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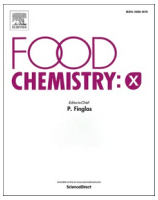

Inhibition effect of non-contact biocontrol bacteria and plant essential oil mixture on the generation of *N*-nitrosamines in deli meat during storage

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ABSTRACT

To reduce the risk of *N*-nitrosamines in deli meat products, this study formulated a novel non-contact *N*-nitrosamines inhibiting preservative IV (NIP-IV) consisting of biocontrol bacteria and plant essential oils (EOs) (*Stenotrophomonas rhizophila* SR-1 + *Paenibacillus provencensis* PP-2 + *Bacillus subtilis* CF-3+ cinnamon EO + grapefruit EO). Luncheon pork, spiced beef, and red sausage were taken as representatives of typical deli meat products and used to validate the effectiveness of NIP-IV in inhibiting *N*-nitroso dimethylamine (NDMA) production. The results showed that NIP-IV restrain protein degradation and lipid oxidation in deli meat products and effectively control microbial activity. Biogenic amines, such as phenethylamine, spermidine, cadaverine, and tyramine, were reduced. The conversion of nitrite to NDMA in deli meats was effectively inhibited by NIP-IV. Volatile organic compounds were the key to excellent NIP-IV non-contact preservation. Butyric acid, 3-methylbutanoic acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde were significantly negatively correlated with NDMA in deli meat products.

1. Introduction

Nitrite is favored as a colorant and colour stabilizer in the deli meat products processing industry (Perea-Sanz et al., 2022). However, the widespread use of nitrite has raised consumer concerns. Excess nitrite could spontaneously react with secondary amines or endogenous amine compounds such as spermidine, cadaverine, and phenylethylamine, ultimately leading to *N*-nitrosamines formation in deli meat products (Drabik-Markiewicz et al., 2011; Hayes et al., 2013; Lu et al., 2022). *N*-nitrosamines would increase the risk of various diseases including cancer. In particular, *N*-nitroso dimethylamine (NDMA) is classified as a class 2 A probable human carcinogen (Akyuz et al., 2016). Therefore, it is crucial to reduce the safety risks associated with deli meat products that inhibit *N*-nitrosamines development during storage.

Some researchers have inhibited NDMA production by adding chemical preservatives to deli meat products (De Mey, De Klerck, et al., 2014; Rywotycki, 2002). As consumer demand for food safety continues to rise, deli meat products containing traditional chemical preservatives have greatly reduced consumers' desire to purchase them. Volatile organic compounds (VOCs) of *Bacillus subtilis* CF-3 could affect the integrity of the cell wall and cell membrane, secretion of cell wall-degrading enzymes, and energy metabolism associated with the tricarboxylic acid cycle in *Monilinia fructicola* (Zhen et al., 2022). *B. subtilis* CF-3 and *Stenotrophomonas rhizophila* have been shown to produce bioactive VOCs, which have a positive impact on fruit preservation (Gao et al., 2018; Rivas-Garcia et al., 2019). Rybakova et al. (2016) showed that VOCs emitted by the *Paenibacillus* genus are widely distributed and exhibit significant biological control capacity, particularly those belonging to the pyrazine class of compounds. Some studies have shown that natural microbial preservatives such as *Lactiplantibacillus plantarum* can degrade the precursors (biogenic amines) of *N*-nitrosamines (Liu et al., 2022; Qin et al., 2023). These studies showed that biocontrol bacteria could inhibit NDMA production. An unpleasant odor is an insurmountable drawback for biocontrol bacteria. Improving the odor of biocontrol bacteria without affecting their activity is therefore important.

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Plant essential oils (EOs) usually have a pleasant aroma, which provides a new solution to the unpleasant odor of biocontrol bacteria. Furthermore, plant EOs could inhibit *N*-nitrosamine production. Sawamura et al. (2005) demonstrated that volatile terpenes in yuzu EO could form derivatives with nitrites, thereby inhibiting the formation of NDMA precursors. In another study, grapeseed extract has been reported to effectively inhibited the formation of *N*-nitrosamines in Western-style smoked sausage (Zhou et al., 2020). Although EOs pose little to no threat to human health as natural preservatives (Konfo et al., 2023), there are no reports on the use of EOs in combination with biocontrol bacteria to inhibit NDMA production. EOs usually function through coating, packaging, or direct addition to processed deli meat products (Mahato et al., 2019). Although EOs are usually pleasantly odorous, their direct addition to deli meat products can negatively affect their quality or sensory attributes (Prakash et al., 2024). Thus, there is an urgent need to develop NDMA inhibitors that do not have to be added to deli meat products. In this study, *N*-nitrosamines inhibition by six biocontrol bacteria was characterized. Typical deli meat products, such as red sausages and spiced beef, were used as experimental meat samples to assess the effects of preservatives. Considering that luncheon pork is one of the most popular processed meat products worldwide, it was also evaluated. Furthermore, eleven plant EOs were screened for non-contact *N*-nitrosamines inhibiting preservatives development.

The objectives of the present study were: (1) to identify the formulation of a non-contact preservative to inhibit NDMA formation; (2) to evaluate the impact of the preservative on the quality of processed meat products through indicators such as biogenic amines, lipid oxidation, protein degradation, and microbial counts; (3) to predict the relationships between VOCs and NDMA formation using bioinformatics analysis. This study could provide a novel approach to reducing the potential risks of N-nitrosamines in deli meat products. This study could provide a novel approach for reducing the potential risk of *N*-nitrosamines in deli meat products.

2. Materials and methods

2.1. Chemicals and samples

Luncheon pork was purchased from Hormel Foods (340 g/can, Austin, USA), spiced beef was purchased from Beijing Yueshengzhai Islamic Food Co., Ltd. (200 g/bag, Beijing, China), and the red sausage was purchased from Jiangsu Shuangyu Food Co., Ltd. (2000 g/bag, Jinjiang, China). The deli meat products used in the experiment were obtained from the same production batch. NDMA standards and a mixture of seven *N*-nitrosamines (EPA 521, 2000 ppm) were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). Bamboo EO, prickly ash EO, grapefruit EO, oregano EO, citronella EO, mustard EO, basil EO, pine needle EO, garlic EO, ginger EO, and cinnamon EO were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). All other chemicals and reagents used were of analytical grade.

The biocontrol bacteria *Paenibacillus provencensis* PP-2 (China General Microbiological Culture Collection Center, CGMCC M 20211109), *Stenotrophomonas rhizophila* SR-1 (China General Microbiological Culture Collection Center, CGMCC M 20211110), and *Microbacterium oxydans* MO-1 were isolated from sturgeon caviar at the Laboratory of High Value Utilization of Aquatic Products, Ocean University of China. *Bacillus subtilis* CF-3 (China Center for Type Culture Collection, CCTCC M 2016125) was first isolated from fermented bean curd and was identified by the Laboratory of Food Safety and Quality Control (School of Life Sciences, Shanghai University, Gao et al., 2016). *Lactiplantibacillus plantarum* AB-1 was kindly provided by Professor Heping Zhang of Key Laboratory of Dairy Biotechnology and Engineering, Inner Mongolia Agricultural University. *Paenibacillus polymyxa* PP-1 (BNCC185335) was purchased from the BeNa Culture Collection (Beijing, China). These biocontrol bacteria were activated in optimal medium at 37 °C for 24 h, inoculated at a concentration of 2 %, and incubated for 48 h. The bacterial powder was prepared by lyophilization (SCIENTZ-10ND, Zhejiang, China) of the bacterial liquid was used for subsequent experiments.

2.2. Preservation experiment

2.2.1. Pretreatment of deli meat products

To better determine the effect of the preservative, excess nitrite was added to deli meat products to enhance of *N*-nitrosamines production. Briefly, 250 mL, 1: 100 (m: v) nitrite solution was added to the deli meat products of 500 g and deli meat products and nitrite was fully mixed through a bowl cutter for 10 min for subsequent experiments. The premises for the preservation experiments were designed specially. As shown in Fig. 1, the preservatives and meat products were placed in separate glassware (d = 60 mm), and non-contact suppression of the premises was performed in a customized refrigerator vacuum drawer (polystyrene, 37 × 32 × 17 cm, Hisense Refrigerator Co., Ltd., Shandong, China). During the accelerated experiments, the drawers were kept at 30 °C for 3 days, and for the preservative formulation screening, they were stored at 4 °C for 10 days. The ratio of pretreated deli meat products to EOs, biocontrol bacteria, and NIP was maintained at 100: 1 (m: m). At the end of the experiment, all samples were stored at −80 °C for subsequent analysis.

