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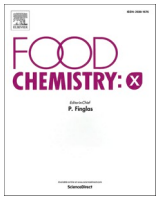

Antimicrobial activity enabled by chitosan-ε-polylysine-natamycin and its effect on microbial diversity of tomato scrambled egg paste

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ABSTRACT

For a long time, food spoilage posed a severe impairment on food safety and public health. Although chemical preservatives are commonly used to inhibit spoilage/ pathogenic microbial growth, the disadvantages of a single target, potential toxicity and high dose of use limit the better use of preservatives. In this research, the combination of natural preservatives: Natamycin (Nat), ε-polylysine (ε-PL), and Chitosan (CS) could achieve an excellent antimicrobial effect including bacteria and fungi, and reduce the usage of a single preservative. Compound preservatives could destroy microbial morphology and damage the integrity of the cell wall/membrane by leakage of protein and alkaline phosphatase (AKP). Besides, high-throughput sequencing revealed that compound preservatives could decrease microbial diversity and richness, especially, *Pseudomonas*, *Acinetobacter*, *Fusarium*, and *Aspergillus*. Therefore, the combination of 1/8 × MIC CS, 1/4 × MIC ε-PL, and 1/2 × MIC Nat can achieve an excellent antibacterial effect, providing new ideas for food preservation.

Introduction

Food spoilage, which is mainly caused by spoilage/ pathogenic microorganisms, poses a severe impairment on food safety and public health (Sun et al., 2022). Generally, natural and chemical preservatives, such as chitosan (Oladzadabbasabadi et al., 2022), organic acid (Unal Turhan, Polat, Erginkaya, & Konuray, 2022), natamycin (Wang, Song, Zhang, Li, & Wang, 2022a) and ε-polylysine (Lin, Gu, Li, Vittayapadung, & Cui, 2018), were used to inhibit microbial growth to delay food spoilage and reduce the occurrence of foodborne illness. However, chemical preservatives may cause adverse effects on public health, such as carcinogenicity (e.g., nitrites/nitrates) and allergies (Kaur & Kaur, 2021). As a result, consumers prefer natural compounds because realize that there is no risk of harmful side effects in consuming healthy food products (Ribes, Fuentes, Talens, & Barat, 2018). Most preservatives are single-target-site antimicrobial agents which cannot simultaneously and effectively inhibit both spoilage/ pathogenic bacteria and fungi (Fones et al., 2020). Therefore, the combination of edible bio-preservatives, which can not only comply with the demands of consumers but also own broad-spectrum antibacterial and antifungal effects, has become a potential and effective antiseptic strategy.

Natamycin (Nat) is a natural polyene macrolide bio fungicide produced by *Streptomyces natalensis* and some other species of *Streptomyces* (Xu et al., 2023). Nat was classified as a Generally Recognized As Safe (GRAS) product by the FDA, Nat has been approved as a food additive in>40 countries (Zhang, Gong, & Liu, 2022). Nat can alter the normal function of ergosterol in the fungal membrane, achieving antifungal activity (Saito, Wang, & Xiao, 2022). It is notable to mention that Nat has been used as an effective antifungal preservative in various food products (Meena, Prajapati, Ravichandran, & Sehrawat, 2021). Wang et al (Wang, Saito, Michailides, & Xiao, 2021a) showed that Nat can significantly inhibit conidial germination and mycelial growth of *Alternaria alternata* (*A. alternata*) and *A. arborescens* and can be a valid tool for reducing postharvest *Alternaria* rot in blueberries. Nevertheless, Nat does not effectively inhibit the growth of bacteria (Wang et al., 2021a). Hence, it is necessary to combine with other antimicrobial agents for improving antimicrobial efficiency. ε-polylysine (ε-PL), a natural product of microbial metabolism, belongs to cationic polypeptide composed of 25–30 L-lysine residues (Chen et al., 2023a). ε-PL could destruct microbial cell wall/membrane, enzymes, etc., thus exhibiting excellent

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antibacterial activity (Bi et al., 2023; Liu, Pei, Han, Feng, & Li, 2015; Zheng, Tang, Yang, Ran, & Li, 2023). Besides, chitosan (CS), a natural polycationic polysaccharide synthesized by the crustaceans such as shrimp and crabs, possesses the positively charged amino groups (NH_3^+) (Chen et al., 2023b). Therefore, due to electrostatic interaction, CS can interact with negatively charged microbial cell membrane and disrupt the integrity of the microbial membrane, thereby inhibiting the growth of microorganisms (Liu, Liao, & Xia, 2023). Therefore, the combination of Nat, ε-PL, and CS not only can make up for the shortcomings of the poor antibacterial of Nat but also can enhance the interaction between preservatives and microbial cells.

Recently, with the increasing popularity, the internationalization of Chinese cuisines has become more and more common. Scrambled egg with tomatoes is one of the representatives of Chinese cuisine. Therefore, based on the theoretical study of the antimicrobial effect and mechanism of preservatives, the effect of preservatives on the microbial diversity of tomato scrambled egg paste was also studied from a practical point of view. In detail, Nat, ε-PL, and CS were used as raw materials to prepare compound preservatives. Then, the antibacterial and antifungal activities of compound preservatives were investigated. Its efficiency on microbial morphology, alkaline phosphatase (AKP) activity, and protein were evaluated. More importantly, the microbial community of tomato scrambled egg paste were analyzed by next-generation sequencing technology, which can more effectively reflect the effect of compound preservatives on microbial diversity.

