivialis* to 54.33 mm in *Pseudomonas fragi*. Amikacin produced smaller inhibition zones, ranging from 20.33 mm in *Pseudomonas fluorescens* to 24.67 mm in *Pseudomonas synxantha* (Table 3).

The uniformly large inhibition zones observed for β-lactam antibiotics are particularly notable in light of increasing reports

of multidrug-resistant *Pseudomonas* spp. in clinical and food-processing environments. Watson *et al.*[32] described resistance to fluoroquinolones and β-lactams in *Pseudomonas* isolates from vacuum-packaged meat, contrasting sharply with the complete susceptibility observed in the present wild game-derived isolates. The comparatively smaller zones observed for amikacin likely reflect differences in diffusion characteristics and antimicrobial mechanisms rather than reduced susceptibility.

3.4.3 *Acinetobacter* species. Three *Acinetobacter* species were tested against imipenem, ciprofloxacin, amikacin, and tobramycin. Imipenem inhibition ranged from 25.67 mm in *Acinetobacter dispersus* to 29.67 mm in *Acinetobacter calcoaceticus*. Ciprofloxacin demonstrated the strongest overall activity in this group, with inhibition zones from 50.67 mm in *A. dispersus* to 53.33 mm in *A. guillouiae*. Amikacin inhibition ranged from 22.33 mm to 29.67 mm, while tobramycin exhibited the widest variability, from 24.33 mm in *A. dispersus* to 33.67 mm in *A. calcoaceticus* (Table 3).

Full susceptibility of *Acinetobacter* spp. to carbapenems and fluoroquinolones is significant given the global spread of carbapenem-resistant *Acinetobacter baumannii* in healthcare settings. Yang *et al.*[27] reported the presence of resistance determinants in *Acinetobacter* isolates from meat-processing environments, albeit at lower prevalence than in clinical settings. The absence of resistance in wild game isolates further supports the hypothesis that resistance emergence is largely driven by anthropogenic antibiotic exposure.

3.4.4 Lactic acid bacteria. Seven lactic acid bacteria (LAB) species, including *Lactococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Lactobacillus*, and *Kurthia*, were evaluated against piperacillin, ceftazidime, imipenem, and tobramycin. Piperacillin inhibition ranged from 25.67 mm in *Carnobacterium divergens* to 28.33 mm in *Kurthia zopfii*. Ceftazidime showed the strongest activity against *C. divergens* (26.67 mm) and the weakest against *Weissella viridescens* (24.33 mm). Imipenem inhibition ranged from 25.67 mm in *C. divergens* to 31.33 mm in *Lactococcus garvieae*. Tobramycin exhibited the narrowest range, with inhibition zones between 26.33 and 27.67 mm (Table 3).

The complete susceptibility of all LAB isolates differs from findings from industrially processed meat products, where LAB frequently harbor transferable resistance genes. Fontana *et al.*[46] reported tetracycline and erythromycin resistance among LAB isolated from vacuum-packaged beef, highlighting the role of food-associated LAB as potential reservoirs of resistance genes. The absence of resistance in LAB isolated from bear meat provides valuable baseline data on natural susceptibility profiles in ecosystems unaffected by anthropogenic antibiotic selection pressure and underscores the importance of wild game as a reference matrix for studying intrinsic bacterial resistance.

3.5 Comparison of antimicrobial activity of monoterpenes and antibiotics

Comparison of inhibition zones obtained for monoterpenes and conventional antibiotics revealed substantial differences in antimicrobial efficacy between these two groups of compounds. While pure monoterpenes produced inhibition zones ranging from 3.33 to 7.33 mm, antibiotics yielded markedly larger zones of 20.33 to 54.33 mm, corresponding to an approximately 6- to 16-fold difference as assessed by the disk diffusion method.

These pronounced differences reflect the fundamentally distinct mechanisms of action of antibiotics and monoterpenes. Antibiotics are synthetic or semi-synthetic molecules designed to target specific intracellular processes such as cell wall synthesis, protein biosynthesis, or DNA replication.[47] In contrast, monoterpenes are natural secondary metabolites that primarily exert nonspecific membrane-disrupting effects, leading to increased membrane permeability and leakage of intracellular contents.[48]

Importantly, all bacterial isolates, including those exhibiting the lowest susceptibility to monoterpenes, remained fully susceptible to all tested antibiotics. This indicates that low monoterpene activity is not related to acquired antibiotic resistance but rather reflects intrinsic tolerance mechanisms, such as reduced membrane permeability, efflux pump activity in Gram-negative bacteria, or protective cell wall structures in Gram-positive species[49]

Despite their lower antimicrobial efficacy compared to antibiotics, monoterpenes offer important advantages for food preservation. At low concentrations, monoterpenes exhibit acceptable sensory properties and may act synergistically with other preservation factors such as refrigeration, vacuum-packaging, or mild acidification.[50] Moreover, their nonspecific mode of action reduces the likelihood of resistance development compared to antibiotics with defined molecular targets,[51] supporting their potential role as complementary natural antimicrobials in sustainable meat preservation strategies. It should be acknowledged that this study was based on meat obtained from a single animal, which represents an inherent limitation. The results may therefore reflect individual variation rather than species-wide characteristics, and should be interpreted with appropriate caution.