2.2.2. Screening of NIPs composition

The composition of the NIPs was selected from Bamboo EO, prickly ash EO, grapefruit EO, oregano EO, citronella EO, mustard EO, basil EO, pine needle EO, garlic EO, ginger EO, and cinnamon EO, *L. plantarum* AB-1, *B. subtilis* CF-3, *P. polymyxa* PP-1, *P. provencensis* PP-2, *S. rhizophila* SR-1, and *M. oxydans* MO-1. The refrigerator drawer with different preservatives and luncheon pork were sealed at 30 °C for 3 days as an accelerated experiment, and the content of NDMA and total *N*-nitrosamines in luncheon pork were used for screening NIPs composition. Two EOs and three biocontrol bacteria were identified as components of NIPs.

2.2.3. Screening of formulation of NIPs

NIP-I comprised a group of two biocontrol bacteria in combination (*B. subtilis* CF-3 + *P. provencensis* PP-2, mass ratio = 1:1). NIP-II comprised a group of two biocontrol bacteria in combination with two EOs (*B. subtilis* CF-3 + *P. provencensis* PP-2 + cinnamon EO + grapefruit EO, mass ratio = 1:1:1:1). NIP-III comprised a group of three biocontrol bacteria in combination (*S. rhizophila* SR-1 + *P. provencensis* PP-2 + *B. subtilis* CF-3, mass ratio = 1:1:1). NIP-IV comprised a group of three biocontrol bacteria in combination with two EOs (*S. rhizophila* SR-1 + *P. provencensis* PP-2 + *B. subtilis* CF-3 + cinnamon EO + grapefruit EO, mass ratio = 1:1:1:1.5:1.5). The EOs and biocontrol bacteria were packaged in a sustained-release filter paper bag (30 × 40 mm, 0.8 mm thick, modified polytetrafluoroethylene composite nonwoven fabric), with a total weight of 2 g (Fig. 1). And the control group is 2 g of 0.9 % NaCl. The refrigerator drawers with different NIPs and deli meat products were sealed at 4 °C for 10 days. The NDMA and nitrite contents in luncheon pork, spiced beef, and red sausage were used to determine the optimal NIP.

2.3. Determination of N-nitrosamines by GC–MS

N-nitrosamines were extracted from samples as described by the Chinese national standard GB 5009.26–2016 and De Mey, De Maere, et al., 2014with slight modifications. 200 g of sample was accurately weighed and add to a 100 mL water and 50 g sodium chloride solution, mixed well in a distillation flask, and checked for airtightness. In a 500 mL flask, 100 mL dichloromethane and a small amount of ice were added to receive the condensate. The outlet of the condenser tube was placed under the surface of the dichloromethane liquid, and the flask

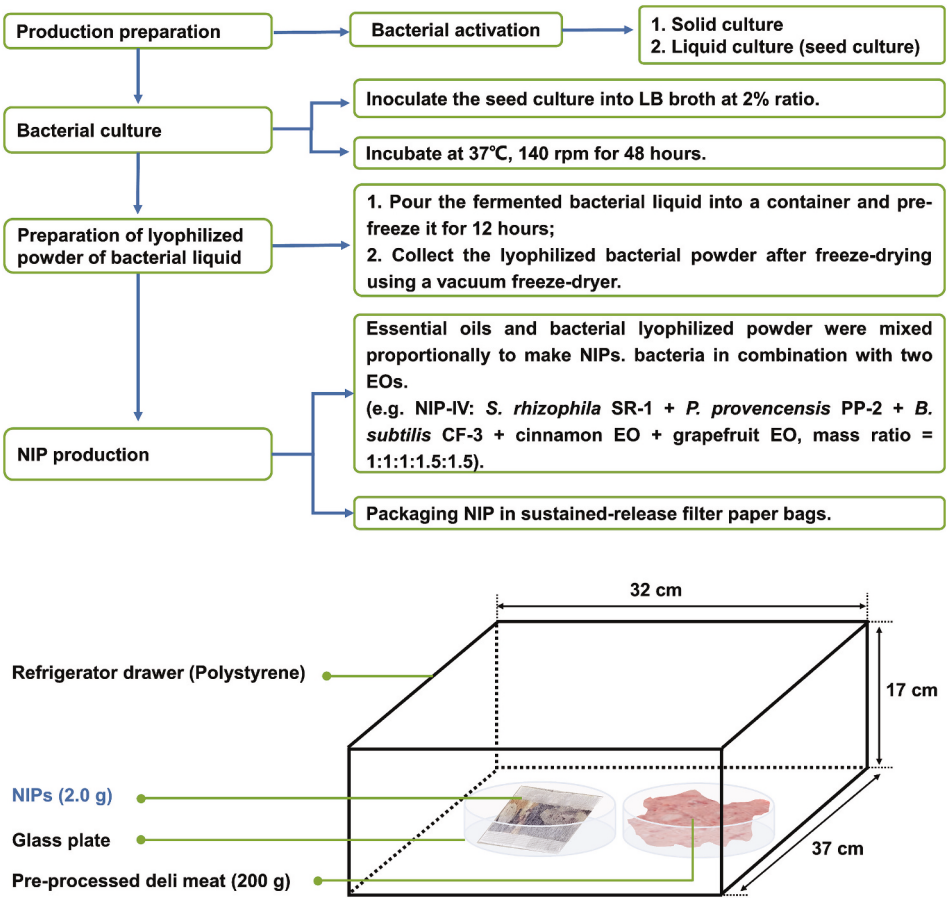

Fig. 1. Schematic representation of the NIPs preparation process and preservation of deli meat products with the NIPs in non-contact mode.

was placed in an ice bath. The distillation apparatus was started and the distillation was heat. After collecting 400 mL of condensate, the heating device was turned off and the distillation was stopped. It was then mixed with 3 mL of 4.6 mol/L sulfuric acid and 20 g of sodium chloride, transferred to a separating funnel, and extracted three times with 150 mL dichloromethane. The combined extracts were dried over 10 g anhydrous sodium sulfate, followed by rotary evaporation (LABOROTA 4000 rotary evaporator, Shandong, China). The dichloromethane extract was concentrated to 5 mL by rotary evaporation on a water bath at 40 °C, then nitrogen blowing was employed for further concentration, and the volume was adjusted to 0.5 mL by accurate dispensing of dichloromethane. The resulting solution was then analyzed utilizing GC–MS. The sample volume was 1 μL.

Determination of the six *N*-nitrosamines (Fig. 2) in deli meat product samples was performed by GC–MS using an Ultra QP 2010 system (Shimadzu, Tokyo, Japan). Chromatographic separation was carried out using a DB-INNOWax capillary column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, CA, USA). Helium (99.99 %) was used as the carrier gas at a constant 1 mL/min flow rate. The injection volume was 1 μL. The injection port was held at 220 °C and used in spitless mode. The GC temperature program was as follows: the temperature was held at 40 °C, increased to 100 °C at a rate of 1 °C/min, increased to 240 °C at a rate of 20 °C/min, and finally held for 2 min. The transfer line for tandem mass spectrometry was maintained at 230 °C. The ion source temperature was maintained at 230 °C. The mass spectrometer was operated in selected ion monitoring mode with an electron energy of 70 eV.

2.4. Determination of nitrite content

Nitrite content was determined using a colorimetric nitrite assay based on the Griess reaction with some modifications (Shao et al., 2021). 5.0 g deli meat products were weighed and placed in a conical flask, which 12.5 mL saturated borax solution and 150 mL water were added at 70 °C. The mixture was heated in boiling water for 15 min and cooled to room temperature. The extract was transferred to a 200 mL volumetric flask, and 5 mL potassium ferrocyanide solution (106 g/L) and 5 mL zinc acetate solution (220 g/L) were added to precipitate the protein. Add water was added to the mark on a 200 mL volumetric flask, the mixture was shaken, and allowed to stand for 30 min to allow for the removal of the upper fat layer. The supernatant was then filtered off, and 2 mL *p*-amino benzene sulfonic acid solution (4 g/L) and 1 mL *N*-(1-naphthyl) ethylenediamine dihydrochloride solution (2 g/L) were added to the supernatant, which was allowed to stand for 15 min. Absorbance at 538 nm was measured and quantified using a standard curve for sodium nitrite.