Methods

Materials

Escherichia coli MC1061 (*E. coli*) and *Staphylococcus aureus* ATCC6538 (*S. aureus*) were obtained from Huayueyang Biotech Co., Ltd. (Beijing, China), *Alternaria alternata* EX2019 (*A. alternata*) and *Botrytis cinerea* bo501 (*B. cinerea*) were stored in the laboratory, Chitosan (CS), ε-polylysine (ε-PL), Natamycin (Nat) and Potassium sorbate (PS) were provided by Shanghai Macklin Biochemical Technology Co., Ltd., agar powder, yeast powder, peptone, and 2.5% glutaraldehyde were provided by Solarbio Technology Co., Ltd. (Beijing, China), Potato Dextrose Agar (Medium) was obtained from Beijing Aoboxing Biotechnology Co. Ltd., Total protein content determination kit and AKP test kit were obtained from Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd.

Determination of minimum inhibitory concentration (MIC) of edible bio-preservatives

The MIC values of CS, ε-PL, and Nat were determined for *E. coli*, *S. aureus*, *A. alternata*, and *B. cinerea* using the two-fold dilution method (Kang et al., 2020). The CS, ε-PL, and Nat were diluted at serial two-fold concentrations ranging from 0 to 1.25 mg/mL. Different concentrations of CS, ε-PL, and Nat were prepared in Luria-Bertani (LB) broth with activated *E. coli* and *S. aureus* ($10^{5\text{-}7}$ CFU/mL), incubation at 37℃ for 24 h in a constant temperature and humidity chamber (Yongguang Medical equipment Co., Ltd., FCD). The lowest concentration of CS, ε-PL, and Nat that inhibited microbial growth was identified as the MIC. Similarly, different concentrations of CS, ε-PL, and Nat were prepared in Potato Dextrose Broth (PDB) with activated *A. alternata* and *B. cinerea* (10^5 spores/mL). All tubes were incubated at 27℃ for 24 h in a constant temperature and humidity chamber (Shanghai Qixin Scientific instrument Co., Ltd., DHP-9052). The lowest concentration of CS, ε-PL, and Nat without any visible growth was defined as MIC.

Compounding of edible bio-preservatives

A checkerboard test was used for compounding of edible bio-preservatives (Bonapace, White, Friedrich, & Bosso, 2000). The antimicrobial combinations of CS + ε-PL, CS + Nat, and ε-PL + Nat were tested in 96-well microtiter trays. Wells not containing edible bio-preservatives served as controls. The concentration of each edible bio-preservative was ranging from 1/8 × MIC to 2 × MIC. A total of 100 μL of bio-preservative solution was added to each well. Then, 100 μL of 10^7 CFU/mL of bacterial suspension or 10^5 spores/mL was added to each well. The well plates were incubated at 37 °C or 27 ℃ for 12 h in constant temperature and humidity chambers. The microbial growth status of each well was observed, and the effect of compound preservatives on microbial growth was recorded and compared. Then, two preservatives with a combined effect were selected to be compounded with the third preservative. The optimal ratio of compounding preservatives was obtained. The MIC of the compounded preservative was also obtained. The following formula was used to calculate the compound strength and determine the antimicrobial effect of compound preservatives.

$$Compound\ strength = \frac{MIC\ of\ preservative\ A\ in\ combination}{MIC\ of\ preservative\ A\ alone} + \frac{MIC\ of\ preservative\ B\ in\ combination}{MIC\ of\ preservative\ B\ alone}$$

Synergy was defined as a compound strength index ≤ 0.5, additivity was defined as a compound strength index > 0.5 to 1, and antagonism was defined as a compound strength index > 1.

Evaluation of the antibacterial activity of compound preservatives

The antibacterial activity of compound preservatives was verified as previously described (Kang et al., 2020). In detail, the bacteria in the exponential growth phase were diluted to $10^5 \sim 10^7$ CFU/mL. Compound preservatives were added to bacterial suspensions to final concentration of 1/2, 1, and 2 × MIC. Then, 200 μL of the mix suspensions were added to a 96-well plate and incubation at 37℃ for 24 h in a constant temperature and humidity chamber. The bacterial growth was measured at 600-nm wavelength by a microplate reader (Bio-Tek ELx800, USA) every two hours. Bacterial suspensions without treatment with compound preservatives served as control.

Evaluation of antifungal properties of compound preservatives

The antifungal activities of compound preservatives were determined using an agar dilution assay. The antimicrobial agents were diluted in a stepwise concentration by Potato Dextrose Agar (PDA). Antimicrobial agents were added to final concentrations (the concentration of compound preservatives was 1 × MIC, and the concentration of other preservatives was the maximum use allowed by the GB 2760–2014) and then poured into a Petri dish. The diluted spore suspension of *A. alternata* and *B. cinerea* were spread separately onto the plate and incubated at 27℃ for 3 days in a constant temperature and humidity chamber to evaluate the antifungal activity of edible bio-preservatives.

SEM analysis

SEM was used to assess the effect of compound preservatives on the morphology of microorganisms. Bacteria suspensions were suspended in PBS (pH = 7.2, 0.01 M/L) to OD_{600} = 0.5. Then, compound preservatives with the final concentration of 1 × MIC were added. Bacterial suspensions that were treated with PBS were served as control. Then, PBS was used to wash bacterial cells three times at 8000 rpm for 5 min, followed by fixed at 4 ℃ with 2.5 % glutaraldehyde for 4 h. Then, PBS was used to wash fixed bacterial cells three times. Subsequently, fixed bacterial cells were dehydrated for 10 min in 30 %, 50 %, 70 %, 80 %, 90 %, 96 %, and 100 % alcohol series, respectively. Bacterial cell morphology was observed by SEM. Similarly, the mycelia of *A. alternata* and *B. cinerea* were used to evaluate the effect of compound

preservatives on microbial morphology.

