American Journal of Biochemistry and Biotechnology

Original Research Paper

Antioxidant and Antibacterial Properties of Essential Oils from *Citrus reticulata* cv. Shatangju Peel for the Preservation of Chilled Pork

[1]Yao Dong, [2]Xintong Zou, [2,3]Hongli Zhou, [2,3]Hongwei Pan and [2,3]Hao Cui

[1]College of Biology and Food Engineering, Jilin Institute of Chemical Technology, Jilin, China
[2]College of Chemistry and Pharmaceutical Engineering, Jilin Institute of Chemical Technology, Jilin, China
[3]Engineering Research Center for Agricultural Resources and Comprehensive Utilization of Jilin Province, Jilin Institute of Chemical Technology, Jilin, China

Article history
Received: 18-05-2024
Revised: 03-07-2024
Accepted: 05-07-2024

Corresponding Authors:
Hao Cui; Hongwei Pan
College of Chemistry and Pharmaceutical Engineering, Jilin Institute of Chemical Technology, Jilin, China
Email: cuihao@jlict.edu.cn; 18629983815@163.com

Abstract: *Citrus reticulata* cv. Shatangju peel essential oil (CrspEo), with three major components D-limonene (88.15%), γ-terpinene (4.59%) and β-myrcene (2.62%), possessed antioxidant activities with DPPH and $ABTS^+$ radical scavenging with the IC_{50} (concentration of CrspEo scavenging 50% of radical) of 9.87±0.12% and 0.48±0.02%, respectively. Meanwhile, CrspEo showed antimicrobial activity in suppressing the growth of *Bacillus pumilus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* with Minimum Inhibitory Concentration (MIC) in the range of 0.04-0.32%. To investigate the preservation performance of CrspEo in chilled pork stored at 4°C, the CrspEo, in the concentrations of 0, 0.5, and 1% (v/v) [denoted as BC, LC, and HC, respectively], were used to treat the chilled pork, which was controlled with 5 mg/mL sodium benzoate solution (PC). The CrspEo treatments showed lower pH, thiobarbituric acid reactive substances, percentage of metmyoglobin, total volatile basic nitrogen, and the total viable count and higher protein solubility than the BC treatment during storage and were consistent with those obtained by the PC treatment. *Yersinia ruckeri* and *Enterobacter agglomerans* were identified in the chilled pork. Both of the two bacteria were sensitive to the CrspEo and the *E. agglomerans* is dominant in the spoilage of the pork samples. The sensory properties of chilled pork treated with CrspEo were not affected and the shelf-life of chilled pork can be extended by CrspEo from 6 days to 9 days. Hence, CrspEo could effectively retard the spoilage of chilled pork.

Keywords: *Citrus reticulata* cv., Shatangju Peel, Essential Oils, Preservation, Sensory Properties, Chilled Pork

Introduction

Chilled pork is fresh meat that is referred to enforce the veterinary system strictly, treated the carcass cooling after slaughter quickly, reduced the temperature to 0-4°C the 24 h, and kept at this temperature in the processing, distribution, and sales process (Wang *et al*., 2006). Compared with frozen meat, it can better maintain the original nutritional value and flavor of fresh meat. Chilled pork is safe and readily accepted by consumers. However, chilled pork deteriorates quickly due to its moisture and unsaturated fat content. The main reason leading to meat spoilage is the proliferation of bacteria in meat, including *Pseudomonas*, lactic acid, and *Enterobacteriaceae* (Samelis *et al*., 2000). Spoilage bacteria can digest proteins and fats in meats, producing small peptides, amino acids, ammonia, acids, and unpleasant odors (Zhi, 2005). Oxidation of unsaturated fats and proteins also causes meat to spoil. The reduction in protein solubility has been reported to be caused by the degeneration of proteins, including myosin (Chan *et al*., 2011; Tironi *et al*., 2010). Myosin degeneration is the principal cause of the generation of unacceptable exudates in pork of inferior quality (Offer and Knight, 1988). All these effects lead to changes in indicators and appearance, including increases in pH, Total Volatile Basic Nitrogen (TVB-N), Thiobarbituric Acid Reactive Substances (TBARS) levels, the conversion of Myoglobin to Metmyoglobin (MetMb), and change in visible color to brown (Ngapo *et al*., 2007).

Plant extracts with antimicrobial and antioxidant activities, such as polyphenols and essential oils (Eos) have been exploited as candidates for the preservation of chilled pork. After treatment with *Morus alba* L. Leaf Ethanolic Extract (MLEE), the quality characteristics, pH, TBARS values, percentage of Metmyoglobin (MetMb%), TVB-N, and the Total Viable Count (TVC) of chilled pork stored at retail conditions for 9 days were more ideal than those of the untreated group. Sensory evaluation of the chilled pork revealed that treated by MLEE did not hurt its sensory characteristics. Therefore, MLEE can prolong the shelf life of chilled pork by 3-6 days (Cui *et al*., 2021b). Van Ba *et al*. (2016) found that water extract from shiitake is a potential preservative for fermented sausages, which can inhibit the increase in pH, the level of lipid oxidation, and the count of spoilage bacteria and improve the lactic acid bacteria count. Pan *et al.* (2022) examined the antimicrobial activity of the Total Flavonoids from *Zizania Latifolia* Bracts (TFZB) against *Escherichia coli* and *Staphylococcus aureus*, respectively. Based on this, 1.0 mg/mL of TFZB has the capacity to markedly enhance the sensory quality of chilled pork by impending microbial growth and thereby preserving its freshness of pork.

Citrus reticulata cv. Shatangju (Crs) is a member of the *Citrus* subfamily. It is named after the village of Shatangkeng, where it was originally grown. Compared to other *Citrus* fruits, Crs has a thin skin, juicy flesh, and sweet taste. This fruit is widely grown in the Guangxi Province in China, with an annual yield of 5 million tons. *Citrus reticulata* cv. peel (Crp), with the properties of nourishing vitality and strengthening the spleen, drying dampness, and dissolving phlegm, was a traditional Chinese medicine and was listed in the Drug and Food Homology directory (Chinese Pharmacopoeia Commission, 2015; Tao, 1955). In addition, Crp has a strong fragrance due to the *C. reticulata* cv. peel Essential oil (CrpEo), is a mixture containing many chemical components that could be distilled with steam, ionic liquids, and fragrances (Bica *et al*., 2011). The dominant components of the *Citrus* species peel Eos were found to be limonene (92.52-97.3%) and b-pinene (1.37-1.82) (Hosni *et al*., 2010). Tao *et al*. (2009) found that the Bingtang sweet orange (*Citrus* sinensis Osbeck) peel Eos inhibited the growth of *S. aureus*, *Penicillium chrysogenum*, *B. subtilis*, *E. coli*, and *Saccharomyces cerevisiae*. Velázquez-Nuñez *et al*. (2013) found that *Aspergillus flavus* could be inhibited by both direct addition and vapor contact of Eos from *C. sinensis* var. Valencia. 'Gannanzao' orange peel Eo exhibited an inhibition effect on the proliferation and migration of HepG2 and HCT116 cells (Liu *et al*., 2019). The inclusion of dietary orange peel Eos in lactating dairy ewes' rations was found to result in an increase in the mean yield of daily milk, an improvement in feed efficiency, an increase in milk fat concentration of saturated fatty acids, a decrease in unsaturated fatty acids concentration and an improved in antioxidant status of milk and blood plasma (Kotsampasi *et al*., 2018). Lv *et al*. (2012) found that oxidative damage in rats with acute otitis media could be reduced by treatment with CrpEo. The antibacterial and antioxidant activities of CrspEo were investigated in order to ascertain its potential for the preservation of chilled pork. In order to expand the applicability of this compound, its effect on pH, TBARS, MetMb%, TVB-N, the TVC, and protein solubility of the pork samples at different storage times was measured. Furthermore, the sensory characteristics and acceptability of the CrspEo-treated pork samples were evaluated.

