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Impact and mechanisms of cinnamaldehyde on putrescine production by *Obesumbacterium proteus* OP216735 during the process of Xinjiang sausage

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ABSTRACT

This study investigated the effects of cinnamaldehyde on putrescine production by *Obesumbacterium proteus* in pure cultures and Xinjiang sausage. Scanning electron microscopy was used to analyze the cell morphology of the test bacteria, and reverse transcription real-time fluorescence quantitative polymerase chain reaction was employed to analyze the gene expression of arginine decarboxylase and ornithine decarboxylase of the test bacteria at different cinnamaldehyde concentrations. Single-molecule real-time sequencing was utilized to identify the species composition of the sausage. Moreover, relevant parameters such as putrescine accumulation and microbial growth were evaluated. The results indicated that cinnamaldehyde inhibited the growth of *O. proteus* by leaking its contents and changing membrane permeability, and significantly reducing putrescine accumulation by inhibiting the expression of arginine decarboxylase (*adiA*) and ornithine decarboxylase (*speF*) genes. The cinnamaldehyde concentration revealed an inverse relationship between gene expression and putrescine accumulation. Besides, cinnamaldehyde significantly reduced the abundance of harmful bacteria (Enterobacteriaceae and *Pseudomonas*), alleviate putrescine content and stimulate the release of amino acids in the sausage. Therefore, adding cinnamaldehyde is an effective method to inhibit putrescine accumulation and reduce the growth of spoilage microorganisms in the sausage.

1. Introduction

Xinjiang sausage is a traditional fermented meat product in Xinjiang, China, known for its popularity due to its rich nutrients and unique flavor[1]. Nevertheless, traditional sausages are susceptible to contamination by various bacteria during production, and their high protein content can contribute to the accumulation of potentially harmful substances like biogenic amines (BAs)[2]. Putrescine, a biogenic amine commonly found in sausages, is a key indicator of food spoilage and can exacerbate the toxic effects of histamine and tyramine, potentially leading to carcinogenic nitrosamine formation[3]. The consumption of high putrescine levels may result in low blood pressure and muscle spasms in the extremities, as well as tumor cell growth promotion[4]. Consequently, effective strategies to regulate putrescine accumulation in sausage are crucial for enhancing their safety and quality.

Putrescine production is mainly associated with *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia*, *Hafnia hives*, and *Lactobacillus delbrueckii*, which were isolated as producing bacteria of putrescine during food fermentation[5-6]. Putrescine can be generated through 2 main pathways: the agmatine deiminase (AGDI) pathway, where arginine is decarboxylated to form agmatine and then deiminated to putrescine, and the ornithine decarboxylase (ODC) pathway, in which arginine is deaminized to ornithine by arginine deiminase (ADI), followed by decarboxylation of ornithine to putrescine[5-6]. Studies have indicated that microorganisms such as *Lactobacillus breve*, *Lactobacillus campylobacter*, *Lactococcus lactis*,

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and *Enterococcus faecalis* produce putrescine through the guanidine butylamine deiminase pathway. Genes involved in this process include the regulatory gene aguR and the polygenic mRNA (operon aguBDAC) co-transcribed by catabolic genes like *aguB*, *aguD*, *aguA*, and *aguC*[7-8]. However, there are currently few studies on the relevant genes in the putrescine production pathway of *Obesumbacterium proteus*.

Putrescine accumulation in fermented sausages is influenced by various factors, including microorganisms with amino acid decarboxylase activity, environmental temperature, humidity, and certain spices added to sausages[9-10]. Cinnamon, a commonly used spice in countries, including China, India, and other Asian countries, is often incorporated into soups and meat products to enhance flavor[11-12]. Cinnamaldehyde, the primary active component of cinnamon, exhibits antibacterial, immunomodulatory, anticancer, and antiangiogenic properties[13-14]. Cinnamaldehyde has been shown to impact the bacterial peptidoglycan layer, alter cell membrane fluidity and selective permeability, resulting in cell deformation and leakage of cell contents[15]. Research indicates that chitosan-modified cinnamaldehyde-loaded liposomes can disrupt cell membrane integrity, causing intracellular component leakage and cell death[16]. The combined use of cinnamaldehyde and thymol can enhance fungal cell membrane permeability and disrupt cell structure[17]. Additionally, cinnamaldehyde can deactivate *Listeria monocytogenes* in minced pork under low temperature conditions by damaging cell membranes and interfering with stress-regulated gene expression[18]. Research also has shown that adding cinnamon bark essential oil microcapsules rich in cinnamaldehyde to ground beef can hinder microbial growth and lipid oxidation and prevent discoloration of ground beef during refrigeration[19]. Consequently, cinnamaldehyde has significant potential for meat products because of its antioxidant and antibacterial effects.

Current research on inhibiting BAs, both domestically and internationally, primarily involves the addition of plant extracts and spices during the fermentation process. However, plant extracts consist of numerous active ingredients with complex functions, and few studies specify the exact active ingredients used. As a result, this study focuses on investigating a specific active ingredient found in spices−cinnamaldehyde. However, to the best of our knowledge, few studies have been conducted on the effect of cinnamaldehyde on putrescine accumulation in *O. proteus* isolated from Xinjiang sausage. Hence, this study aimed to elucidate the mechanism by which cinnamaldehyde affects putrescine production by measuring the effects of cinnamaldehyde on microorganisms, gene expression, and putrescine accumulation. The findings of this study are expected to provide a theoretical basis for the application of cinnamaldehyde in fermented foods, ultimately enhancing their safety.