Leakage of protein and alkaline phosphatase activity

The bacteria in the logarithmic growth period were washed and then suspended in sterilized 0.85% NaCl to 1 × 10^8 CFU/mL. Then, compound preservatives with final concentrations of 1/2, 1, and 2 × MIC were added, incubated at 37℃ in a thermostatic shaker (Shanghai Zhicheng Analytical instrument Co., Ltd., ZHWY103B), 170 rpm for different times and centrifuged at 6000 g, 10 min. After filtration by 0.22 μm membrane, the leakage of protein and the AKP activity in the supernatant was determined by a total protein content determination kit and an AKP test kit, respectively.

Measurement of peroxide value of tomato scrambled egg paste during storage

Tomato scrambled egg paste with compound preservatives and potassium sorbate were prepared separately using our previous method (Li et al., 2023). Among them, the preservatives were added before bagging. Samples not containing edible bio-preservatives were used as control. All treatment tomato scrambled egg paste were sealed and sterilized in a water bath (92–95 ℃, 30 min). The peroxide value was determined based on GB 5009.227–2016.

Measurement of moisture content of tomato scrambled egg paste during storage

The moisture content of tomato scrambled egg paste was determined using an electronic moisture tester. About 5 g of the sample was placed in the sample rack and dried until a constant weight was obtained according to the corporate standard Q/XXCJ-JS-2021 of Xinjiang Xiaochu Food Co., Ltd.

$$moisture\ content\ (\%) = \frac{(m_2 - m_1)}{m_2} \times 100\%$$

among them, m_2: sample weight before drying, m_1: sample weight after drying.

Measurement of tomato scrambled egg paste microorganisms during storage

Aerobic plate count was measured based on GB 4789.2–2022. Coliforms were measured based on GB 4789.3–2016. Molds and yeasts were measured according to GB 4789.15–2016.

Sample DNA extraction, PCR amplification, and sequencing

Tomato scrambled egg paste with CS, ε-PL, Nat, compound preservatives and PS were prepared separately using our previous method (Li et al., 2023). Among them, the preservatives were added before bagging (the final concentration of compound preservatives was 1 × MIC, and the final concentration of other preservatives was the maximum use allowed by the GB 2760–2014. With no preservatives added as control. All treatment tomato scrambled egg paste were sealed and sterilized in a water bath (92–95 ℃, 30 min). After placing the bulging bag (37 ℃, 6 months), the E.Z.N.A.® soil DNA Kit (Omega Biotek, Norcross, GA, U.S.) was used to extract total microbial genomic DNA. 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) served as primer pairs to amplify the hypervariable region V3-V4 of the bacterial 16S rRNA gene. The fungal primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) were used to amplify the ITS1 region of fungal ITS rRNA genes. PE libraries were constructed by TruSeq™ DNA Sample Prep Kit (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China). Then, PE libraries were sequenced on the Illumina Hiseq platform by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Statistical and bioinformatics analysis

Flash software (Version 1.2.11) and Qiime software (Version 1.9.1) were utilized to analyze raw statistics. Uparse (Version 7.0.1090) was utilized to cluster operational taxonomic units (OTUs) with a 97% similarity cutoff. The Silva (Release 138 https://www.arb-silva.de) and Unite (Release 8.0 https://unite.ut.ee/index.php) databases were utilized to analyze the taxonomy of each 16S rRNA and ITS rRNA gene sequence, respectively. Mothur software (Version 1.30.2) was utilized to analyze Alpha diversity. R language (Version 3.3.1) was utilized to analyze the community bar and community heat map.

All experiments were repeated three times. The results were reported as means ± standard deviation (SD).

Results and discussions

Antimicrobial activity of preservatives

MIC of edible bio-preservatives

The antimicrobial activity of CS, ε-PL, and Nat were verified for further compounding of preservatives. The MIC values of CS, ε-PL, and Nat were determined for *E. coli*, *S. aureus*, *A. alternata*, and *B. cinerea* using the two-fold dilution method. As shown in Table S1, Nat exhibited the weakest inhibitory effect on *E. coli* and *S. aureus* and the best inhibitory effect on *A. alternata* and *B. cinerea*. This is attributed to the fact that Nat acts specifically on ergosterol in the fungal membrane, so it has no inhibitory effect on bacteria (Saito et al., 2022). By contrast, CS exhibited better antibacterial properties. The possible reason is that positively charged CS could be more effectively combined with bacteria to improve the antibacterial effect (Liu et al., 2023). Among them, ε-PL exhibited the best inhibitory effect on both bacteria and fungi. The MIC to bacteria and fungi was as low as 0.00625 mg/mL. The main reason was attributed to not only that ε-PL could destruct microbial cell wall/membrane and enzymes, but also that ε-PL could stimulated intracellular reactive oxygen species accumulation (Li et al., 2019). However, the weak mechanical of ε-PL may lead to its poor adsorption on food products and cannot achieve better preservation (Wang et al., 2021b). Therefore, the combination of Nat, ε-PL, and CS was expected to achieve an excellent broad-spectrum antibacterial effect and reduce the dose of a single preservative used.