Materials and Methods

Materials, Microbial and Instruments

The *Longissimus thoracis et lumborum* chilled pork (from the pig aged approximately 180 days and weighing 95-100 kg) was provided for testing on the same day of slaughter by a local market, Huazheng Chilled Pork Retailer number Ji B 0031. The *Citrus reticulata* cv. Shatangju (Crs) were purchased in the supermarket and were identified by Prof. Xianpu Ni of Shenyang Pharmaceutical University. Dry *Citrus reticulata* cv. Shatangju peel samples, bearing voucher no. Crsp-01 was submitted for deposition at the Jilin Institute of Chemical Technology (Jilin, Jilin, China). The bacterial strains, *B. pumilus* ATCC 700814, *B. subtilis* ATCC 6633, *C. albicans* ATCC 14053, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *S. cerevisiae* ATCC 9763, were obtained from http://www.bzwzw.com/index.php. *Yersinia ruckeri* and *Enterobacter agglomerans* were isolated from spoiled pork. Muller-Hinton Broth (MHB) was obtained from Oxoid Ltd. (London, UK). Sabouraud Dextrose Broth (SDB) was purchased from Merck KGaA (Darmstadt, Germany). Plastic wrap was obtained from Yessel of Nanjing Yuehuo Home Furnishings Co., Ltd (2022, Jiangsu, China). GC-MS instrument was Shimadzu GCMS-QP-2010 plus (Japan). Multiskan was Molecular Devices SpectraMax M4 (USA). The microbial identification analysis system was Qingdao Juchuang Environmental Protection Group Co. LTD, JC-W21 (China).

Analyzation of the Chemical Composition of the CrspEo by GC-MS

Fresh Crsp were shade-dried at room temperature. CrspEo was extracted by steam distillation under the optimal conditions as an extraction time of 138.71 min, a soak time of 199.45 min, and the ratio of solid to liquid of 1:8.94 and stored at 4°C in darkness (Cui *et al*., 2021a). GC-MS was performed as previously reported (Cui *et al*., 2018). A GC-MS instrument was utilized in accordance with the following conditions: The Rxi-5MS fused silica column, with a length of 30 m, internal diameter of 0.32 mm, and a film thickness of 0.25 µm, was used. Helium was employed as the carrier gas, with a purity of 99.999%. The flow rate was set at 3.0 mL min^{-1}. The heating program was set to a column temperature of 50°C (held for 3 min), increased to 100°C at a rate of 50°C min^{-1}, to 200°C at a rate of 8°C min^{-1}, and to 290°C at a rate of 100°C min^{-1}, which was maintained for 10 min. The injector and interface temperatures were set at 280 and 230°C, respectively, with a pressure of 117.6 kPa. The Eos components were identified by comparing the Kovats index and mass spectra of known components in the mass spectrometry database (National Institute of Standards and Technology) (Adams, 2007). The relative quantity of the compounds was determined from the Flame Ionization Detector (FID) peak areas without the application of the FID response factor correction. This value was subsequently expressed as a percentage.

Antioxidant Activities of CrspEo

DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging assay of CrspEo was performed according to the methodology outlined by Dong *et al*. (2015) with appropriate modifications. Two milliliters of CrspEo solution in the concentration of 2.50-12.50% in 95% ethanol was added to an equal volume of a 0.1 mmol/L DPPH ethanol solution (95%). The absorbance of the mixture was determined at 517 nm (A_i) following a 30 min period of incubation in the dark. The control (A_0) and blank (A_j) were obtained from the systems containing the DPPH solution without the sample and containing just the ethanol, respectively. Vitamin C (VC, in concentrations ranging from 1-5 μg/mL) was chosen as a positive control. The DPPH Radical Scavenging Activity (DPPH RSA) was expressed by the following equation:

$$DPPH\ RSA\ (\%) = \frac{A_0 - (A_i - A_j)}{A_0} \times 100$$

The results were expressed as the IC_{50}, which represents the concentration of CrspEo that scavenges 50% of the DPPH radical.

$ABTS^+$ Radical Scavenging Activity

The $ABTS^+$ Radical Scavenging Activity ($ABTS^+$RSA) of CrspEo was monitored by the method of Wiriyaphan *et al.* (2012) which was represented by the values of IC_{50} (mg/mL). The $ABTS^+$ radical solution was diluted to an absorbance of 0.70±0.02 at 734 nm. The CrspEo solution was adjusted in a concentration gradient from 0.25-1.25% (v/v) in 95% ethanol and stored in the dark. Two hundred microliters of different concentrations of the ethanol solution of CrspEo were mixed with 3 mL of $ABTS^+$ radical solution. The absorbance of the mixture was determined at 734 nm (A_S) following the completion of the reaction in the dark. In the control group, CrspEo was replaced by a 20% ethanol solution (A_0). VC at concentrations of 1.0, 3.0, 5.0, 7.0, and 9.0 μg/mL, were used instead of the CrspEo in the system as a positive control group. $ABTS^+$ RSA of CrspEo was expressed by the following equation:

$$ABTS^+\ RSA\ (\%) = \frac{A_0 - A_S}{A_0} \times 100$$

Evaluation of Antibacterial Activities

The Minimum Inhibitory Concentration (MIC) was determined by the micro-dilution method (reduced by 0.5-fold) to represent the *in vitro* antibacterial activity (Cui *et al*., 2018). *B. pumilus*, *B. subtilis*, *C. albicans*, *E. coli*, *S. aureus*, and *S. cerevisiae* were selected as the test microorganisms. CrspEo was diluted with the 20% DMSO and its final concentration was fixed in the range of 0.01-0.64% in each well of the 96 well plate. A single control well, devoid of CrspEo, was utilized as a negative control. The 96 well plate was incubated at 28°C (two fungus species on SDB) or at 37°C (four bacteria species on MHB for 24 h and then the optical densities of this mixture (absorbance) were measured at 620 nm by a Multiskan. The method was also employed for the assessment of the antibacterial activity of CrspEo against the microbes isolated from meat samples in the section "Microbiological analysis of chilled pork" and sodium benzoate was a positive control.

Experiment with the Preservation of Chilled Pork

Sample Preparation

The CrspEo was diluted to the concentration of 0% (v/v) CrspEo solution Blank Control, (BC), 0.5% CrspEo solution (Low Concentration CrspEo; LC) and 1.0% CrspEo solution (High Concentration CrspEo; HC) with 20% ethanol (Zhang *et al*., 2016). The 10 pork samples from 5 pigs were randomly divided into 180 portions weighing 35 g ± 1 g. The samples in each treatment were sprayed as a group with the solution of HC, LC, BC, and the 5 mg/mL sodium benzoate Positive Control, (PC) (in the amount of approximately 1 mL/100 cm^2) (Bellés *et al*., 2017). Chilled pork samples from each treatment were packaged in plastic wrap (GB 10457, 2009) and stored at refrigeration (4°C) for 0, 3, 6, and 9 days in the same refrigerated cabinet at the Huazheng chilled pork retailer. Three samples were used for each treatment and storage time, with each treatment repeated three times. This resulted in a total of 144 samples (3×4×4×3). Each pack contained three meat samples. A further 36 samples were prepared for sensory evaluation, including 12 samples for sensory characteristics and 24 samples (equally divided between the BC and HC groups) for the duo-trio discrimination test.

pH Determination

According to the report of Lu *et al.* (2013), chilled pork can be divided into three categories according to its pH range, namely, 5.6≤ pH ≤6.2 was I-class fresh meat, 6.3≤ pH ≤6.6 was II-class acceptable meat and pH >6.7 was spoiled meat. Fifteen milliliters of sterilized distilled water was added to the homogenate of 5 g of treated chilled pork at 0, 3, 6, and 9 days. The pH of the filtrate of the mixture was determined using a digital pH meter at room temperature (25°C). The standard solutions were potassium hydrogen phthalate (with a pH of 4.00 at 25°C) and mixed phosphate (with a pH of 6.68 at 25°C).

Lipid Oxidation

The extent of lipid oxidation was assessed using the TBARS assay, as described by Cui *et al*. (2021b). After immersion in 10 mL of 20% trichloroacetic acid for 1 h, 10 g of pork sample was homogenized and stirred for 10 min. The resulting mixture of 2 mL of the supernatant and 2 mL of the 0.02 mol/L thiobarbituric acid was boiled in water for 30 min. Following a period of cooling to room temperature, the absorbance at 532 nm (A_{532}) of the samples was measured. The results were calculated using 1, 1, 3, and 3-tetramethoxypropane as a standard curve, with the data expressed as milligrams of malondialdehyde per kilogram of chilled pork.