2.5. Measurement of protein degradation

Total volatile basic nitrogen (TVB-N) was measured according to the procedure described by Zhang et al. (2019). 10.0 g deli meat products were separately weighed into a distillation tube, and 75 mL water was added. The mixture was shaken using a vortex mixer (Haimen Kylin-Bell Lab Instruments Co., Ltd., Jiangsu, China) to homogenize the sample evenly in the sample solution and was macerated for 30 min. Then 1 mL, 0.32 mol/L H_3BO_3, and 20 μL of a mixed indicator (ethanolic solution of methyl red mixed with ethanolic solution of bromocresol green, v: v = 1: 5) were added to the central inner chamber of a Conway diffusion dish.

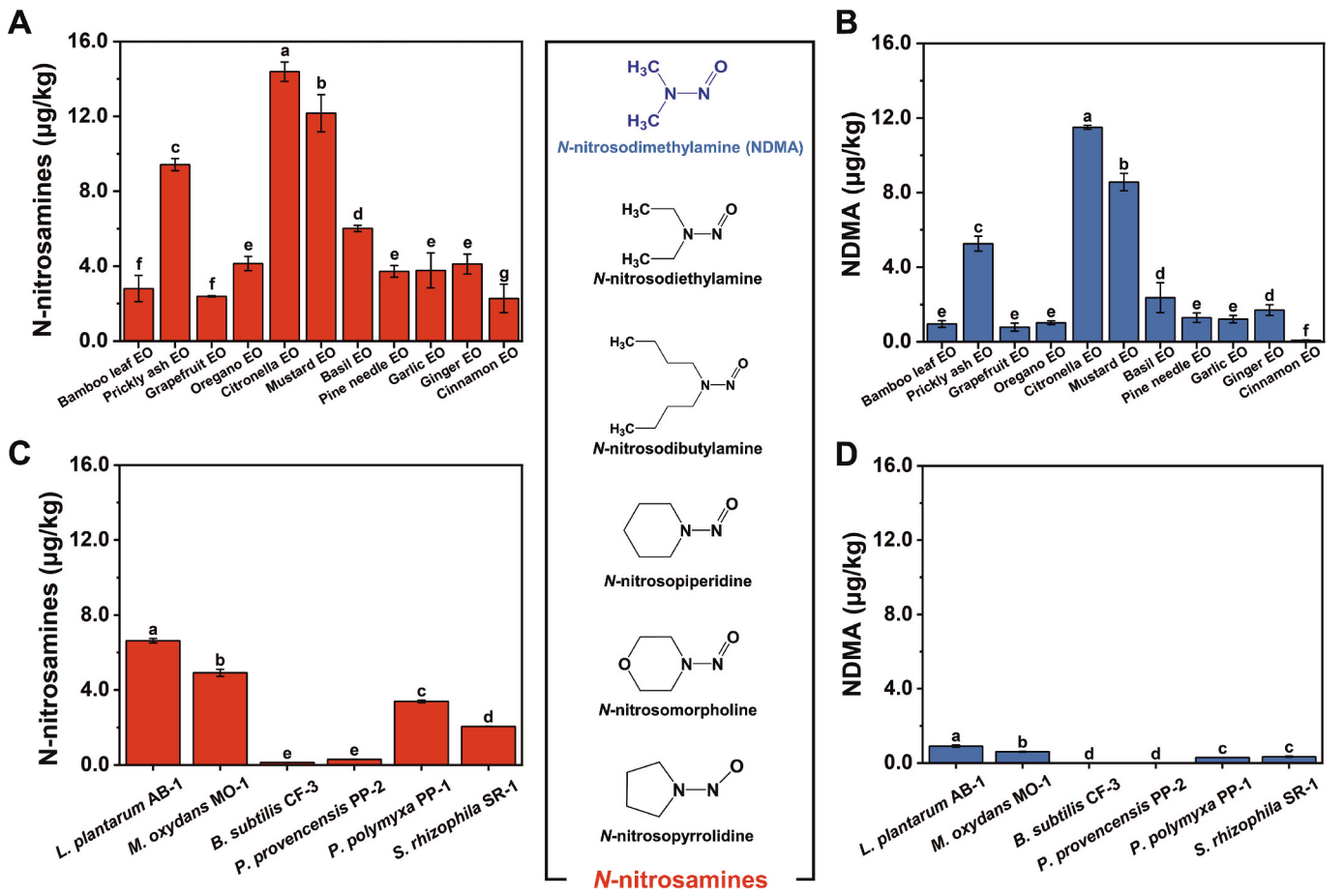

Fig. 2. *N*-nitrosamine (A) and NDMA (B) contents in luncheon pork treated with 11 EOs. *N*-nitrosamine (C) and NDMA (D) content in luncheon pork treated with six biocontrol bacteria. Different letters indicate $p < 0.05$ between different groups.

1 mL of the filtrate and 1 mL saturated K_2CO_3 solution were quickly added to the outer chamber of the dish, covered with a frosted glass lid to ensure thorough mixing, and then placed in an oven at 37 °C for 2 h. The sample was titrated with 0.01 mol/L HCl until purple-red. The TVB-N content was calculated by the following equation:

$$\text{TVB} - N = 0.14 \times (V_1 - V_2) \times 100 \times 100/m[\text{mg}/100\ \text{g}] \quad (1)$$

where V_1 is the volume of the sample used for titration (mL), V_2 is the volume of the control used for titration (mL), 100 is the dilution factor, and m is the mass of the sample (g).

2.6. Measurement of lipid oxidation

The method used to determine the thiobarbituric acid reactive substance (TBARS) value was described by Panahi and Mohsenzadeh (2022). 5 g deli meat products were added to 50 mL of a trichloroacetic acid mixture (containing 3.75 g trichloroacetic acid and 0.05 g disodium EDTA) and placed on a constant-temperature shaker at 50 °C for 30 min, then filtered. The filtrate was accurately pipetted into a 25 mL stoppered cuvette, and 5 mL of the trichloroacetic acid mixture was added. The reaction was performed in water at 90 °C for 30 min. 150 μL of the filtrate was combined with 150 μL 2-thiobarbituric acid, and the absorbance of the filtrate at a wavelength of 538 nm was measured and quantified against a standard curve using 1,1,3,3-tetraethoxypropane as the standard. The TBARS value was calculated using the following equation:

$$\text{TBARS value} = c \times V \times 1000/m \times 1000 \quad (2)$$

where c is the concentration of malondialdehyde in the specimen solution determined from the standard curve (μg/mL), V is the volume of the sample solution (mL), 1000 is the dilution factor, and m is the mass of the sample (g).

2.7. Determination of microbial abundance

The determination of microbial abundance follows the guidelines specified in the Chinese National Standard GB 4789.2–2022. 25 g deli meat products after pretreatment with NIP-IV were placed in a sterile plastic bag with 225 ml of sterile saline and shaken thoroughly. Subsequently, a series of dilutions of the resulting samples were performed in a sterile saline solution. Inoculate aliquots of appropriate dilution fluids onto plate count agar (PCA). For the total viable count analysis, the PCA plates were incubated at 36 °C for 48 h. At the end of the incubation period, the surviving microbial colonies were counted, and the results were reported as logarithmic values in colony forming units per gram (log CFU/g). All experiments were performed in triplicates to ensure reliable results.

2.8. Antimicrobial testing of NIP-IV towards E. coli and S. aureus

The purpose of this step was to detect the microbial safety of NIP-IV. Verification of foodborne pathogens (*E. coli* and *S. aureus*) in NIP-IV was performed using the oscillation culture method according to the Chinese National Standard GB/T 21510–2008. 1 g NIP-IV and 100 % skimmed white cotton cloth (control sample) were weighed into triangular flasks. Fresh cultures of nutrient agar medium slants of *E. coli* and *S. aureus* from the third to eighth generations were prepared for 18–24 h. The

cultures were then diluted with 0.03 mol/L phosphate buffer to the appropriate concentration (approximately 10^5 CFU/mL) to prepare ready-made bacterial suspensions. Then, 95 mL phosphate-buffered saline containing 0.1 % Tween-80 and 5 mL of the ready-made bacterial suspensions were added to triangular flasks. Triangular flasks containing the control and test samples were fixed on a shaker in a thermostatic shaking incubator and shaken at 150 rpm for 4 h at a reaction temperature of 37 °C. Live bacteria were conducted in the samples before and after shaking using the flat colony counting method.