Antimicrobial activity of compound preservatives

Based on broad-spectrum antimicrobial properties of compound preservatives, the best ratio of the compound preservative was obtained as 1/8 × MIC CS + 1/4 × MIC ε-PL + 1/2 × MIC Nat (Table. S2-S6). And this was used as the MIC of the compound preservative. Then, agar dilution assay was used to prove the antimicrobial activity of compound preservatives against *E. coli*, *S. aureus*, *A. alternata* and *B. cinerea*. It is noteworthy to mention that compound preservatives exhibit excellent antimicrobial activity compared with single preservative (Fig. 1A). Furthermore, without compound preservatives, *E. coli* and *S. aureus* showed rapid growth and reached a stabilization stage after incubation 22 and 20 h, respectively (Fig. 1B-C). However, bacterial growth was completely inhibited after treated with 1 × MIC compound preservatives, showing compound preservatives exhibit excellent bactericidal effect. Besides, 1/2 × MIC compound preservatives also exhibit inhibitory effect on both *E. coli* and *S. aureus*. The possible reason was that electrostatic interaction between CS and microorganisms could capture more bacteria and fungi (Chen et al., 2023b). Then, ε-PL and Nat worked on cell membrane/ wall and ergosterol in the fungal membrane, respectively (Liu et al., 2015; Saito et al., 2022). And ultimately, compound preservatives lead the death of both bacteria and fungi. Therefore, the combination of Nat, ε-PL and CS showed excellent broad-spectrum antimicrobial effect.

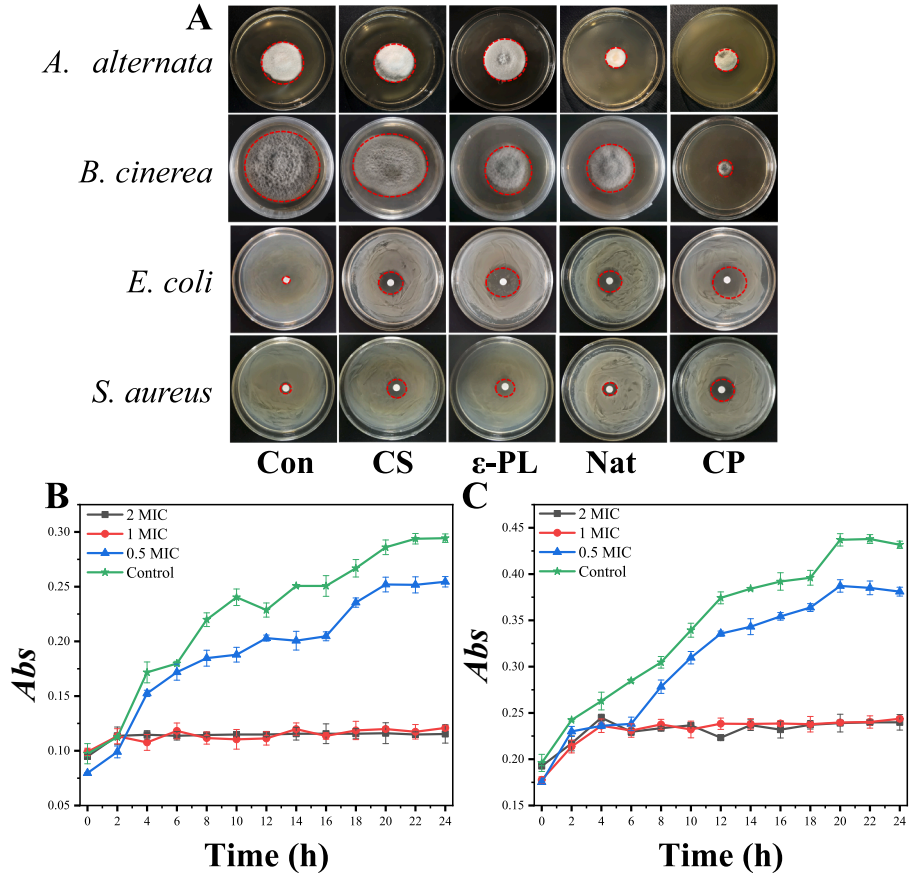

Fig. 1. Antimicrobial activity of compound preservatives: (A) Images of the colonies formed by *A. alternata*, *B. cinerea*, *E. coli* and *S. aureus* after exposed to preservatives. Con: control, CS: chitosan, ε-PL: ε-polylysine, Nat: natamycin, CP: compound preservatives; (B) Effect of compound preservatives on the growth curve of *E. coli*; (C) Effect of compound preservatives on the growth curve of *S. aureus*.

Antimicrobial mechanisms of compound preservatives

Effect of compound preservatives on microbial morphology

The morphology of microbial before and after treatment with compound preservatives was observed by SEM. Without compound preservatives, *E. coli* cells kept a complete and typical rod-like shape morphology; however, the bacterial cells became rough and wrinkled after compound preservatives were treated (Fig. 2A-B). And *S. aureus* showed a spherical-shape and intact cell walls without the interference of compound preservatives, once it comes into contact with compound preservatives, the cells were distorted and wrinkled membranes (Fig. 2C-D). As well as in bacteria, without the treatment of compound preservatives, the mycelia of *B. cinerea* and *A. alternata* remained a complete and robust morphology, showing smooth surfaces. On the contrary, the mycelia of *B. cinerea* and *A. alternata* were deformed and collapsed after treatment with compound preservatives (Fig. 2E-H). It was found that Nat, ε-PL, and CS pose different degrees of adverse effects on the cell membrane of microorganisms (Liu et al., 2015; Liu et al.,