MetMb% Content

The content of MetMb in chilled pork was extracted and quantified in accordance with the methodology outlined by Pogorzelska *et al*. (2018), with minor modifications. Five grams of chilled pork samples were homogenized with 5 mL of 0.04 mol/L potassium phosphate buffer (PBS), followed by centrifugation at 1000 r/min for 15 min at 4°C. The absorbance of the supernatant was determined at the wavelengths of 525 nm (A_{525}), 572 nm (A_{572}), and 700 nm (A_{700}). The MetMb% was expressed according to the following equation:

$$MetMb\% = \frac{1.395 - (A_{572} - A_{700})}{A_{525} - A_{700}} \times 100\%$$

TVB-N Value

The TVB-N value was determined in accordance with the methodology outlined in GB 5009.228 (2016). One hundred milliliters of distilled water were added to the homogenate of 10 g of chilled pork. The mixture was filtered after standing for 30 min. Ten milliliters of the mixture, 5 mL of the filtrate, and 5 mL of a 10 g/L of magnesium oxide suspension in ethanol were distilled for 5 min. Ten milliliters of a solution of 20 g/L boronic acid containing 0.5 mL of indicators (the 1.0 g/L of methyl red and 0.5 g/L methylene blue in ethanol), were added to the condensed liquid. The 0.01 mol/L hydrochloric acid (V_1 in mL) was used as an aqueous standard to titrate directly to a titration endpoint with the blue-violet color. The reaction system devoid of chilled pork constituted the blank control, consuming V_2 (mL) of the standard aqueous. The TVB-N value (X, mg/100 g) was calculated using the following equation:

$$X(\text{mg}/100\text{ g}) = \frac{(V_1 - V_2) \times 0.01 \times 14 \times 100}{m \times 5}$$

As outlined in the report by Lu *et al*. (2013), the TVB-N content of chilled meat was classified into three groups: ≤15 mg/100 g was classified as fresh meat of I-class, ≤20 mg/100 g was deemed acceptable meat of II-class and >20 mg/100 g was identified as spoiled meat.

Microbiological Analysis of Chilled Pork

TVC was determined according to the procedure of GB 4789.2 (2016). On days 0-9, the supernatant of 3 g chilled pork from each group in 27 mL of sterilized PBS homogenate was diluted at a 10-fold gradient. In order to perform a colony count, 1 mL of two adjacent-concentration diluents, in which the colonies were present at a concentration range of 30-300 cfu/mL, were distributed on the plate count agar and incubated at 37°C for 48 h. The number of colonies of the chilled pork sample was calculated using the following equation:

$$C = \frac{\Sigma N}{(c_1 + 0.1 \times c_2) \times f}$$

where, C represents the number of the colonies of the chilled pork, $\sum N$ is the sum of the number of colonies in the plate, c_1 is the number of plates with the first dilution (low dilution ratio), c_2 is the number of plates with the second dilution (high dilution ratio) and f is the dilution factor (first dilution).

The results of the microbiological analysis were expressed in terms of $\log_{10}$ cfu/g. As described by Lu *et al*. (2013), $\log_{10}$ cfu/g were divided into three sections ($\log_{10}$ cfu/g <4, 4≤ $\log_{10}$ cfu/g ≤6, $\log_{10}$ cfu/g >6) to relate the quality of the chilled pork (I-class, II-class and deteriorated meat). The microorganisms isolated from the meat samples were identified by biochemical analysis using a Microbial Identification Analysis System as the process of Fig. S1 (Collins *et al*., 1989; Benson, 2002; Zaved *et al*., 2008).

Total Protein Solubility

According to Joo *et al*. (1999), the measurement of Total Protein Solubility (TPS) was employed as an indicator of protein denaturation. TPS was analyzed in accordance with the methodology outlined by Farouk and Swan, (1998). The homogenate of 2 g of chilled pork in 20 mL of cold 1.1 mol/L potassium iodide solution in 0.1 mol/L PBS (pH 7.4) was subjected to centrifugation at 6000 r/min for 15 min, after which the supernatant was employed in the determination of protein content in accordance with the methodology outlined by Torten and Whitaker (1964).

The percentage of TPS in the chilled pork sample was calculated using the formula:

$$TPS(\%) = \frac{P_S}{P_T} \times 100$$

where, *TPS* is the total protein solubility; P_T and P_S, expressed in mg/mL, are the total protein concentration and the protein concentration of the supernatant of the chilled pork sample, respectively.

SDS-PAGE Analysis of Muscle Proteins

According to Ryu *et al*. (2005), the denaturation of Sarcoplasmic Proteins (SPs) could be a more reliable indicator of muscle quality, and the precipitation of SPs on Myofibrillar Proteins (MPs) was associated with pork color. SPs and MPs were prepared using the method of Johansson *et al*. (1994). One gram of the chilled pork sample was homogenized in 10 mL of 25 mmol/L potassium PBS (pH 7.4). The supernatant was filtered through a 0.45 μm filter and used as SP. The precipitate was washed twice with 10 mL of PBS. The remaining precipitate was homogenized with 10 mL of mixed solution (1.1 mol/L potassium iodide solution and 0.1 mol/L PBS in a ratio of 1:1) for 1 min. MP was obtained after filtration through a 0.45 μm filter.

After heating for 5 min at 100°C, the SP and MP were loaded at a final concentration of 2 mg/mL on a 5% stacking and 10% separating gel. The electrophoresis of 20 μL proteins was performed on stacking gels (10 mA) and separating gels (20 mA). Standard protein markers (Premixed Protein Marker, Takara) were used as molecular weight standards.

Sensory Evaluation

The PC, HC, and BC samples were used for sensory evaluation. The chilled pork samples were used for sensory evaluation. The panelists were trained according to Sensory analysis (2015) before the analysis. The sensory evaluation was presented in the descriptive test by the sensory characteristics and in the discrimination test by the duo–test. In all three replicate experiments, the same panelists were involved in the sensory analysis.

Sensory Characteristics

Twenty grams of chilled pork in the PC, HC, and BC treatments were used for the descriptive evaluation of odor, color, elasticity, and viscosity. After the evaluation, the samples were boiled in 100 mL of sterile distilled water for 20 min and the broth and its clarity were checked. Table 1 shows how the attributes were defined and standardized for the sensory analysis. The quantified descriptors were odor (0 = the smell of the meat is weak or absent and with or without a slight odor of orange peel and it has a peculiar odor to 10 = unique odor of fresh meat with or without the slight odor of orange peel, no miscellaneous odor), color (0 = the color is grayish brown, with dark to 10 = the color is bright red, with luster), elasticity (0 = the tissue is loose and cannot be recovered after pressing to 10 = it has complete shape, compact tissue, not loose and can be recovered after pressing), viscosity (0 = very sticky to 10 = appearance is not dry, hands are not sticky) and characteristics of the broth (0 = the broth was cloudy with a flocculent precipitate to 10 = transparent and clear, with a rich meaty flavor). The sensory score of 6 is the lower limit of acceptability. The sensory was performed by A nine-member panel, trained according to GB 5009.228 (2016) evaluated the samples in terms of the senses.

Discrimination Test

After 6 days of storage, the chilled pork samples from the BC and HC groups were cut into 54 pieces weighing 5 g ± 0.2 g. Four polyethylene trays, numbered 1, 2, 3, and 4, were used to hold 18 pieces of BC, 18 pieces of HC, 36 pieces of BC, and 36 pieces of HC, respectively. Trays 1 and 2 were set as the reference group and coded as A_{rg} and B_{rg}. Trays 3 and 4 were designated as A and B and coded with unique random three-digit numbers (Table S1). The first sample submitted in each group was the control sample as follows: A_{rg}AB, B_{rg}AB, A_{rg}BA, and B_{rg}BA. Thirty-six evaluators were selected to assess the sensory quality of chilled pork by evaluating the odor, color, elasticity, and viscosity using the duo-trio test (GB/T 17321, 2012). The evaluation was performed according to the design in Table S1. α represents the probability of inferring the sensory differences when they were not present.