2.9. Determination of biogenic amines

The biogenic amine content in luncheon pork, red sausage, and spiced beef treated with NIP-IV was determined by Chen et al. (2023). Briefly, the biogenic amines were quantified using a Shimadzu LC-20 A HPLC system (Shimadzu, Tokyo, Japan). 20 μL sample of the filtrate was injected into a C18 column (4.6 mm × 150 mm × 5 μm; Agilent Technologies, CA, USA) at a column temperature of 35 °C. A gradient elution program with a mixture of 0.01 mol/L ammonium acetate as solvent B and 90 % acetonitrile as solvent A was used. The gradient program started at 60 % A and ramped to 85 % A at 22 min and 100 % A at 25 min. Subsequently, the gradient was switched to 60 % A until the end of the run at a flow rate of 1 mL/min. The concentrations of biogenic amines in the samples were calculated by comparison with the standard concentrations.

2.10. Determination of VOCs by GC–MS

The VOCs were performed by headspace solid-phase microextraction combined with GC–MS. The method used to determine VOCs was described by Contarino et al. (2019). Biocontrol bacteria in lyophilized powder form (0.5 g) and EOs (0.5 mL) were added to the internal standard dimethyl heptanone-3, which was placed in a 20 mL headspace bottle and rapidly sealed separately. Similarly, a 0.5 g mixture of EOs and biocontrol bacteria from NIP-IV was added to a headspace bottle containing dimethyl heptanone-3, and immediately sealed. The sample was allowed to equilibrate at 40 °C for 30 min and then inserted into the upper space of the headspace bottle with a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane extraction head. After extraction at 40 °C for 30 min, the extraction head was inserted into the inlet of the GC–MS system and desorbed at 250 °C for 5 min. QP2010 SE gas chromatograph-mass spectrometer (Shimadzu, Tokyo, Japan) was used and was coupled with an HP-INNOWax column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Santa Clara, CA, USA). The programmed column temperature started at 60 °C and was then increased to 160 °C at 5 °C/min, increased to 250 °C at 6 °C/min, and maintained at 250 °C for 3 min. The injection port temperature was 250 °C. Helium was used as the carrier gas and the injection volume was 1 μL. MS was performed with an ion source temperature of 230 °C, mass range of m/z 35–500, and electron ionization energy of 70 eV.

2.11. Statistical analysis

The data were analyzed using Origin 2022 software (OriginLab Corporation, Northampton, MA, USA) and SPSS Statistics 25.0 (SPSS, Chicago, IL, USA), with $p < 0.05$. Pearson correlation analysis was used the free online platform of the Majorbio Cloud Platform (https://www.majorbio.com) for clustering heat map plotting.

3. Results and discussion

3.1. Effects of EOs and biocontrol bacteria inhibit N-nitrosamines production in luncheon pork

The nitrosamine content of luncheon meat treated with EO is shown in Fig. 2A. The cinnamon EO group showed significantly lower *N*-nitrosamines content of 2.27 μg/kg than the other EO groups ($p < 0.05$). The grapefruit EO group also showed good inhibitory effects, with an *N*-nitrosamine content of 2.39 μg/kg. Nitrosamines include a range of compounds, such as NDMA, *N*-nitroso diethylamine, *N*-nitroso dibutylamine, and *N*-nitroso piperidine. Among them, NDMA is the most threatening *N*-nitrosamine for human health, therefore, it was additionally evaluated. The results in Fig. 2B show that cinnamon EO and grapefruit EO treatments significantly reduced NDMA in luncheon pork to 0.08 μg/kg and 0.79 μg/kg, respectively. The *N*-nitrosamine in the *P. provencensis* PP-2 group and *B. subtilis* CF-3 group were only 0.13 μg/kg and 0.29 μg/kg, respectively, which were lower than EOs group ($p < 0.05$, Fig. 2C). The NDMA was also lower than that in the EOs group (Fig. 2D). These results indicated that the biocontrol bacteria appeared to inhibit the production of *N*-nitrosamines more effectively. In subsequent experiments, two types of EO and three types of biocontrol bacteria were used for the development of NIP: cinnamon EO, grapefruit EO, *S. rhizophila* SR-1, *B. subtilis* CF-3, and *P. provencensis* PP-2.

3.2. NIP-IV inhibited the production of NDMA in deli meat products

Four NIPs were prepared based on different ratios of EOs and biocontrol bacteria, namely NIP-I, NIP-II, NIP-III, and NIP-IV. Xie et al. (2023) showed that NDMA is derived from the reaction between nitrite or nitrous oxides and nitrosable substrates, such as secondary amines. Therefore, the nitrites of deli meat products were evaluated first. NIPs could effectively inhibit the conversion of nitrite to NDMA in luncheon pork, spiced beef, and red sausage. The nitrite contents in the three deli meat products showed an increasing trend ($p < 0.05$, Fig. 3A-3C). Fig. 3D-3F shows that NDMA in the three deli meat products significantly decreased after treatment with different NIPs. In luncheon pork, the NDMA contents of NIP-I, NIP-II, NIP-III, and NIP-IV were 0.32 μg/kg, 0.23 μg/kg, 0.06 μg/kg, 0.01 μg/kg, which were all significantly lower than control group, 12.47 μg/kg (Fig. 3D, $p < 0.05$). A limit of 10 μg/kg total volatile *N*-nitrosamines has been set for cured deli meat products in the USA (USDA, 2005). Although excess nitrite was added to amplify the reaction in this study, the NDMA contents in luncheon pork, spiced beef, and red sausage were well below 3.0μg/kg after treatment with the four NIPs. These results show that NIPs could be considered as effective NDMA inhibitors. Significantly, NIP-III did not appear to be as good or even as good as NIP-I and NIP-II in inhibiting nitrite transformation, and the levels of nitrite in NIP-III treated red sausages were slightly lower than those in the control group. However, NIP-III still exhibited a better NDMA reduction effect. The compositions of NIP-III and NIP-IV were further analyzed. As shown in section 2.2.3, EOs were additionally added in NIP-IV than NIP-III. These results suggest that the inhibition of biocontrol bacteria was effective in inhibiting NDMA production, but they could be slightly less effective than EOs in inhibiting nitrite reactions. In addition, these EOs were effective in ameliorating the unpleasant odor of the biocontrol bacteria. Therefore, NIP-IV was identified as the best preservative.

3.3. NIP-IV inhibited protein degradation, lipid oxidation, microbial abundance, and biogenic amines in deli meat products

Lipid oxidation is one of the critical reactions responsible for the deterioration of deli meat product quality. The TBARS value indicates the degree of lipid oxidation in the meat (Zhang et al., 2019). After treatment with NIP-IV, the TBARS values of spiced beef and red sausage were significantly reduced (Fig. 4A, $p < 0.05$). In luncheon pork, the TBARS value of the NIP-IV group was not significantly lower than control group. TVB-N indicates the degree of protein degradation, which is another important indicator for evaluating the meat quality (Bekhit et al., 2021). Fig. 4B demonstrates that TVB-N was significantly reduced by 44.59 % after NIP-IV was treated with luncheon pork, 17.79 % in spiced beef, and 9.30 % in red sausage ($p < 0.05$). These results show that NIP-IV could be effective in reducing protein degradation and lipid