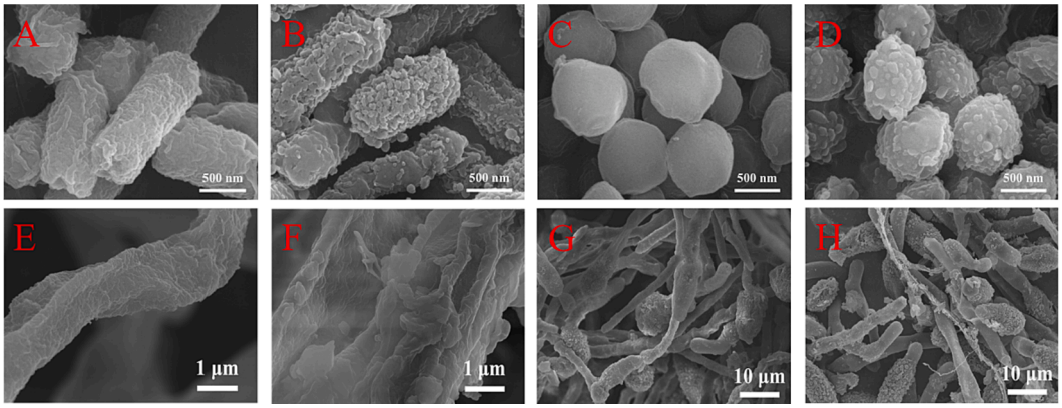

Fig. 2. Effect of compound preservatives on microbial morphology: (A) SEM of *E. coli*; (B) SEM of *E. coli* treated with compound preservatives; (C) SEM of *S. aureus*; (D) SEM of *S. aureus* treated with compound preservatives; (E) SEM of the mycelia of *B. cinerea*; (F) SEM of the mycelia of *B. cinerea* treated with compound preservatives; (G) SEM of the mycelia of *A. alternata*; (H) SEM of the mycelia of *A. alternata* treated with compound preservatives.

2023; Saito et al., 2022). Therefore, compound preservatives showed excellent antimicrobial activities and exerted their antimicrobial effect by destroying the cell membrane and releasing the contents.

Effects of compound preservatives on microbial wall/membrane integrity

In order to further confirm the impact of compound preservatives on the microbial wall/membrane integrity, the protein concentrations and AKP activity with or without compound preservatives treatment were monitored. Generally, leakage of intracellular contents such as proteins is a key symbol of membrane damage (Kang et al., 2020). After being treated with compound preservatives for 2 h, the protein content of *E. coli* and *S. aureus* increased dramatically, reaching peak values of 6.17 μg/mL and 6.25 μg/mL when exposed to 2 × MIC compound preservatives for 10 h and 8 h, respectively (Fig. 3A-B). Similarly, compared with the control group, compound preservatives at 2 × MIC increased the protein concentration in *B. cinerea* and *A. alternata* by 69.40% and 73.47%, respectively (Fig. 3C-D). The results further showed that the membrane integrity of bacteria and fungi can be destroyed by compound preservatives leading to content leakage.

Alkaline phosphatase (AKP) is one of the important enzymes in microorganisms. Due to AKP will be released only when the cell wall/membrane was damaged, the damage to the cell wall/membrane could be tracked by extracellular AKP activity (Lin et al., 2018; Yang et al., 2016). Notably, compared with the control group, the AKP activity was increased by 5-fold and 4.95-fold in *E. coli* and *S. aureus* when treated with 2 × MIC compound preservatives for 8 h, respectively (Fig. 4A-B). Moreover, the AKP activity was increased by 3.37-fold and 3.09-fold after 2 × MIC compound preservatives treatment of *B. cinerea* and *A. alternata* for 16 h, respectively (Fig. 4C-D). These results indicated that compound preservatives can damage microbial wall/membrane integrity, release protein and important enzymes in cells, and ultimately bacterial/ fungal death. Lan et al (Lan, Zhang, Liu, Chen, & Xie, 2019) also reported that ε-PL could lead to membrane rupture and the increase of AKP content. Nat could target fungal cell membrane ergosterol (Gong et al., 2019), ε-PL could damage microbial membranes (Lin et al., 2018), and CS could strengthen the interaction between compound preservatives and microorganisms (Liu et al., 2023). Accordingly, various modes of action of Nat, ε-PL, and CS against bacteria and fungi could encourage antimicrobial activities of compound preservatives.

Comparison of spoilage microbiota composition in tomato scrambled egg paste in different treatment groups

Tomato scrambled egg paste, which is rich in nutrients, such as carbohydrate, proteins, vitamins, and minerals, was chosen as the representatives of Chinese cuisine to further investigated the antimicrobial effect of preservatives. Spoilage/ pathogenic bacteria and fungi could easily grow and multiply in tomato scrambled egg paste which has been sealed and sterilized in a water bath (92–95 ℃, 30 min) (Table. S7). It was found that compound preservatives displayed little effect on the physicochemical quality of tomato scrambled egg paste (Fig. S1-2). During storage, the colonies number remains very low level, meeting the needs of industrial production (Table. S8). However, microorganisms in products are complex and variable. And changes in diversity and community are important factors affecting the flavor and quality of the product. Therefore, next-generation sequencing technology was used to analyze the microbial diversity of tomato scrambled egg paste after storage, which further reflect the antimicrobial and preservation property of compound preservatives.