Table 1: Standards for evaluating the sensory quality of chilled pork

Project	A (8.0-10.0)	B (6.0-7.9)	C (0-5.9)
Odor	The odor of fresh meat with or without a slight odor of orange peel, no miscellaneous odor	Fresh meat smell with or without the slight odor of orange peel, less miscellaneous odor	The smell of meat is weak or absent, with or without a slight of orange peel and it has a peculiar odor
Color	The color is bright red, with luster	The color is oxblood red	The color is grayish brown, with dark
Elasticity	It has a complete shape and compact tissue is not loose and can be recovered after pressing	Tissue is not compact and can recovered after pressing for some time	The tissue is loose and cannot be recovered after pressing
Viscosity	Appearance is not dry, not sticky hands	Dry appearance, slightly sticky hands	Very sticky
Characteristics of broth	Transparent and clear, with rich meat flavor	Slightly cloudy, a small amount of flocculent, light meat flavor	The broth was cloudy with a flocculent precipitate

The numbers in brackets show the range of scores on this sensory evaluation scale

Statistical Analyses

Statistical analysis was performed on experimental data by analysis of variance using the general linear model procedure, using SPSS (version 19.0) statistical software for Windows 10. Data were expressed as mean ± standard error. Treatments (BC, LC, HC, and PC) and storage times (0, 3, 6, and 9 days) were considered as fixed factors and the replications of the experiments as a random term. For sensory data, panelists and sessions were considered as a random factor as well. Duncan's multiple-range tests were used to determine the significance of means for multiple comparisons. $p<0.05$ was considered significant.

Results

Eos Yield and Characterization

As shown in Table 2, 22 components were identified as the constituents of CrspEos. The Eos mainly consisted of D-limonene (88.15%), γ-terpinene (4.59%), and β-myrcene (2.62%). These four components accounted for 95.36% of the total Eos. The major components of the CrspEos were similar to the reported Eos of the orange peel, but the other components were slightly different (Liu *et al*., 2019; Yang *et al*., 2017). The result may be because the oranges came from different places and at different times.

Antioxidant Activity

As illustrated in Table 3, the *in vitro* antioxidant activities of CrspEo are reflected in its IC_{50} values. The DPPH radical scavenging activity of CrspEo was observed to increase with increasing concentration within the range of 2.50-12.50%. The IC_{50} value of CrspEo was 9.87±0.12% and that of VC, the positive control, was 4.72±0.02 μg/mL. Similarly, the radical scavenging activity for $ABTS^+$ was increased by increasing the concentration of CrspEo from 0.25-1.25% with the IC_{50} value of 0.48±0.02% and the IC_{50} value of VC was 7.23±0.10 μg/mL.

Table 2: Relative percentage composition of CrspEo

No.	Retention time (min)	CAS	Compounds	Molecular formula	Area %
1	3.999	58037-87-9	bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	$C_{10}H_{16}$	0.12
2	4.156	80-56-8	α-pinene	$C_{10}H_{16}$	1.04
3	5.364	28634-89-1	β-thujene	$C_{10}H_{16}$	0.22
4	5.434	127-91-3	β-pinene	$C_{10}H_{16}$	0.34
5	6.22	123-35-3	β-myrcene	$C_{10}H_{16}$	2.62
6	6.567	99-83-2	α-phellandrene	$C_{10}H_{16}$	0.04
7	7.108	536-50-5	methyl-p-tolyl carbinol	$C_9H_{12}O$	0.03
8	7.272	527-84-4	2-isopropyl toluene	$C_{10}H_{14}$	0.35
9	7.955	5989-27-5	d-limonene	$C_{10}H_{16}$	88.15
10	8.642	3338-55-4	β-cis-ocimene	$C_{10}H_{16}$	0.08
11	8.95	99-85-4	γ-terpinen	$C_{10}H_{16}$	4.59
12	10.222	29050-33-7	4-carene	$C_{10}H_{16}$	0.18
13	10.745	115-99-1	linalool formate	$C_{11}H_{18}O_2$	0.13
14	15.495	112-31-2	decanal	$C_{10}H_{20}O$	0.09
15	21.89	586-63-0	isoterpinolene	$C_{10}H_{16}$	0.19
16	23.68	3856-25-5	copaene	$C_{15}H_{24}$	0.03
17	24.568	515-13-9	b-element	$C_{15}H_{24}$	0.90
18	27.959	54211-14-2	7-methylenenorcarane	C_8H_{12}	0.04
19	29.407	23986-74-5	germacrene D	$C_{15}H_{24}$	0.23
20	30.468	75023-40-4	helminthogermacrene	$C_{15}H_{24}$	0.10
21	31.261	502-61-4	α-farnesene	$C_{15}H_{24}$	0.45
22	31.468	17699-14-8	α-cubebene	$C_{15}H_{24}$	0.07

Table 3: IC_{50} of *in vitro* antioxidant

	DPPH				$ABTS^+$			
	CrspEo		VC		CrspEo		VC	
	C[1] (v/v) (%)	RSA[2] (%)	C (m/v) (μg/mL)	RSA (%)	C (v/v) (%)	RSA (%)	C(m/v) (μg/mL)	RSA (%)
A	2.50	33.21±0.58	1.00	3.90±0.88	0.25	39.51±1.45	1.00	10.65±1.15
	5.00	37.42±0.67	3.00	32.36±0.88	0.50	51.70±0.67	3.00	19.94±2.08
	7.50	43.35±0.88	5.00	57.16±0.88	0.75	60.42±1.45	5.00	38.03±1.15
	10.00	51.59±0.67	7.00	80.31±0.58	1.00	73.53±1.45	7.00	47.21±1.20
	12.50	57.51±0.67	9.00	89.67±0.67	1.25	82.02±1.15	9.00	61.09±1.15
B	IC_{50} (%)	9.87±0.12	IC_{50} (μg/mL)	4.72±0.02	IC_{50} (%)	0.48±0.02	IC_{50} (μg/mL)	7.23±0.10

C = Concentration of CrspEo or VC; [2]RSA = Radical Scavenging Activity

Antibacterial Activity

Six species of microorganisms, *B. pumilusis*, *B. subtilis*, *C. albicans*, *E. coli*, *S. aureus*, and *S. cerevisiae*, were employed to ascertain the antibacterial efficacy. The results were represented by the MIC which was measured as 0.32, 0.04, 0.16, 0.32, 0.16, and 0.08%.

Effect of CrspEo on the Preservation of Chilled Pork

pH Value

As illustrated in Fig. 1, pH, one of the critical quality characteristics, was increased in all four treatment groups of chilled pork by prolonging the storage time. In the BC treatment, there was a notable increase in pH from 5.65±0.03 on day 0-6.35±0.04 on day 6, exceeding the pH limit of the I-class and reaching 7.04±0.04 on day 9, which is indicative of deteriorated meat. The rate of pH increase in the CrspEo treatments was slower than that in the BC group. Similar to the PC group, the pH of the LC group increased to 6.23±0.03 by day 9, which remained in the I-class. The pH of the HC group was lower than the values of the PC group, which remained in theI-class.

TBARS Value

A summary of the changes in TBARS values during the storage period is presented in Fig. 2. The TBARS values showed a significantly increasing trend in all four groups at all of the storage times. However, the values of the HC and LC treatments exhibited a gradual decline, ultimately reaching levels that were significantly lower than those of the PC treatment during the storage periods. The TBARS values of the BC treatment showed the fastest increasing trend from day 3-9 and a significant difference with the other groups was observed.

MetMb% Value

As illustrated in Fig. 3, the MetMb% exhibited a notable increase at the different storage times in the order of BC > LC > PC > HC. The MetMb% of the BC treatment had the fastest increasing trend, while the MetMb% of the HC treatment demonstrated the lowest values across all treatments. While the MetMb% of the HC treatment demonstrated the lowest values across all treatments. The LC and PC increased in the same trend and showed no difference except for the value on day 6.

The TVB-N Value

The TVB-N values are presented in Fig. 4. The TVB-N values of the four treatments demonstrated an upward trajectory. The value for BC increased to 16.87 mg/100 g ± 0.40 mg/100 g (>15 mg/100 g) on day 6, corresponding to fresh meat of class II and to 20.56±0.53 mg/100 g on day 9 for the deteriorated meat. Meanwhile, the values of the other three groups increased to more than 15 mg/100 g in the order of LC > PC > HC, indicating that the meat samples in these groups were the II-class fresh meat.