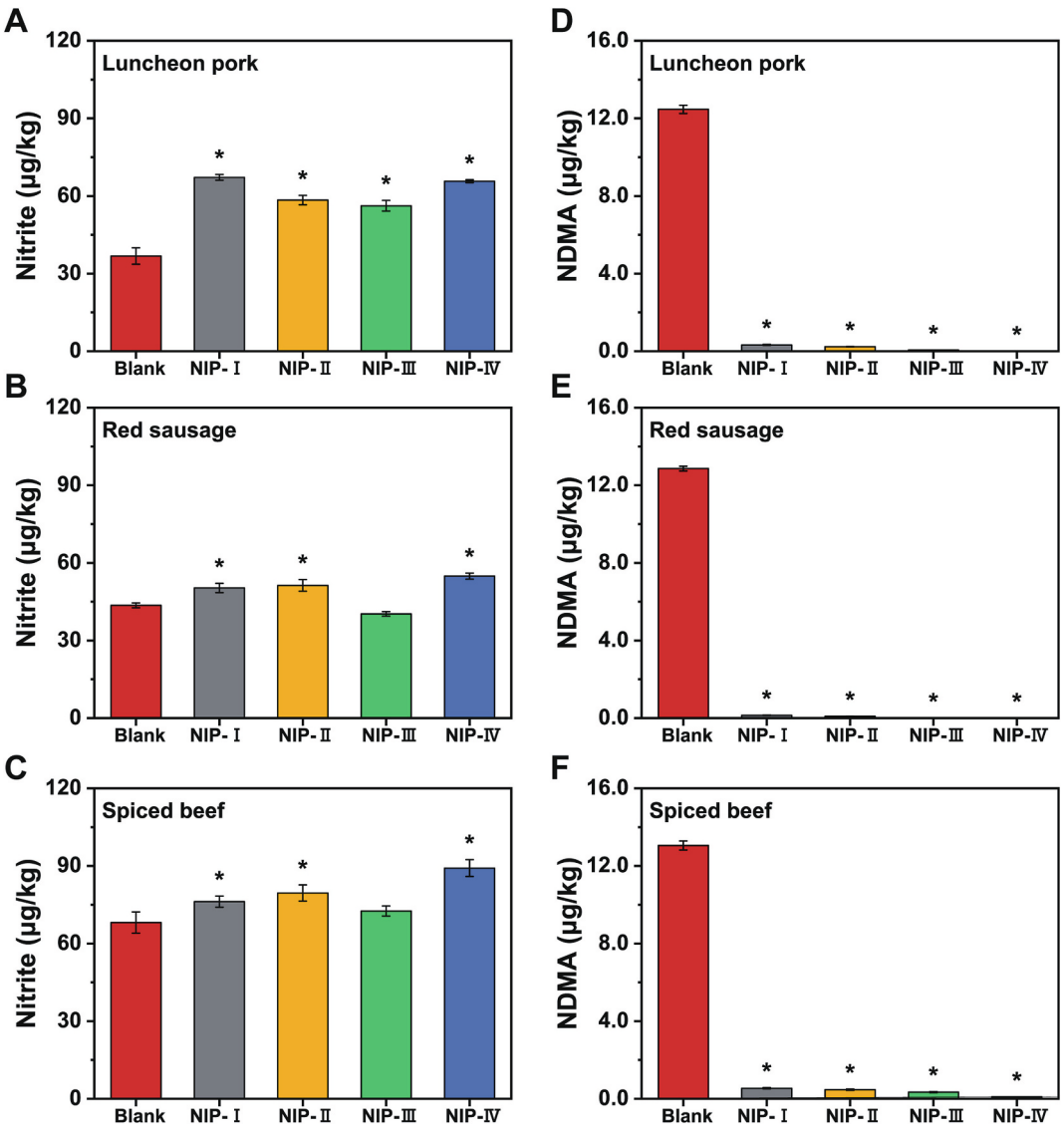

Fig. 3. Changes in nitrite and NDMA in luncheon meat (A, D), red sausage (B, E), and five-spice beef (C, F) after different NIPs treatments. "*" indicated $p < 0.05$ compared to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oxidation in deli meat products. Protein degradation and lipid oxidation are closely associated with bacterial growth and propagation (Wu et al., 2019). Fig. 4C presents the effect of NIP-IV on microbial abundance in luncheon pork, spiced beef, and red sausage. NIP-IV significantly reduced microbial abundance in the three deli meat products, and the most significant effect was observed in red sausage, 21.96 % ($p < 0.05$). *E. coli* and *S. aureus* are the most common foodborne spoilage and pathogenic bacteria in model systems in daily food, thus the biological safety of the NIP-IV itself had been evaluated. As expected, NIP-IV exhibited high bacterial inhibition rates of 99.9 % for *E. coli* and 99.5 % for *S. aureus* (Fig. 4D and E). Therefore, NIP-IV demonstrated a relatively high level of biological safety and exerted its effects through non-contact means, rendering it readily acceptable to consumers and holding promising prospects for widespread application.

Secondary amines, in addition to nitrite, are important precursors for inducing NDMA production. Secondary amines are derived partly from the excessive oxidation of proteins and partly from the cyclization and deamination reactions of biogenic amines present in meat (Xie et al., 2023). Wójcik et al. (2021) showed that biogenic amines are generated by the oxidative degradation of proteins or catalyzed by the secretion of amino acid decarboxylases by enzyme-producing microorganisms. In luncheon pork, spiced beef, and red sausage, treatment with NIP-IV could significantly inhibit the generation of phenethylamine ($p < 0.05$, Fig. 4F) and spermidine (Fig. 4G). Cadaverine has no direct adverse effects on human health; however, it may play an important role in food poisoning. This was due to its ability to potentiate the histamine toxicity through the inhibition of diamine oxidase and histamine methyltransferase. It can also react with nitrite to form carcinogenic nitrosamines (Shakila et al., 2001). NIP-IV significantly reduced cadaverine levels in the three deli meat products ($p < 0.05$, Fig. 4H). Although tyramine levels were low in the samples, NIP-IV was still effective in inhibiting tyramine production, especially in luncheon pork ($p < 0.05$, Fig. 4I). These results are the same as those shown in Fig. 4B, indicating that NIP-IV effectively inhibited the protein oxidation in the three deli meat products, and thus effectively inhibit the production of NDMA.

3.4. NIP-IV reduced NDMA in deli meat products through VOCs

Unlike the traditional methods of inhibiting NDMA production in deli meat products, such as the direct addition of ascorbic acid or probiotics, NIP-IV does not come into direct contact with the ingredients