Alpha diversity

In an ecosystem, the diversity and richness of species were primarily reflected by alpha diversity (Zhou, She, Zhu, & Zhou, 2022). Therefore, the effect of compound preservatives on the alpha diversity of tomato scrambled egg paste was investigated by Shannon index and Chao index. Shannon index indicated community diversity and the Chao index indicated community taxon richness. Higher Shannon and Chao index indicated higher community diversity and greater microbial richness. As shown in Fig. 5A-B, the Shannon index (0.09 ± 0.03) and Chao index (126.85 ± 32.74) of compound preservatives treatment group were not significantly different from that of CS and ε-PL treatment group, but was far below than that of not treated with preservatives (control) (1.22 ±

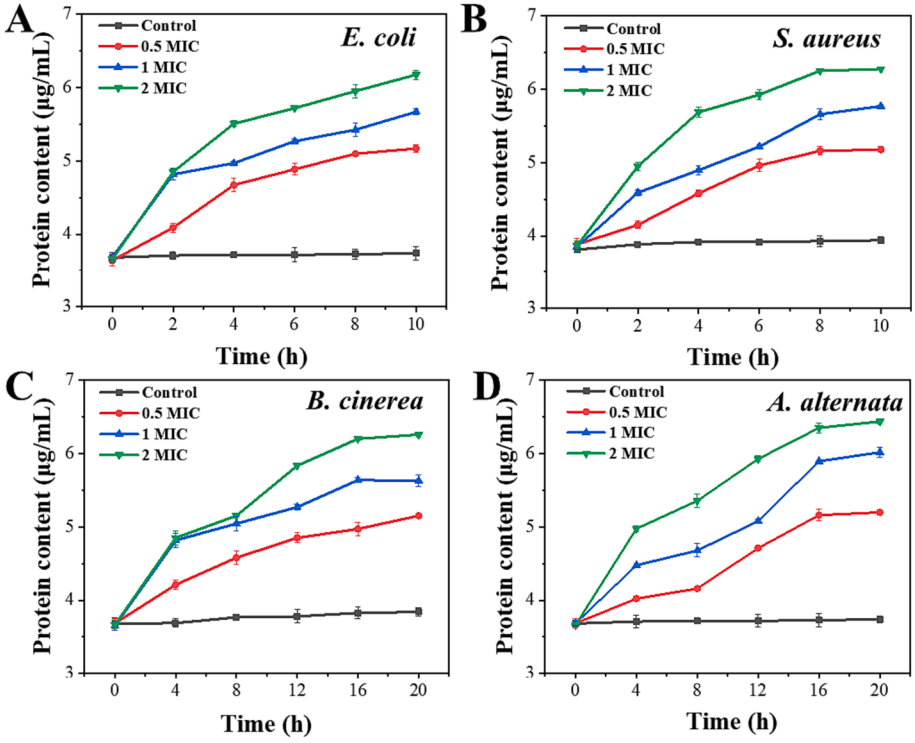

Fig. 3. Determination of the release of cell contents: Leakage of protein (A) *E. coli*; (B) *S. aureus*; (C) *B. cinerea*; (D) *A. alternata*.

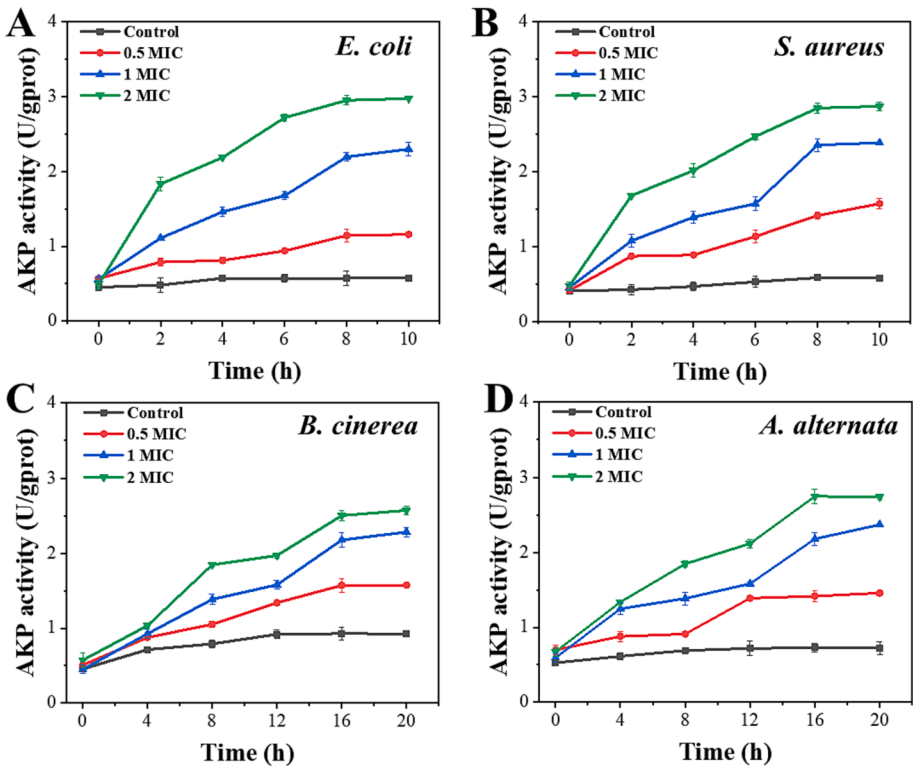

Fig. 4. Determination of the release of cell contents: Alkaline phosphatase activity (A) *E. coli*; (B) *S. aureus*; (C) *B. cinerea*; (D) *A. alternata*.