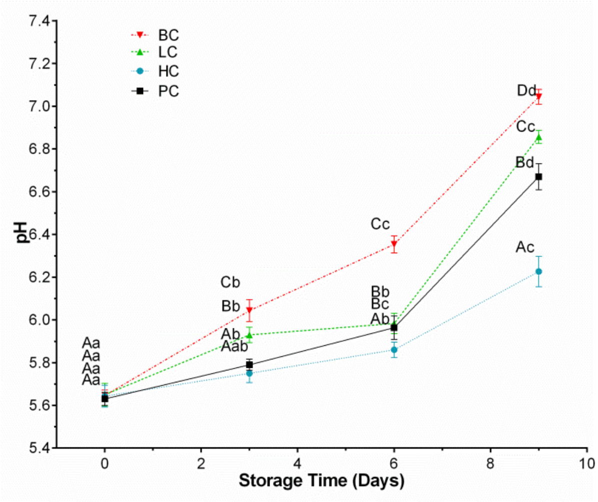

Fig. 1: Mean values (± standard error) for pH of chilled pork in different treatments during different storage times. The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

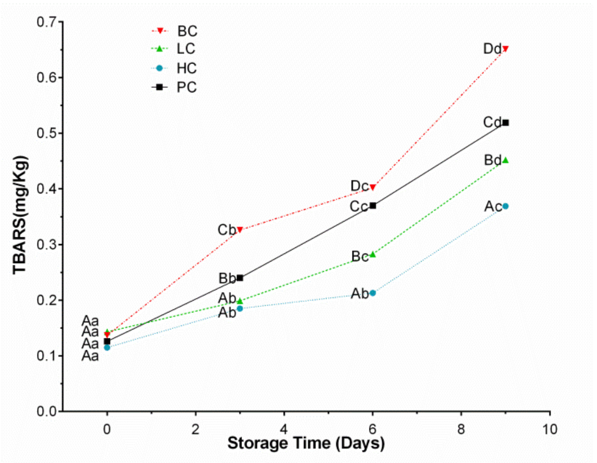

Fig. 2: Mean values (± standard error) for TBARS of chilled pork in different treatments during different storage times. The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

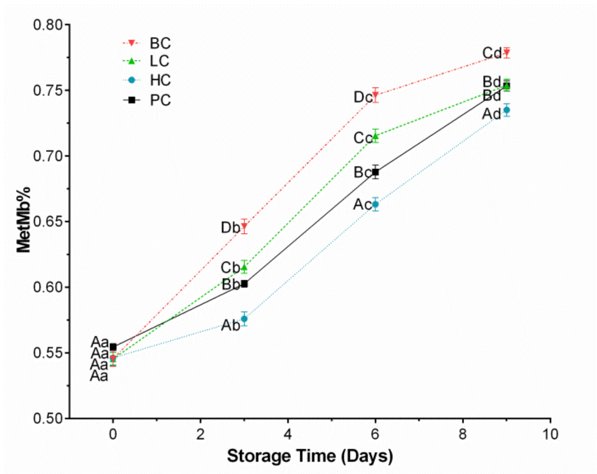

Fig. 3: Mean values (± standard error) for MetMb% of chilled pork in different treatments during different storage times. The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

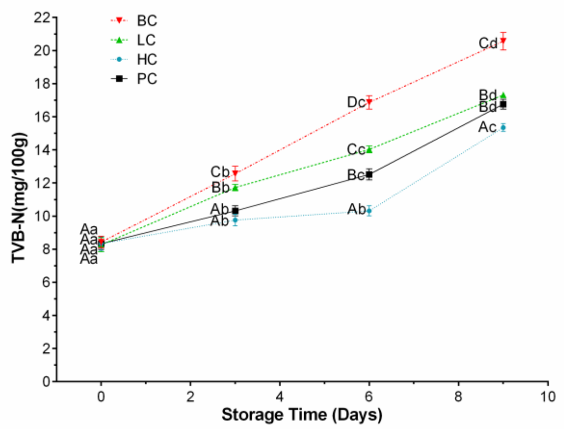

Fig. 4: Mean values (± standard error) TVB-N pH of chilled pork in different treatments during different storage times. The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

Microbiological Analysis

The outcomes of the microbiological examination are illustrated in Fig. 5. The number of bacteria increased significantly in all four treatments. The logarithm of the TVC of the fresh meat samples was 3.45 (<4) on day 0. In the BC treatment, the value increased to 4.53±0.05 on day 3 (in the range of 4-6) and reached 6.81 ± 0.11 on day 9 (>6). The results of the BC treatment exhibited a notable divergence from those of the other three groups, with values below 4 on day 3 and in the range of 4-6 on days 6 and 9. Two types of bacteria were isolated and biochemically characterized. The results in Table 4 showed that they were *Yersinia ruckeri* and *Enterobacter agglomerans*. The MIC of CrspEo against *Y. ruckeri* and *E. agglomerans* were 0.16 and 0.32%, those values of sodium benzoate were 2.5 and 1.25 mg/mL, respectively. Fig. 6 shows the proportions of the two types of bacteria and *E. agglomerans* is dominant in the overall trend. In the BC group, on days 0, 3, and 6, *Y. ruckeri* accounts for 30% and *E. agglomerans* accounts for 70%, while on day 9 the proportion is 60 and 40%, respectively. In the PC group, the proportion of *Y. ruckeri* increased from 30-39%, while the proportion of *E. agglomerans* decreased accordingly. In the LC and HC groups, the proportion of *E. agglomerans* gradually increased over time, reaching 78 and 88% respectively on the 9 days. The number of microorganisms was negatively correlated with the sensitivity of the two types of microorganisms to the preservative.

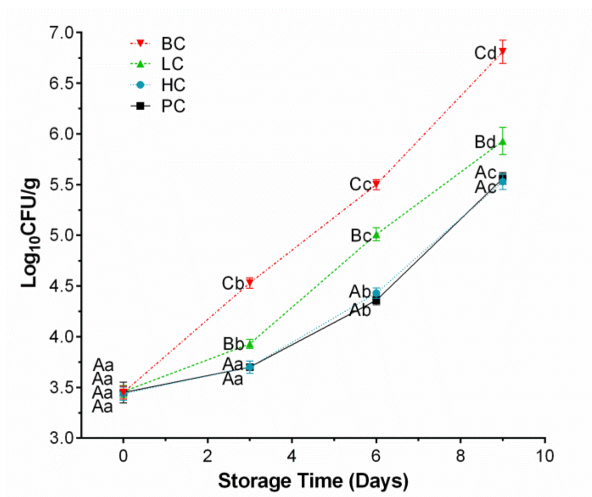

Fig. 5: Mean values (± standard error) for the total number of bacterial colonies of chilled pork in different treatments during different storage times. The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

Table 4: Biochemical characterization of bacterial isolates

	Yersinia ruckeri	*Enterobacter agglomerans*		*Yersinia ruckeri*	*Enterobacter agglomerans*
Gram	G^-	G^-	Oxidase	-	-
Ornithine	+	-	Indole	-	-
Lysine	+	-	Methyl Red	+	+
Arginine	-	-	Phenylalanine	-	-
OF	+	+	Nitrate reductase	+	+
Sorbitol	-	+	Voges-Prokauer	+	+
Adonitol	-	-	Cellobiose	-	-
Rhamnose	-	+	Citrate	-	-
Xylose	+	+	Trehalose	-	+
Saccharose	-	+	Maltose	-	-
Malonate	-	+	Esculin	-	+
Mannitol	+	+	Raffinose	-	+
Arabitol	+	+	Urease	-	-

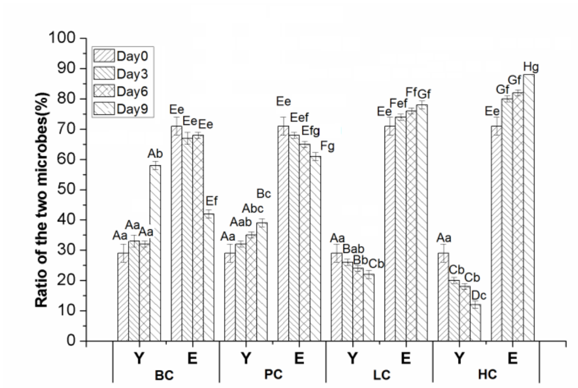

Fig. 6: Changes in the percentage of *Yersinia ruckeri* and *Enterobacter agglomerans* in chilled pork in different treatments during different storage times; The data represented by A and B, which are presented in the same storage period with different superscripts, are significantly different (p<0.05). Similarly, the data represented by a and b, which are presented in the same treatment with different superscripts, are also significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