4 Conclusion

This study presents the first systematic characterization of microbial succession in brown bear (*Ursus arctos*) shoulder meat during refrigerated vacuum storage and the first evaluation of the effects of pure monoterpenes applied *via* alginate coatings on microbial dynamics of bear shoulder meat. Culture-based enumeration demonstrated that application of monoterpene-enriched alginate coatings tended to result in higher LAB counts, which reached higher counts in treated samples (5.09–5.16 log CFU g^{-1}) compared to the control (4.92 log CFU g^{-1}). This represents a shift in the meat microbiota toward higher LAB counts, although whether this translates into beneficial effects on meat quality cannot be concluded from the present data, as sensory properties, pH, volatile compounds, and texture were not assessed in this study. Obligate aerobic *Pseudomonas* spp. were eliminated by vacuum-packaging in all experimental groups and remained below the detection limit from day 3 onward. MALDI-TOF MS

identification revealed 36 bacterial species associated with bear shoulder meat, belonging to four major groups: Enterobacteriaceae (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Citrobacter braakii* and others), *Pseudomonas* (*P. fluorescens*, *P. fragi*, *P. lundensis*, *P. synxantha*, *P. taetrolens*, *P. trivialis*), Acinetobacter (*A. calcoaceticus*, *A. dispersus*, *A. guillouiae*), and lactic acid bacteria (*Lactococcus lactis*, *Lactococcus garvieae*, *Leuconostoc mesenteroides*, *Lactobacillus sakei*, *Weissella viridescens*, *Carnobacterium divergens*, *Kurthia zopfii*). Microbial succession during vacuum storage was characterized by initial Enterobacteriaceae dominance (50% on day 0, 71% on day 3), complete elimination of obligately aerobic genera (*Pseudomonas* and *Acinetobacter*) by day 3, and a progressive shift toward LAB dominance (35% on day 7). Disk diffusion assays established a clear hierarchy of monoterpene antimicrobial activity, with the oxygenated monoterpene D-carvone exhibiting the highest efficacy (inhibition zones 3.33–7.33 mm), followed by sabinene and limonene, while bicyclic hydrocarbons α-pinene and β-pinene showed the weakest activity. These results confirm the strong dependence of antimicrobial efficacy on monoterpene chemical structure, with markedly higher activity of oxygenated compounds. All 36 bacterial species isolated from bear shoulder meat demonstrated complete susceptibility to all tested antibiotics, with inhibition zones ranging from 20.33 to 54.33 mm. This provides baseline data describing natural antibiotic susceptibility of brown bear meat microbiota unexposed to anthropogenic antibiotic selection pressure. The absence of acquired resistance in all tested species, including genera commonly associated with multidrug resistance (*Pseudomonas*, *Acinetobacter*, *Serratia*), has important implications for environmental monitoring of antimicrobial resistance and provides reference data for comparison with microbiota from intensive animal production systems. Overall, this study demonstrates that monoterpenes applied *via* alginate coatings function as selective modulators of microbial succession, promoting LAB dominance rather than exerting broad nonspecific antimicrobial inhibition. The application of MALDI-TOF MS enabled the first species-level resolution of bacterial diversity in brown bear shoulder meat and precise monitoring of storage-associated microbial dynamics. These findings provide a foundation for future research focused on optimization of monoterpene-based coatings for controlled aging, quality enhancement, and bioprotection of bear shoulder meat.

Ethics approval and consent to participate

This study did not involve live animals or experimental animal procedures. The bear meat used in this study was obtained from a legally hunted brown bear (*Ursus arctos*) under regulated wildlife management practices in accordance with Slovak Game Management Act (Act No. 274/2009 Coll.) and the Act on Nature and Landscape Protection (Act No. 127/2024 Coll.). The meat was sourced from a licensed game processor and complied with EU hygiene standards for wild game (Regulation EC No 853/2004). No ethics committee approval was required as the study involved only post-mortem tissue analysis of legally obtained game meat.

Author contribution

Miroslava Kačániová: conceptualization, data curation, writing – original draft, methodology, investigation, formal analysis, supervision, project administration, funding acquisition. Guiguo Zhang: data curation, writing – original draft, formal analysis. Minhang Qiao: review & editing, data curation, formal analysis. Qing Li: Data curation, writing – original draft, Formal analysis. Wenhui Wang: data curation, writing – original draft, formal analysis. Yunke Yu: data curation, writing – original draft, formal analysis. Peter Haščík: review & editing, supervision, methodology. Joel Horacio Elizondo-Luevano: review & editing, supervision, methodology. Stefania Garzoli: methodology, review & editing, supervision. Alessandro Bianchi: visualization, software, data curation, methodology, investigation, formal analysis, writing – original draft, review & editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting this article have been included within the article.

Acknowledgements

The authors thank grant VEGA 1/0059/24 “Chemical properties and biological activity (*in vitro*, *in vivo* and *in situ*) of plant volatile mixtures, their main components and inclusion systems. This research was funded by the grant APVV-20-0058 “The potential of the essential oils from aromatic plants for medical use and food preservation. The authors used Claude (Anthropic) for English language editing and formatting assistance during manuscript preparation. All content was reviewed and verified by the authors, who take full responsibility for the publication.

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