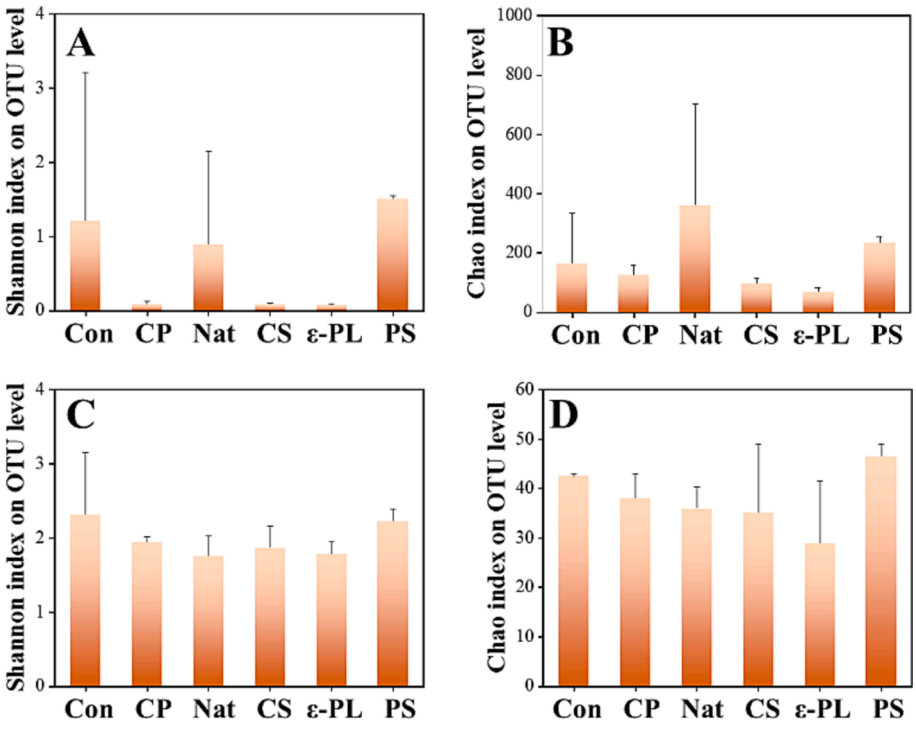

Fig. 5. The alpha diversity of tomato scrambled egg paste: (A) Shannon index of bacteria; (B) Chao index of bacteria; (C) Shannon index of fungi; (D) Chao index of fungi. Con: control, CP: compound preservatives, Nat: natamycin, CS: chitosan, ε-PL: ε-polylysine, PS: potassium sorbate.

1.99 and 165.18 ± 170.15) and positive control group (PS treatment group). Notably, compared with Nat treatment group, both Shannon index and Chao index of compound preservatives treatment group were decreased greatly. These results demonstrated that compound preservatives could reduce the diversity and the richness of bacteria in tomato scrambled egg paste. Similarly, according to fungal Shannon index and Chao index (Fig. 5C-D), it was found that compound preservatives could decrease the diversity and the richness of fungi community.

Comparison of bacterial community in tomato scrambled egg paste in different treatment groups

The bacterial community composition of tomato scrambled egg paste was analyzed by 16S rRNA gene sequencing. At the phylum level, the proportion of *Actinobacteriota* (68.04%), *Proteobacteria* (23.19%), *Firmicutes* (6.06%), and others (1.60%) were the predominant phylum in the tomato scrambled egg paste that not treated with preservatives (control) (Fig. 6A). And the proportion of *Actinobacteriota* (90.64%), *Proteobacteria* (5.83%), and *Cyanobacteria* (1.51%) were the predominant phylum in the Nat treatment group. However, in the compound preservatives treatment group, *Actinobacteriota* (99.13%) was the predominant phylum (Fig. 6A). The results indicated that compound preservatives could decrease the diversity of bacterial community and bacterial abundance. Notably, the antibacterial effect of the compound preservatives was the same as that of CS and ε-PL. It was shown that the combination of Nat, ε-PL, and CS exhibit excellent antibacterial effects and could greatly reduce the usage of preservatives. And PS cannot inhibit the growth of bacteria compared with compound preservatives.

In addition, the top 20 species in all groups in terms of total abundance at the genus level were compared. The compound preservatives treatment group had the lowest bacterial richness, compared with both the control group, the Nat group, and the PS group (Fig. 6B). And *Rhodococcus* was the main genus in all the groups, but in general, *Rhodococcus* is non-pathogenic and *Rhodococcus* could produce biosurfactants that are applicable in the food-processing industries (Cappelletti et al., 2020; Nazari et al., 2022). It's been discovered that *Rhodococcus*, *Pantoea*, *Pseudomonas*, *Clostridium_sensu_stricto_1*, and *Acinetobacter* were the top 5 genera in the control group. Among them, *Pseudomonas* and *Acinetobacter* played a vital role in causing food putrefaction and deterioration and can create economic havoc via spoiling lots of food products (Pinu, 2016; Sun et al., 2022). It is noteworthy to mention that the abundance of *Pseudomonas* and *Acinetobacter* in the compound preservatives treatment group was significantly decreased. The possible reasons were that compound preservatives could directly inhibit the growth of food spoilage, or compound preservatives disrupt the balance of the flora leading to microbial interactions and ultimately inhibiting spoilage bacteria.

Comparison of fungal community in tomato scrambled egg paste in different treatment groups

In addition to bacteria, fungi also played a determining role on the quality of food (Bernardi, Garcia, & Copetti, 2019; Fones et al., 2020). So, the fungal community composition of tomato scrambled egg paste was investigated. *Basidiomycota* and *Ascomycota* were the main phylum in all the groups (Fig. 6C). A more complicated fungal community of the control group was discovered indicating that preservatives could decrease the number of fungal species. Furthermore, the species of the top 20 genera in the heat map of the fungal community were selected for analysis. It is not difficult to know that like other preservatives, compound preservatives could reduce the fungal abundance and the diversity of the fungal community (Fig. 6D). Among the identified fungal species, *Apiotrichum* was the most dominant fungus in all groups, showing that preservatives used in this research made no effect on *Apiotrichum* growth. Notably, *Russula*, *Fusarium*, *unclassified_f_Nectriaceae*, and *Aspergillus* were expressed a lower level in the compound preservatives treatment group, compared with control group (Fig. 6D).