Protein Solubility

Figure 7 illustrates the changes in protein solubility across the various treatment groups at different storage times. The proportion of soluble protein was observed to decrease in each treatment group with the prolongation of storage time. The proportion of soluble protein in fresh meat samples was 94.08±0.99% on day 0, which decreased most significantly to 58.78±2.23% in the BC group on day 9, followed by 63.86±2.02% in the LC group and the lowest to 71.96±1.74% and 73.55±5.68% in the HC and PC groups (p<0.05). At the same storage time, the proportion of soluble protein decreased significantly on day 3-81.45±1.98% in the BC group, 87.87±2.64% in the LC group, and approximately 91.31±2.02%, and 90.79±2.19% in the HC and PC groups (p<0.05). The decrease continued on day 6, similar to that on day 3. On day 9, the order of soluble protein was BC < LC < HC and PC (no significant difference was found between HC and PC groups, p<0.05). Fig. 8 shows the electrophoresis of the changes in MP (7 bands) and SP (13 bands) in different treatment groups in the patterns of SDS PAGE. As reported by Grujić *et al*. (2018), the bands of MP and SP were assigned as the 1, myosin heavy chain; 2, c-protein; 3, actin; 4, tropomyosin-1; 5, tropomyosin-2; 6-7, myosin light chains; 8, phosphorylase b; 9, pyruvate kinase; 11, phosphoglucose isomerase; 12, enolase; 14, creatine kinase; 15, aldolase; 16, glyceraldehyde phosphate dehydrogenase; 17, lactate dehydrogenase; 18, phosphoglycerate mutase; 19, triosephosphate isomerase; 20, myokinase. As time progressed, the brightness of the protein bands decreased and bands No.3, 4, and 5 (actin, tropomyosin-1, and tropomyosin-2) in MP changed significantly. In particular, on day 9, the proteins in the range from 29-44.3 kDa became significantly flatter and two additional bands were observed. In SP, the most obvious change was in band 20 (myokinase).

Sensory Evaluation and Analysis

The sensory analysis results are shown in Table 5. Samples were evaluated for odor, color, elasticity, viscosity, and flavor of HC, PC, and BC treatments on days 0, 3, 6, and 9 as the standard in Table 1.

All sensory characteristics decreased significantly over time in all three treatments (p<0.05). The HC treatment of chilled pork, with a slight odor of orange peel had a significant effect on sensory acceptance. Already on days 3, 6, and 9, the panelists noticed differences in the sensory indices between the treatments to varying degrees. The odor score of the BC treatment fell below 6, which was unacceptable, while the values of PC and HC scores were above 6. The addition of CrspEo affected the color changes of chilled pork (Fig. 9). The color gradually changed from red to a macroscopic dark red-brown, especially in the BC and LC treatments on day 9. The color scores in the HC treatment were all acceptable. BC scores significantly differed from PC and HC scores on day 3 in terms of odor, elasticity, and flavor. From day 0 to day 6, the values of sensory analysis scores of the HC and PC groups were similar and acceptable. On day 9, the scores for odor, color, elasticity, and flavor of HC were higher than those of PC. The sensory evaluation by the duo trio test was performed as GB/T 17321 (2012) and 31 out of the 36 panelists could accurately discriminate the HC and BC-treated chilled pork, which meant that the value of α <0.001 indicated the sensory differences between the samples at 6 days. Therefore, the CrspEo had some fresh-keeping effect on chilled pork and was acceptable to consumers and distinguishable from untreated samples.

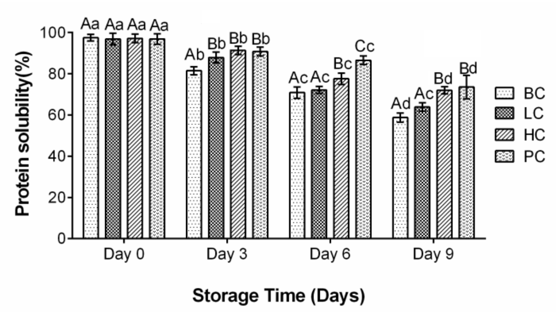

Fig. 7: Effect of CrspEo on protein solubility in chilled pork; The values marked with different letters (a-d) within the same treatment are found to be significantly different (p<0.05). The values marked with different letters (A-D) for the same storage period are significantly different (p<0.05). BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution

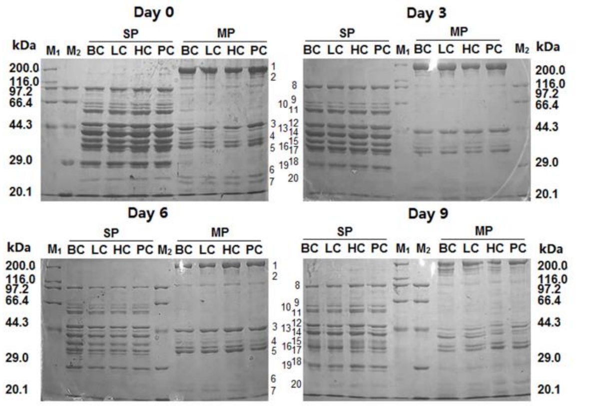

Fig. 8: Patterns of SDS-PAGE of sarcoplasmic and myofibrillar protein from chilled pork treated with different levels of CrspEo at different storage times. SP, sarcoplasmic protein; MP, myofibrillar protein; M_1, Premixed protein marker (High, Takara); M_2, Premixed protein marker (Low, Takara). BC, 0% CrspEo solution; LC, 0.5% CrspEo solution; HC, 1% CrspEo solution; PC, 5 mg/mL sodium benzoate solution. 1, myosin heavy chain; 2, c-protein; 3, actin; 4, tropomyosin-1; 5, tropomyosin-2; 6-7, myosin light chains; 8, phosphorylase b; 9, pyruvate kinase; 11, phosphoglucose isomerase; 12, enolase; 14, creatine kinase; 15, aldolase; 16, glyceraldehyde phosphate dehydrogenase; 17, lactate dehydrogenase; 18, phosphoglycerate mutase; 19, triosephosphate isomerase; 20, myokinase

Table 5: Sensory indexes of chilled pork treated with CrspEo during storage for 9 days

		PC	BC	HC
Odor	0	9.77± 0.02^{Aa}	9.67±0.03^{Aa}	9.87±0.02^{Aa}
	3	9.27± 0.03^{Ba}	8.77±0.03^{Bb}	9.33±0.04^{Ba}
	6	8.33± 0.03^{Ca}	7.40±0.06^{Cb}	8.20±0.05^{Cc}
	9	6.13± 0.03^{Da}	5.07±0.08^{Db}	6.57±0.04^{Dc}
Color	0	9.93± 0.01^{Aa}	9.70±0.04^{Aa}	9.80±0.03^{Aa}
	3	9.33± 0.03^{Ba}	8.93±0.04^{Bb}	9.33±0.03^{Ba}
	6	8.30±0.03^{Ca}	7.50±0.06^{Cb}	7.87±0.04^{Cc}
	9	6.07±0.09^{Da}	5.43±0.05^{Db}	6.77±0.07^{Dc}
Viscosity	0	9.77±0.02^{Aa}	9.77±0.03^{Aa}	9.83±0.03^{Aa}
	3	9.37±0.04^{Ba}	8.83±0.06^{Bb}	9.27±0.05^{Ba}
	6	7.70±0.03^{Ca}	7.40±0.06^{Cb}	7.53±0.03^{Cc}
	9	6.33±0.09^{Da}	5.50±0.05^{Db}	6.33±0.06^{Da}
Elasticity	0	9.77±0.03^{Aa}	9.67±0.03^{Aa}	9.73±0.04^{Aa}
	3	8.73±0.04^{Ba}	8.07±0.05^{Bb}	8.53±0.04^{Bc}
	6	7.60±0.04^{Ca}	7.17±0.05^{Cb}	7.47±0.04^{Cc}
	9	6.00±0.04^{Da}	5.63±0.06^{Db}	6.37±0.04^{Dc}
Flavor	0	9.63±0.04^{Aa}	9.40±0.07^{Aa}	9.43±0.04^{Aa}
	3	8.53±0.06^{Ba}	8.10±0.08^{Bb}	8.43±0.05^{Ba}
	6	7.62±0.06^{Ca}	6.87±0.05^{Cb}	7.37±0.04^{Cc}
	9	5.83±0.04^{Da}	4.33±0.05^{Db}	6.23±0.03^{Dc}

BC, 0% of CrspEo solution; LC, 0.5% of CrspEo solution; HC, 1% of CrspEo solution; PC, 5 mg/mL of sodium benzoate solution. The presence of different superscripts (a, b, c) in the same row indicates a statistically significant difference between the days displayed (p≤0.05). The use of different superscripts (A, B, C) in the same column indicates the presence of significant differences under varying treatment conditions (p≤0.05).