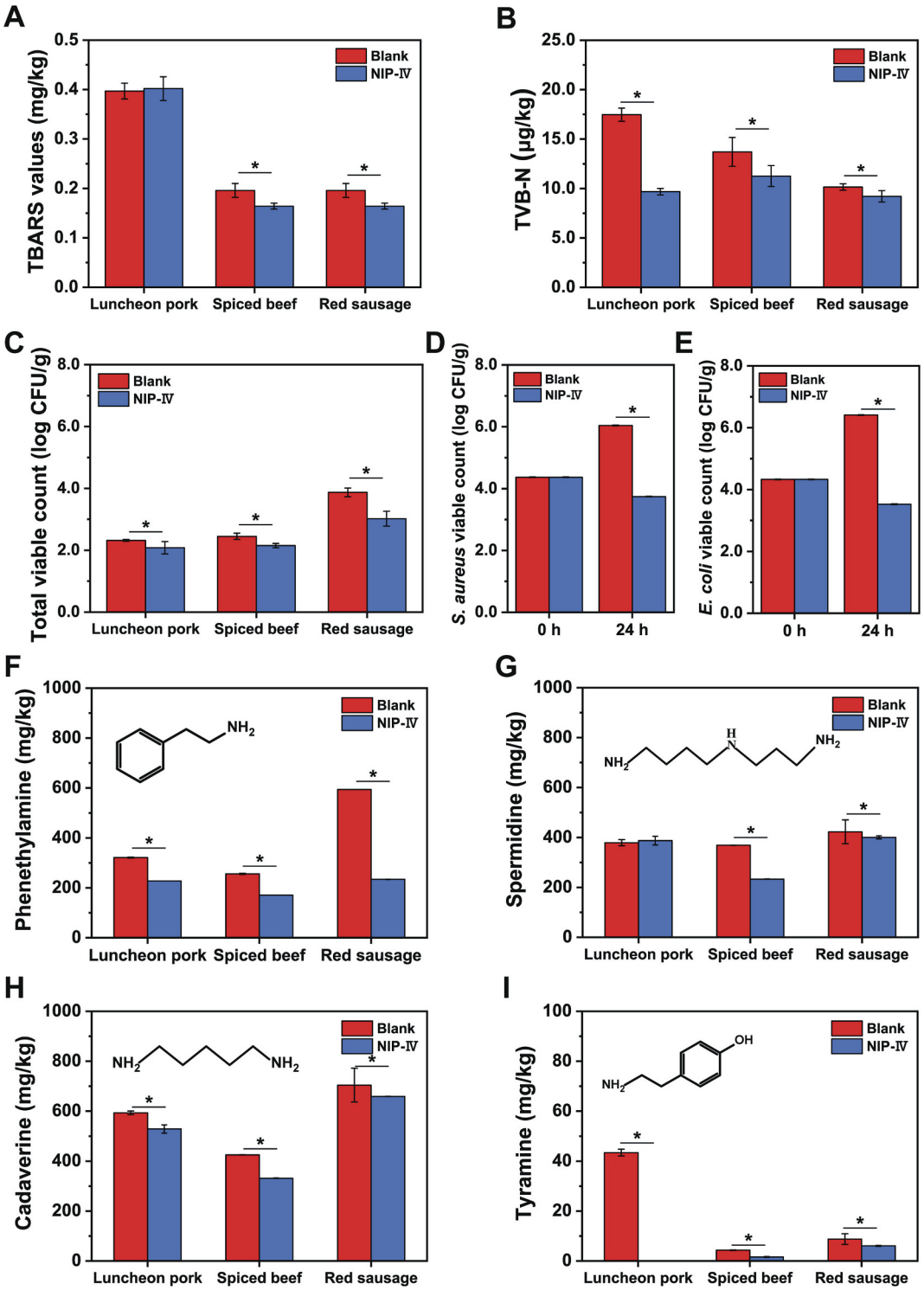

Fig. 4. Evaluation of the preservation effect of NIP-IV. Changes in TBARS value (A), TVB-N (B), and microbial abundance (C) after NIP-IV treatment of luncheon meat, five-spice beef, and red sausages. Biosafety of NIP-IV by detection of *E. coli* (D) and *S. aureus* (E). Effect of NIP-IV treatment on biogenic amines in luncheon meat, five-spice beef, and red sausage, including phenethylamine (F), spermidine (G), cadaverine (H), and tyramine (I). "*" indicated $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and is not directly involved in the metabolism of proteins and lipids in meat. However, NIP-IV can produce many VOCs, which provides a novel direction for exploring the mechanism of NIP-IV. As shown in Table 1, 38 VOCs, including 12 acids, 8 alcohols, 5 aldehydes, and 5 olefins, were detected in NIP-IV using GC–MS. To further understand the detailed sources of VOCs in NIP-IV, the VOCs of the two EOs and three biocontrol bacteria that constitute NIP-IV were determined (Table 1). The VOCs of the biocontrol bacteria were mainly acids, alcohols, and pyrazines, whereas the VOCs of the EOs were mainly alcohols, olefins, aldehydes, and ketones (Fig. 5A). The highest concentration of acetic acid was observed in *S. rhizophila* SR-1, followed by butyric acid, which can degrade *N*-nitrosamines under low pH conditions (Xiao et al., 2018). Rybakova et al. (2016) demonstrated that pyrazine derivatives produced by *Paenibacillus* have high antimicrobial activity. The concentration of

Table 1
The concentrations of VOCs in NIP-IV, *S. rhizophila* SR-1, *P. provencensis* PP-2, *B. subtilis* CF-3, cinnamon EO, and grapefruit EO.

Category	Compounds	CAS	Concentration (μg/g)					
			NIP-IV	*S. rhizophila* SR-1	*P. provencensis* PP-2	*B. subtilis* CF-3	Cinnamon EO	Grapefruit EO
Acids	Acetic acid	64-19-7	34.2 ± 5.99^{c}	47.50 ± 9.23^{b}	23.36 ± 4.33^{d}	76.03 ± 6.91^{a}	/	/
	Propionic acid	1078-61-1	/	6.69 ± 1.43a	2.93 ± 0.25^{b}	0.77 ± 0.09^{c}	/	/
	Butyric acid	107-92-6	5.02 ± 0.78^{b}	45.81 ± 9.81^{a}	0.59 ± 0.08^{d}	1.60 ± 0.17^{c}	/	/
	Isobutyric acid	79-31-2	/	14.00 ± 0.78^{a}	2.31 ± 0.63^{c}	8.37 ± 1.06^{b}	/	/
	2-Methylbutyric acid	116-53-0	/	/	4.69 ± 0.22^{a}	/	/	/
	3-Methylbutanoic acid	503-74-2	9.29 ± 1.44^{b}	/	/	94.62 ± 21.23^{a}	/	/
	Salicylic acid	118-58-1	1.52 ± 0.70^{b}	/	/	/	5.05 ± 0.41^{a}	/
	trans-Cinnamic acid	140-10-3	3.40 ± 1.19^{b}	/	/	/	5.40 ± 0.29^{a}	/
	Hexanoic acid	142-62-1	0.59 ± 0.25^{b}	/	/	/	0.72 ± 0.33^{a}	/
	Benzoic acid	65-85-0	5.21 ± 1.47^{b}	/	/	10.01 ± 2.69^{a}	/	/
	Benzeneacetic acid	103-82-2	1.52 ± 0.02^{b}	/	/	4.53 ± 0.61^{a}	/	/
	Phenylacrylic acid	621-82-9	2.10 ± 0.05^{b}	/	/	5.94 ± 0.89^{a}	/	/
Alcohols	Ethanol	64-17-5	1.08 ± 0.23^{c}	8.19 ± 0.45^{b}	11.70 ± 2.71^{a}	11.60 ± 1.03^{a}	/	/
	Isopropyl alcohol	67-63-0	/	/	/	0.51 ± 0.03^{a}	/	/
	2,3-Butanediol	513-85-9	/	1.11 ± 0.01^{b}	2.59 ± 0.27^{a}	/	/	/
	L-α-Terpineol	10482-56-1	3.84 ± 0.44^{b}	/	/	1.99 ± 0.04^{c}	/	13.46 ± 0.22^{a}
	Propylene glycol	57-55-6	/	0.68 ± 0.15^{b}	1.62 ± 0.41^{a}	/	/	/
	3-Methylthiopropanol	505-10-2	/	/	0.52 ± 0.07^{a}	/	/	/
	4-Methyl-5-thiazole ethanol	137-00-8	/	/	0.55 ± 0.01^{a}	/	/	/
	4-Penten-2-ol	625-31-0	/	0.29 ± 0.02^{b}	0.52 ± 0.03^{a}	/	/	/
	1-Octanol	111-87-5	1.52 ± 0.06^{c}	/	/	3.23 ± 0.96^{b}	/	11.24 ± 0.15^{a}
	Benzyl Alcohol	100-51-6	2.28 ± 0.55^{a}	/	/	/	0.47 ± 0.11^{b}	/
	Trans-p-mentha-2,8-dienol	42370-41-2	0.85 ± 0.20^{a}	/	/	/	/	/
	Cis-p-Mentha-2,8-dien-1-ol	22771-44-4	0.54 ± 0.03^{a}	/	/	/	/	/
	Linalool	78-70-6	7.44 ± 1.15^{b}	/	/	/	/	21.94 ± 0.16^{a}
	3-Phenyl-2-propen-1-ol	104-54-1	0.02 ± 0.01^{b}	/	/	/	0.49 ± 0.13^{a}	/
	1-Nonanol	143-08-8	/	/	/	/	/	0.62 ± 0.08^{a}
	1-Decanol	112-30-1	/	/	/	/	/	0.79 ± 0.19^{a}
	Citronellol	106-22-9	/	/	/	/	/	1.27 ± 0.11^{a}
	(*Z*)-3,7-dimethyl-2,6-Octadien-1-ol	106-25-2	/	/	/	/	/	1.09 ± 0.02^{a}
	(*Z*)-4-Hexen-1-ol	928-91-6	/	/	/	/	/	2.10 ± 0.10^{a}
	Trans-α, α,4-trimethyl-cyclohexanemethanol	5114-00-1	/	/	/	/	/	1.06 ± 0.03^{a}
	2-Phenylethanethiol	4410-99-5	/	/	6.28 ± 0.33^{a}	5.90 ± 0.01^{b}	/	/
	Phenylethanol	60-12-8	/	/	0.48 ± 0.02^{a}	/	/	/
Esters	Methyl acetate	79-20-9	0.42 ± 0.01^{c}	1.75 ± 0.54^{b}	1.76 ± 0.40^{b}	4.87 ± 0.28^{a}	/	/
	Methyl butyrate	623-42-7	/	0.13 ± 0.01^{a}	/	/	/	/
	Methyl-2-hydroxypropionate	547-64-8	/	/	/	0.45 ± 0.10^{a}	/	/
	L-alanine methyl ester	114041-77-9	/	/	/	0.71 ± 0.21^{a}	/	/
	Isopropenyl acetate	108-22-5	/	/	1.59 ± 0.74^{a}	/	/	/
	Benzyl formate	104-57-4	0.85 ± 0.16^{a}	/	/	/	0.44 ± 0.08^{b}	/
	Methyl salicylate	119-36-8	0.74 ± 0.11^{a}	/	/	/	0.40 ± 0.12^{b}	/
	Propanedioic acid, diethyl ester	105-53-3	/	/	/	/	5.73 ± 0.26^{a}	0.89 ± 0.11^{b}
	Formic acid, hexyl ester	629-33-4	/	/	/	/	/	0.92 ± 0.07^{a}
Pyrazines	2,3-Diethyl-5-methylpyrazine	18138-04-0	/	/	1.21 ± 0.05^{a}	/	/	/
	2,5-Dimethyl-3-(3-methylbutyl) pyrazine	18433-98-2	/	/	/	1.09 ± 0.21^{a}	/	/
	2,5-Dimethylpyrazine	123-32-0	1.81 ± 0.39^{c}	0.20 ± 0.01^{d}	7.38 ± 1.02^{a}	2.18 ± 0.07^{b}	/	/
	2,6-Dimethylpyrazine	108-50-9	/	/	3.83 ± 0.91^{a}	0.26 ± 0.01^{b}	/	/
	2-Ethyl-6-methylpyrazine	13925-03-6	/	/	0.52 ± 0.01^{a}	/	/	/
	2-Isobutyl-3-methylpyrazine	13925-06-9	/	/	1.24 ± 0.07^{a}	/	/	/
	3-Ethyl-2,5-dimethylpyrazine	13360-65-1	0.52 ± 0.07^{d}	0.98 ± 0.03^{c}	6.14 ± 0.87^{b}	7.50 ± 2.01^{a}	/	/
	Methylpyrazine	109-08-0	/	/	1.28 ± 0.02^{a}	/	/	/
	Trimethyl pyrazine	14667-55-1	/	/	4.38 ± 0.01^{a}	/	/	/
Aromatics	1,3-Dimethylbenzene	108-38-3	/	0.23 ± 0.01^{b}	/	0.51 ± 0.17^{a}	/	/
	Phenol	108-95-2	/	/	/	0.58 ± 0.01^{a}	/	/
	Paraxylene	106-42-3	/	0.13 ± 0.01^{b}	/	0.32 ± 0.21^{a}	/	/
	Toluene	108-88-3	/	0.65 ± 0.21^{c}	1.24 ± 0.07^{a}	0.96 ± 0.01^{b}	/	/
	Ethylbenzene	1077-16-3	/	0.20 ± 0.01^{c}	0.55 ± 0.01^{b}	0.71 ± 0.16^{a}	/	/