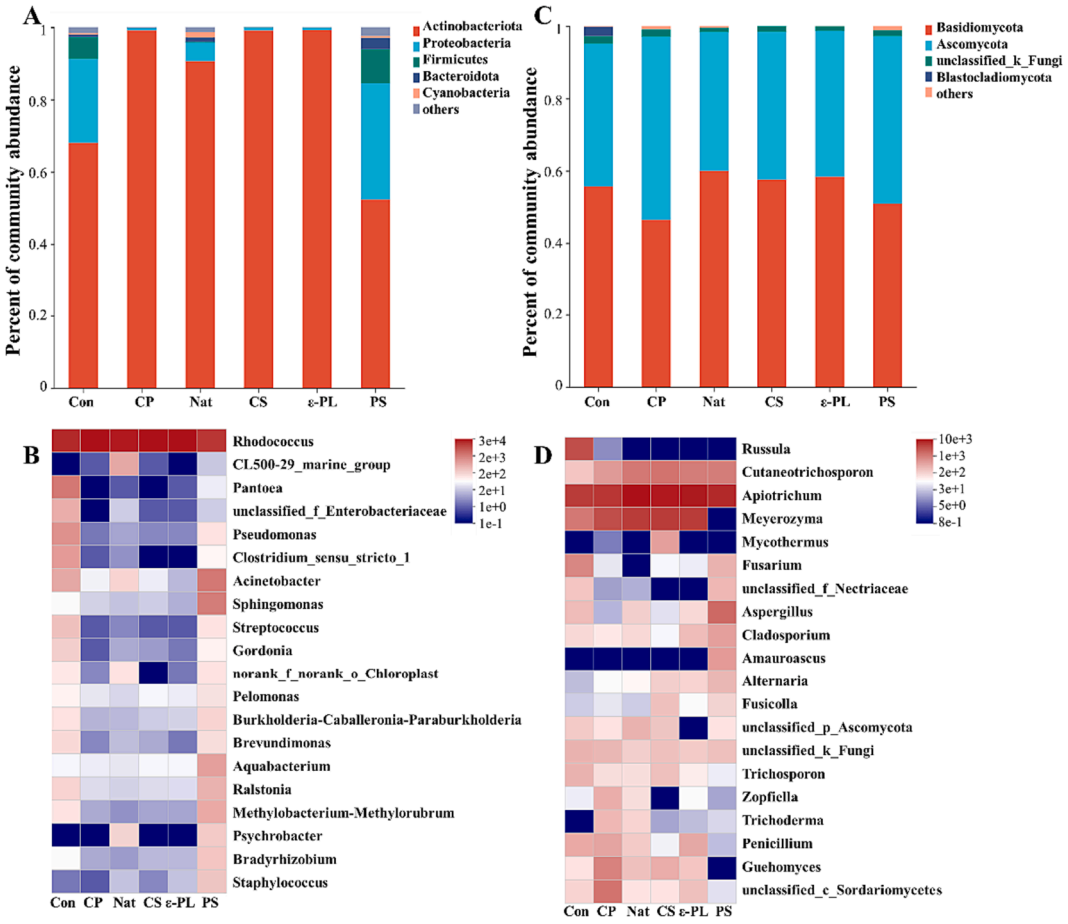

Fig. 6. Distribution of spoilage bacterial and fungal microbiota in tomato scrambled egg paste: (A) Percent of bacterial community abundance on phylum level; (B) Heat map of bacterial community (top 20 genera); (C) Percent of fungal community abundance on phylum level; (D) Heat map of fungal community (top 20 genera). Con: control, CP: compound preservatives, Nat: natamycin, CS: chitosan, ε-PL: ε-polylysine, PS: potassium sorbate.

It is well-known that *Fusarium* and *Aspergillus* are the typical spoilage/ pathogenic microorganisms in the spoilage of foodstuffs and in the production of mycotoxins (Misiou & Koutsoumanis, 2022; Taniwaki, Pitt, & Magan, 2018). Mycotoxins produced by them own the most acute and chronic toxicity, such as genotoxicity, carcinogenicity, and immunotoxicity (Gurikar, Shivaprasad, Sabillón, Nanje Gowda, & Siliveru, 2023; Wang, Wang, & Sha, 2022b). Therefore, compound preservatives could directly or indirectly inhibit the growth of spoilage/ pathogenic fungal in the tomato scrambled egg paste and ameliorate fungal community structure.

Conclusion

In conclusion, compound preservatives exhibit excellent broad-spectrum antimicrobial effect compared with single preservative. Compound preservatives mainly act on cell wall/membrane and damage cell wall/membrane integrity, which leading to the leakage of protein and AKP. Notably, microbial diversity and richness could decrease by compound preservatives. Especially, typically spoilage/ pathogenic microorganisms such as *Pseudomonas*, *Acinetobacter*, *Fusarium* and *Aspergillus* were inhibited by compound preservatives. The research might provide a novel strategy for preserving the food.

CRediT authorship contribution statement

Wanfeng Wu: Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Yaru Li:** Conceptualization, Investigation, Data curation, Writing – review & editing. **Xiaoyu Zhu:** Data curation, Writing – review & editing. **Liang Wang:** Conceptualization, Writing – review & editing. **Jiayi Wang:** Formal analysis, Writing – review & editing. **Yanan Qin:** Writing – review & editing. **Minwei Zhang:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Chunshan Yu:** Writing – review & editing. **Chunmei Gou:** Writing – review & editing. **Xiaoqin Yan:** Writing – review & editing.

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

All data needed to evaluate the conclusions in the paper are present in the paper and/or the supplementary information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100872.

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