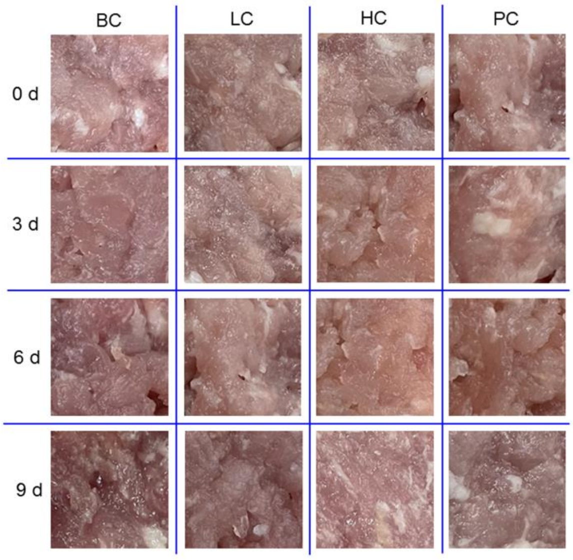

Fig. 9: Photographs of chilled pork treated with CrspEo. BC, 0% CrspEo solution; LC, 0.5% CrspEo solution; HC, 1% CrspEo solution; PC, 5 mg/mL sodium benzoate solution

Discussion

The fundamental factors in the preservative effect of natural preservatives are their antioxidant and antibacterial activities. As expected, CrspEo exhibited antioxidant activities in radical scavenging of DPPH and $ABTS^+$ and antibacterial activities against eight types of microorganisms. The compositional characteristics of CrspEo determine its biological activity. The three main compounds, including D-limonene, γ-terpinene, and β-myrcene, accounted for 95.36% of the total CrspEo. D-limonene was found to have broad-spectrum antibacterial activity and potent antibacterial activity against many bacteria and fungi (Aggarwal *et al*., 2002). Chutia *et al*. (2009) found that limonene at a concentration of 0.1% had an apparent inhibitory effect on some fungi, such as *Rhizoctonia solani* and *Fusarium oxysporum*. The Eo of Myrtle growing in Northeastern Algeria, with the major compounds of α-pinene (55%), 1,8-cineole (33,42%), and limonene (33,42%), showed antibacterial effectiveness against twenty Gram-negative bacteria (Badra *et al*., 2016). The Eo from pink pepper consisting of β-myrcene (41%), β-cuvebene (12%), and limonene (9%), showed antibacterial activity against *S. aureus* and *Listeria monocytogenes* with MICs of 0.68 and 1.36 mg/mL, respectively (Dannenberg *et al*., 2019). Shah and Mehta (2018) found that D-limonene showed significant antioxidant activity in different systems with the IC_{50} in DPPH (384.73 μmol/L), $ABTS^+$ (603.23 μmol/L), FRAP (-589.85 μmol/L), iron chelation (-18475.5 μmol/L) and hydroxyl radical scavenging (442.75 μmol/L), respectively. Myrcene, in the concentration of 40.5 mg/mL, showed significant antioxidant activity of DPPH free radical scavenging capacity with an inhibition rate of 29.22±6% (Xanthis *et al*., 2021).

The main causes of fresh meat spoilage and quality loss are microbial contamination and lipid/protein oxidation. Therefore, the preservative activity of CrspEo on the chilled pork under retail conditions was evaluated based on the antibacterial and antioxidant activities. According to the results of the antioxidant and antibacterial activities (the IC_{50} and the maximum MIC), concentrations of 0, 0.5, and 1.0% of CrspEo were selected to treat the chilled pork. The values of pH, TVB-N, TBARS, MetMb%, TVC, and protein solubility were used as the quality indicators. With increasing storage time, the pH tends to increase due to the effect of microorganisms on protein degradation and amines production (Xiao *et al*., 2020). Therefore, the pH of the CrspEo treatments was significantly lower than that of the BC treatment. At the same time, the increase in TBARS value during storage is related to the accumulation of lipid oxidation products. (Chouliara *et al*., 2007). The antioxidant activity of CrspEo inhibits this accumulation and prevents the increase in TBARS levels. Oxymyoglobin (MbO_2) is formed when myoglobin progressively combines with oxygen in the air and MbO_2 is progressively oxidized to metmyoglobin (MetMb). MetMb levels have been reported to correlate with lipid oxidation (Estévez and Cava, 2004). The formation of MetMb is inhibited because CrpsEo has excellent antioxidant activity. TVB-N is a significant indicator of meat freshness, predominantly resulting from the action of spoilage bacteria or amino acid decarboxylase during storage. It was thus observed that the TVB-N values of the samples treated with CrspEo were markedly lower than those of the control samples. The overall trend of pH and TVB-N was identical. The CrspEo-treated groups reduced the number of bacteria compared to the untreated pork samples, which directly contributed to the antibacterial activity of CrspEo. The bacteria in the pork samples were identified as *Y. ruckeri* and *E. agglomerans*, which have been reported as detectable bacteria in meat (Zhu *et al*., 2022; Özdemir and Arslan, 2015). The changing trend of microorganisms showed that *E. agglomerans* was the main strain causing spoilage in untreated meat samples at 0, 3, and 6 days, and *Y. ruckeri* played this role at 9 days. *E. agglomerans* was the main strain in the preservative-treated group. These two bacterial species are sensitive to the CrspEo, which may be one of the most direct reasons for preservation.

CrspEo prevented the decrease in the soluble protein ratio and the effect was concentration dependent MP and SP degeneration was evident in all treatment groups at different storage times. The decrease in protein solubility of the CrspEo treatment was inhibited to some extent. Myosin represents approximately 45% of the total MPs and is the primary structural protein in muscle and it has been proposed that SP degeneration may be a better indicator of muscle quality (Schiaffino and Reggiani, 1996; Choi *et al*., 2006; Ryu *et al*., 2005). Lopez-Bote *et al*. (1989) found that precipitated protein led to a color change in the sarcoplasm. The color of the meat undergoes a transformation, becoming a dark red-brown as a result of low light reflectance and the formation of metmyoglobin (Hui, 2001).

The pH, TVB-N, and TVC were classified into different levels based on their values in the treated meat, which correlated with the sensory analysis results. In particular, the log10 cfu/g of LC reached 5.01 (more than 4) on day 6, indicating that the meat was of a quality that would be classified as second-class fresh. BC, HC, and PC treatments were used, for sensory evaluation. After 6 days, HC treatment resulted in quality characteristics similar or superior to those of PC treatment. The BC treatment resulted in indices in the range of spoiled meat on day 9, with all sensory analysis values falling below 6, indicating unacceptability. On the other hand, PC and HC treatments met class I or II standards, with sensory analysis values exceeding 6 in the order of HC > PC. This trend was consistent with the pH, TBARS, MetMb%, and TVB-N values of the two groups after 6 days.