(*continued on next page*)

Table 1 (*continued*)

Category	Compounds	CAS	Concentration (μg/g)					
			NIP-IV	*S. rhizophila* SR-1	*P. provencensis* PP-2	*B. subtilis* CF-3	Cinnamon EO	Grapefruit EO
	M-xylene	108-38-3	/	/	0.48 ± 0.03[a]	/	/	/
	1-Methyl-2,4-pentadienyl-benzene	64234-49-7	0.70 ± 0.14[a]	/	/	/	/	/
	Hexa-2,4-dienylbenzene	79482-86-3	0.43 ± 0.07[a]	/	/	/	/	/
Aldehydes	3-Methylbutyraldehyde	590-86-3	0.16 ± 0.08[b]	0.59 ± 0.01[a]	/	0.23 ± 0.02[c]	/	/
	Acetaldehyde	75-07-0	/	/	/	0.45 ± 0.02[a]	/	/
	Benzaldehyde	100-52-7	3.82 ± 0.16[b]	/	/	3.28 ± 0.13[b]	82.57 ± 7.45[a]	/
	2-Butenal	4170-30-3	/	/	/	/	0.72 ± 0.09[a]	/
	3-Phenyl-2-propenal	104-55-2	1.05 ± 0.32[c]	/	/	/	8.65 ± 0.13[b]	8.88 ± 13.79[a]
	Benzeneacetaldehyde	122-78-1	1.02 ± 0.09[b]	/	/	/	6.03 ± 0.66[a]	/
	(*E*)-Cinnamaldehyde	14371-10-9	12.93 ± 1.78[b]	/	/	/	212.84 ± 30.11[a]	/
	Nonanal	124-19-6	/	/	/	/	/	1.06 ± 0.15[a]
	2-Hexenal	505-57-7	/	/	/	/	/	2.14 ± 0.42[a]
	(*R*)-3,7-dimethyl-6-Octenal	2385-77-5	/	/	/	/	/	1.25 ± 0.10[a]
	Decanal	112-31-2	/	/	/	/	/	5.64 ± 0.50[a]
	Octanal	124-13-0	/	/	/	/	/	3.81 ± 0.22[a]
	(*E*)-3,7-dimethyl-2,6-Octadienal	141-27-5	/	/	/	/	/	1.66 ± 0.11[a]
Ketones	Acetone	67-64-1	/	1.82 ± 0.87[b]	/	2.63 ± 0.43[a]	/	/
	4-Phenyl-3-buten-2-one	122-57-6	0.32 ± 0.02[a]	/	/	/	0.41 ± 0.14[a]	/
	Acetophenone	98-86-2	/	/	/	/	2.57 ± 0.14[a]	/
	2-chloro-Acetophenone	532-27-4	/	/	/	/	7.26 ± 1.42[a]	/
	2-Hydroxy-1-phenyl-ethanone	582-24-1	/	/	/	/	2.69 ± 0.24[a]	/
	Coumarin	91-64-5	/	/	/	/	4.89 ± 0.68[a]	/
	2-Pyrrolidone	616-45-5	/	0.52 ± 0.03[c]	1.28 ± 0.02[a]	/	/	/
Olefins	D-limonene	5989-27-5	261.99 ± 30.36[c]	/	/	0.51 ± 0.01[b]	/	986.61 ± 230.16[a]
	α-Pinene	7785-70-8	2.61 ± 0.86[c]	/	/	/	/	11.34 ± 0.12[a]
	β-Phellandrene	555-10-2	2.20 ± 1.02[c]	/	/	/	/	8.39 ± 0.28[a]
	3-Carene	13466-78-9	1.45 ± 0.60[b]	/	/	/	/	5.21 ± 0.20[a]
	β-Myrcene	123-35-3	17.01 ± 4.15[b]	/	/	/	21.81 ± 6.25[a]	13.52 ± 3.31[c]
	(+)-4-Carene	29050-33-7	/	/	/	/	/	0.73 ± 0.11[a]
	3,7-Dimethyl-1,3,6-octatriene	13877-91-3	/	/	/	/	/	1.92 ± 0.26[a]
Ethers	Dimethyl disulfide	624-92-0	0.12 ± 0.01[c]	/	0.52 ± 0.05[a]	0.39 ± 0.01[b]	/	/
	Dimethyl trisulfide	3658-80-8	/	/	/	0.32 ± 0.02[a]	/	/
Other	Acetonitrile	75-05-8	0.62 ± 0.12[c]	1.27 ± 0.05[b]	1.24 ± 0.35[b]	1.92 ± 0.10[a]	/	/
	Acetamide	60-35-5	0.08 ± 0.01c	0.29 ± 0.00b	0.59 ± 0.08a	/	/	/

Note: Different superscript letters within the same row indicate significant differences among samples ($p < 0.05$); /: undetected.

2,5-dimethylpyrazine in *P. provencensis* PP-2 was 7.38 μg/g, the highest among the three biocontrol bacteria. In another study, *B. subtilis* was shown to possess antimicrobial activity, such as inhibition of *S. aureus* and *E. coli* (Coutts et al., 1965). The pyrazine concentration in *B. subtilis* CF-3 was 10.93 μg/g. Dimethyl disulfide and dimethyl trisulfide were detected in *B. subtilis* CF-3 at concentrations of 0.39 μg/g and 0.32 μg/g, respectively. There was a study shown that dimethyl disulfide, dimethyl trisulfide, and other sulfur-containing VOCs derived from *B. subtilis* showed antifungal activity (Gotor-Vila et al., 2017). The VOCs of NIP-IV are a combination of EOs and biocontrol bacteria, the abundance of VOCs could be one of the reasons for the good preservation function of NIP-IV. Significantly, the VOCs in NIP-IV were mainly derived from EOs and VOCs from biocontrol bacteria at concentrations greater than 10 μg/g, such as acetic acid, linalool, and benzaldehyde.