The plant Eo has been demonstrated to possess antioxidant and antibacterial properties, which have been linked to its ability to preserve meat products. For example, Huang *et al*. (2021) discovered that *Weissella*, *Lactobacillus*, *Staphylococcus*, and *Leuconostoc* constituted the most prevalent microbiota during fermentation in sausages. Thyme Eo treatment can inhibit the accumulation of biogenic amines and alter the relative abundance of the microbiota, thereby enhancing the quality of sausages during the fermentation process. Šojić *et al*. (2023) discovered that the Eo of *Origanum majorana* L., *Satureja hortensis* L., and *Satureja montana* L. reduced bacterial growth and the formation and accumulation of biogenic amines. The *Satureja hortensis* L. Eo treatment resulted in the lowest *Enterobacteriaceae* count and total biogenic amine concentration and proved an effective method for processing fresh turkey sausage, enhancing both safety and shelf life. The addition of black pepper Eo resulted in a notable enhancement of the quality of pork loins stored at 4°C for 9 days (Zhang *et al*., 2016). The Eo of *Juniperus communis* L. has been demonstrated to possess notable antioxidant activity (Tomović *et al*., 2020), thereby suggesting its potential utilization as a partial substitute for sodium nitrite in the production of dry fermented sausages. Similarly, Fasseas *et al*. (2008) reported that the use of oregano and sage Eo resulted in reduced oxidation of pork and beef ground meat samples. Chouliara *et al*. (2007) demonstrated that the combined effect of oregano Eo and modified atmosphere packaging had an additive preservative effect, extending the shelf life of fresh chicken meat stored at 4°C. In addition to the traditional indicators for checking the freshness of meat, new inspection methods have been reported recently to give us new insights. For example, Suwarno *et al*. (2023) detected harmful gases and alcohols in meat products using a Microcontroller and Gas Sensor. In addition, the properties of a bioactive substance can be changed by preparing it as an active membrane. To illustrate, the membrane film comprising 30% nano chitosan resveratrol and the film membrane of nano chitosan demonstrated remarkable resilience to pH fluctuations (25% each) across a range of solvents, including DMSO, 0.9% NaCl, PBS, and aquadest. The swelling of the film membrane of NCHR was notably effective in PBS and 0.9% NaCl solvents, with the most significant alterations in the permeability of the film membrane of NCHR observed in the 0.9% NaCl solvent (average of 10%), followed by DMSO solvents (average of 9%), PBS (average of 7%) and aquadest solvents (average of 4%). The film membrane of NCHR exhibited superior biodegradation properties in the presence of 0.9% NaCl and PBS, as compared to the solvents DMSO and aquadest. All solvents with varying concentrations of each film membrane of NCHR and the film membrane of nano chitosan film without resveratrol demonstrated robust bacteriostatic effects for 24 and 48 h (Gani *et al*., 2023). In conclusion, HC treatment was found to be effective in the preservation of chilled pork, thus demonstrating the potential of CrspEo as a preservation method for chilled pork products.

Conclusion

The peel of the popular fruit *Citrus reticulata* cv. Shatangju contains a large number of Eos (CrspEo). The components of CrspEo were subjected to analysis by GC-MS, resulting in the identification of 22 components. The predominant constituents of CrspEo were found to be terpenoid and aldehyde groups, including D-limonene (88.15%), γ-terpinene (4.59%) and β-myrcene (2.62%). These three components accounted for 95.36% of CrspEo. The strong scavenging effect of the CrspEo on DPPH and $ABTS^{+}$ radical, as well as the inhibitory effect on the six test microorganisms, suggests that the CrspEo has strong antioxidant activity and a broader antimicrobial spectrum. The parameters of pH, TBARS, MetMb%, TVB-N, and number of microorganisms of the CrspEo-treated chilled pork, which reflect the quality of the sample, were lower than those of the untreated samples. *Yersinia ruckeri* and *Enterobacter agglomerans* were found in deteriorated chilled pork. CrspEo showed excellent antibacterial activity against both microbes. The incorporation of CrspEo resulted in a significant extension of the shelf life of chilled pork, from a previously observed duration of 6-9 days, while simultaneously maintaining the sensory quality of the product. It can therefore be concluded that CrspEo is a suitable candidate for use as a natural preservative in the chilled pork industry. It can also increase the utilization of granulated *Citrus reticulata* cv. Shatangju peel and reduce resource wastage.

Acknowledgment

We would like to express our sincerest gratitude to the esteemed editors and anonymous reviewers for providing invaluable feedback and suggestions for improvement. We would like to express our gratitude to the anonymous experts and reviewers for their invaluable feedback, which has enhanced the quality of this study.

Funding Information

This study was supported by the department of science and technology of Jilin province, grant number YDZJ202401511ZYTS, the Department of Education of Jilin Province, grant number JJKH20240320KJ, the Technology Bureau of Jilin City, grant number 20240504005, and the Jilin Institute of Chemical Technology, Grant number 2020076.

Author's Contributions

Yao Dong: Software, validation, investigation, resources, written original drafted preparation, visualization, project administration and funded acquisition.

Xintong Zou: Methodology, validation, formal analysis, investigation, and written review and editing.

Hongli Zhou: Visualization.

Hongwei Pan: Data curation, written review and editing, supervision, and project administration.

Hao Cui: Conceptualization, data curation and written original drafted preparation.

Ethics

This article is an original manuscript, all authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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Supplementary Materials

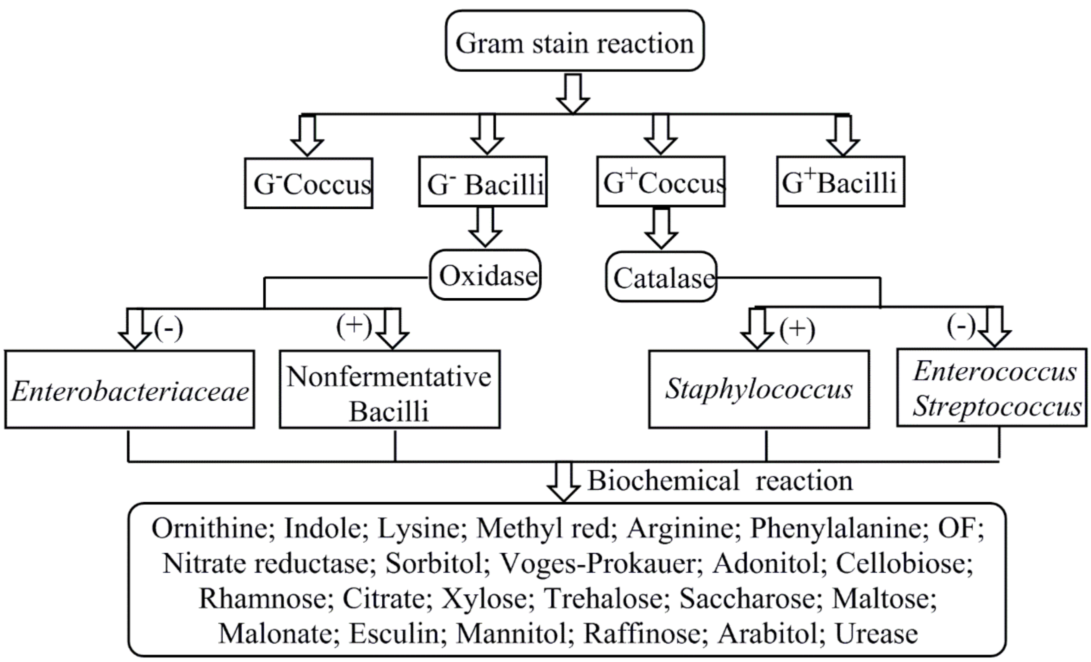

Fig. S1: Microbial identification flowchart

DOI: 10.3844/ajbbsp.2024.226.242

Table S1: The code of delivery container in Duo trio test

Panelists	Code of samples			Panelists	Code of samples		
1	A_{rg}	A-521	B-381	19	A_{rg}	A-521	B-381
2	A_{rg}	B-381	A-521	20	B_{rg}	B-972	A-684
3	B_{rg}	A-684	B-972	21	B_{rg}	A-684	B-972
4	B_{rg}	B-972	A-684	22	B_{rg}	B-972	A-684
5	B_{rg}	A-684	B-972	23	A_{rg}	A-521	B-381
6	A_{rg}	B-381	A-521	24	A_{rg}	B-381	A-521
7	A_{rg}	A-521	B-381	25	A_{rg}	A-521	B-381
8	B_{rg}	B-972	A-684	26	A_{rg}	B-381	A-521
9	B_{rg}	A-684	B-972	27	B_{rg}	A-684	B-972
10	A_{rg}	A-521	B-381	28	B_{rg}	B-972	A-684
11	B_{rg}	B-972	A-684	29	A_{rg}	A-521	B-381
12	A_{rg}	B-381	A-521	30	B_{rg}	B-972	A-684
13	B_{rg}	A-684	B-972	31	B_{rg}	A-684	B-972
14	B_{rg}	B-972	A-684	32	A_{rg}	B-381	A-521
15	A_{rg}	A-521	B-381	33	B_{rg}	A-684	B-972
16	A_{rg}	B-381	A-521	34	B_{rg}	B-972	A-684
17	B_{rg}	A-684	B-972	35	A_{rg}	A-521	B-327
18	A_{rg}	B-381	A-521	36	A_{rg}	B-381	A-521

A: means the CrpEos treated samples which was coded in 521 and 684; B: means the water treated samples which was coded in 381 and 972; A_{rg} and B_{rg} were the reference group of A and B samples, respectively