Variable importance in projection (VIP) scores reflect the differential contributions of VOCs across components. Five compounds from NIP-IV had significant VIP scores >1, which were butyric acid, 3-methylbutanoic acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde (Fig. 5B). In addition to butyric acid, 3-methylbutanoic acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde were ROAV >1 (Fig. 5C). D-limonene in NIP-IV was mainly derived from grapefruit EO, and its ROAV was as high as 7705.59. Sweet, orange, and citrus were the main odor characteristics of D-limonene. This gave NIP-IV a pleasant sweet citrus aroma and reduced the odor of the biocontrol bacteria. (*E*)-Cinnamaldehyde in NIP-IV was primarily sourced from cinnamon EO, with a ROAV of 2.16, contributing to the sweet and spiced aroma of NIP-IV. These results indicate that unpleasant odors originating from biocontrol bacteria were effectively masked. Cinnamon EO contains phenolic compounds that possess bactericidal or bacteriostatic effects. These constituents attack phospholipids in cell membranes, leading to increased permeability and cytoplasm leakage, or interact with enzymes located on the cell wall, thus prolonging the shelf life of meat products (Kim et al., 2013). Pearson's correlation analysis was used to correlate the five essential VOCs with NDMA and nitrite. The results in Fig. 5D show that they all showed a significant positive correlation with nitrite ($p < 0.001$) and a significant negative correlation with NDMA ($p < 0.001$) in luncheon pork, spiced beef, and red sausage. Terpenes react with nitrites to form terpene derivatives. Sawamura et al. (2005) demonstrated that D-limonene from yuzu EO can reduce NDMA. The electronegative (*E*)-cinnamaldehyde interferes with electron transfer-related biological processes and reacts with nitrogen-containing components, such as proteins and nucleic acids, thereby inhibiting the formation of NDMA (Gupta et al., 2008). These evidences suggest that butyric acid, 3-methylbutyric acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde are critical compounds for the inhibition of nitrite conversion to NDMA in NIP-IV. Although the mechanism underlying the inhibition of NDMA production by these compounds remains to be further explored, there is no doubt that the combined use of EO and

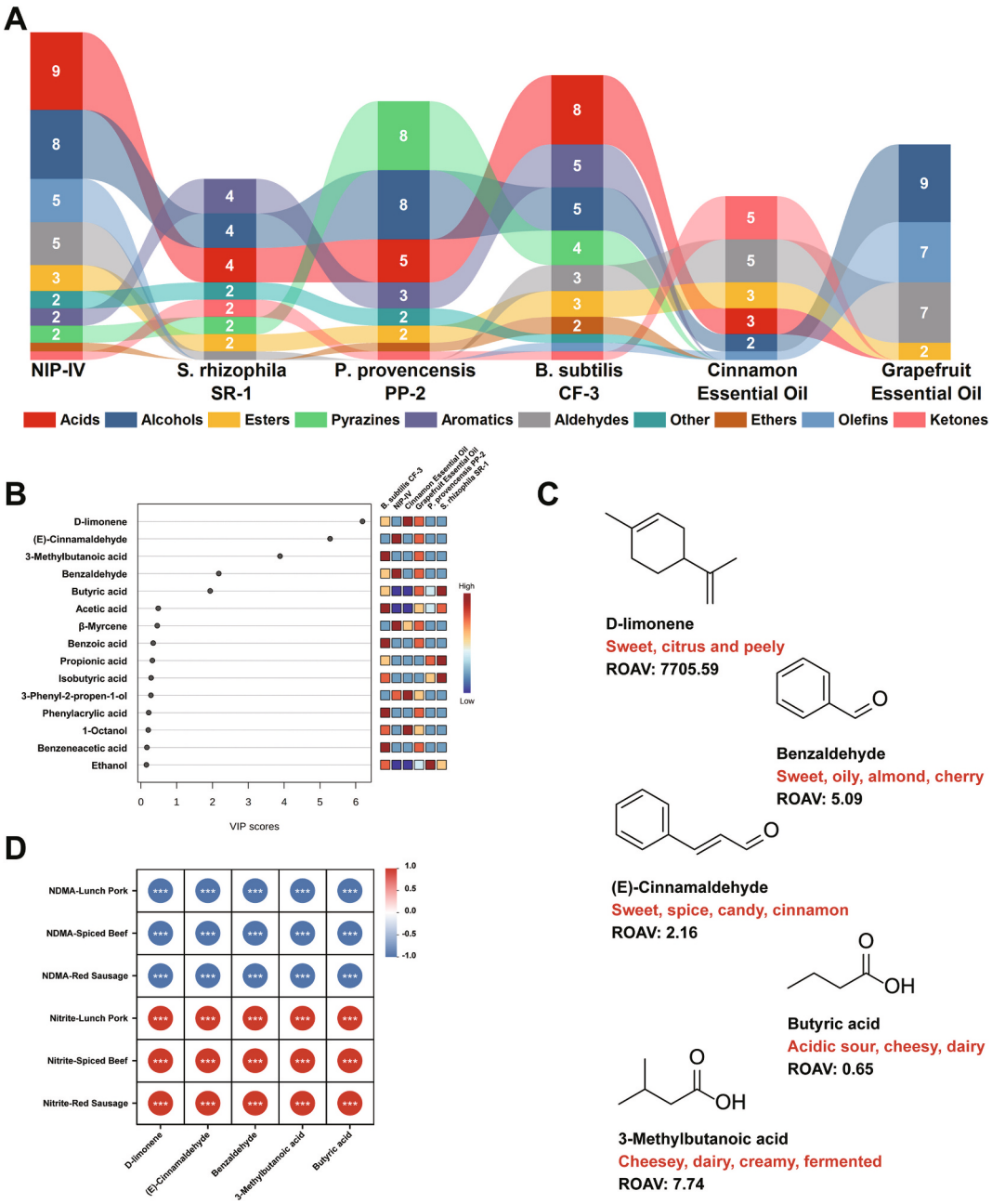

Fig. 5. Details of VOCs in NIP-IV. Composition of VOCs in NIP-IV, *S. rhizophila* SR-1, *P. provencensis* PP-2, *B. subtilis* CF-3, cinnamon EO, and grapefruit EO (A). VIP scores for VOCs in NIP-IV (B). Details of compounds with VIP scores of >1 (C). Butyric acid, 3-methylbutanoic acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde with nitrite and NDMA were determined using Pearson's correlation analysis ($-1 \leq r \leq 1$, D). "***" indicated $0.0001 < p \leq 0.001$.

biocontrol bacteria in a non-contact mode of preservation could effectively inhibit NDMA production and minimize the impact of preservatives on the flavor of deli meat products flavor.

4. Conclusions

A novel preservative, NIP-IV, was developed to inhibit the NDMA generation in deli meat products. NIP-IV was composed of *S. rhizophila* SR-1 + *P. provencensis* PP-2 + *B. subtilis* CF-3 + cinnamon EO + grapefruit EO (mass ratio = 1:1:1:1.5:1.5). NIP-IV could reduce protein degradation and lipid oxidation in deli meat products. Due to its non-contact with food and natural ingredients, it exhibits a high level of biological safety. VOCs were the key pathways by which NIP-IV played a non-contact inhibitory role, and butyric acid, 3-methylbutanoic acid, benzaldehyde, D-limonene, and (*E*)-cinnamaldehyde were significantly negatively correlated with NDMA production in deli meat products. This study provided new insights into reducing the potential risk of NDMA in deli meat products and offered a new reference for the application of biocontrol bacteria in food preservation.

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CRediT authorship contribution statement

Ke Li: Writing – original draft, Methodology, Data curation, Conceptualization. **Guixin Han:** Writing – review & editing. **Shixue Lu:** Investigation, Formal analysis. **Xinxing Xu:** Visualization, Validation, Investigation. **Hao Dong:** Formal analysis. **Haiyan Wang:** Formal analysis. **Fulei Luan:** Formal analysis. **Xiaoming Jiang:** Formal analysis. **Tianhong Liu:** Supervision, Resources, Formal analysis. **Yuanhui Zhao:** Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing 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

Data will be made available on request.

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