2. Materials and methods

2.1 Bacterial strain and culture condition

O. proteus was isolated from commercial Xinjiang sausage. The sequences were deposited in the GenBank database under accession number OP216735. *O. proteus* OP216735 was cultured in Brain Heart Perfusion Solution media at 37 ℃ supplemented with 100 mmol/L arginine (Sigma, California, USA)[20]. A total of 5 groups were prepared as follows: a control batch without arginine (batch CK), a batch containing arginine and 0% cinnamaldehyde (batch AZ), a batch with added arginine and minimum inhibitory concentration (MIC) cinnamaldehyde (batch AO), a batch with added arginine and 1/2 MIC cinnamaldehyde (batch AT), and a batch with added arginine and 1/4 MIC cinnamaldehyde (batch AF). The MIC of cinnamaldehyde is 0.625%. Each group was inoculated with 1% overnight cultures of *O. proteus*. Sampling (2 mL) was performed every 2 h for 24 h. Growth was monitored by measuring the absorbance at 600 nm using a BioTek multimode reader. Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) was used to quantify gene expression, and high-performance liquid chromatography (HPLC) was used to determine putrescine accumulation[2].

2.2 Cell membrane permeability and cell morphology analysis

Membrane permeability was assessed using neighboring nitrobenzene *β-D*-galactose glucoside (ONPG) as outlined by Li et al.[21]. In summary, a logarithmic phase bacterial suspension was treated with ONPG (25 mmol/mL) and cinnamaldehyde. The suspension was then incubated at 37 °C, with samples taken at various time intervals. Subsequently, the absorbance of the supernatant was measured at 420 nm using a spectrophotometer. Changes in cell morphology were observed by scanning electron microscopy (SEM) and conducted by Zhan et al.[22] with slight modifications. Two concentrations of cinnamaldehyde (0, MIC) were accurately weighed and sterilized under ultraviolet light for 15 min. Then 5.0 mL of a logarithmic phase bacterial suspension of the test bacteria was added, treated at 37 °C for 10 min, and washed thrice with sterile water. The experimental bacteria were collected, and the untreated test bacteria were used as a negative control. The bacteria were fixed in 2.5% glutaraldehyde for 4 h and centrifuged at 12 000 r/min (4 °C) for 5 min. The supernatant was discarded, bacteria were collected, and 30%, 50%, 70%, 80%, 90%, and 100% ethanol were added, followed by dehydration for 10 min. Finally, wet bacterial cells were obtained, pre-frozen at –20 °C, and freeze-dried to obtain the experimental samples. The dried samples were adhered to the metal sample stage with double-sided tape, and a scanning electron microscope (SU8000, Hitachi Limited, Japan) was used to observe changes in the test bacteria before and after cinnamaldehyde treatment at 5 kV.

2.3 RT-qPCR analysis

The cells were centrifuged after 12 h of microbial growth to harvest them. RNA was extracted according to the method described by Linares et al.[23]. RNA quality was assessed using agarose gel electrophoresis and a micro nucleic acid analyzer. Subsequently, the RNA samples were reverse-transcribed into cDNA using NovaBio HyperScript™ III RT SuperMix for qPCR and gDNA Remover (Bio-Rad) following the manufacturer's instructions. Amplification was conducted using the specific primers listed in Table 1, with the Butler gene 16S rRNA serving as the internal reference[24]. RT-qPCR conditions included an initial denaturation at 95 ℃ for 30 s, followed by 45 cycles of denaturation at 95 °C for 10 s, and annealing extension at 60 °C for 30 s. The melting curve was generated using the default program for the Stratagene MX 3000 sequence inspection system (Agilent Technologies, California, USA). The target gene expression was determined using the $2^{-\Delta\Delta Ct}$ relative quantitative method[25].

Table 1
Primers used in this work.

Gene	Primer	Sequence (5′→3′)	Description	Product length (bp)
speF	*speF*-F *speF*-R	AAGGTGAAAGCGGAAAACGC TCGATGGTTGCTGGAACGAA	ODC	115
adiA	*adiA*-F *adiA*-R	ATCAGCGGTTGGTCGTGAAT TGTGATCGAGCAACGAACCA	Arginine decarboxylase	101
16S rRNA	16S rRNA-F 16S rRNA-R	GTCTGCAACTCGACTCCATGA CTTTTGCAACCCACTCCCATG	Internal reference	121

2.4 Preparation of sausage sample

Xinjiang sausage was prepared following the method of Lu et al.[1]. Raw horse meat (80% lean meat, 20% fat meat, ethanol was applied to the meat surface, burned with fire, and then irradiated under a UV lamp for 30 min) was cut into cubes, and ingredients were added (2.5% salt, 0.01% nitrite, 2.0% white sugar, 0.1% monosodium glutamate, 0.2% ginger powder, 0.1% star anise, 0.1% pepper, 0.15% pepper powder, and 1% liquid smoke), marinated at 4 °C for 1 day, and finally enema to become horsemeat sausage. The sausage-making groups were as follows: group K was the blank group, group R was added with cinnamaldehyde, group O was inoculated with *O. proteus*, and OR group was inoculated with *O. proteus* and cinnamaldehyde. The added concentration of cinnamaldehyde is 0.313%. The amount of *O. proteus* added to the sausages was 1×10^3 CFU/g. Sausage samples were collected at different stages of fermentation (0, 3, 7, 14, and 28 days) to analyze the microbial composition, bacterial diversity, and putrescine.

2.5 Bacterial counts

The microbial counts were determined according to the method described by Lu et al.[1]. Under sterile conditions, scissors were used to cut 10 g of sausage sample. The horsemeat sausage samples were cut into pieces and placed in 90 mL of sterile physiological saline. The mixture was shaken at 200 r/min for 30 min at room temperature to prepare a series of dilutions. MRS medium was used to culture lactic acid bacteria (LAB, 30 °C). MSA medium was used to culture Gram-positive catalase-positive cocci (GCC+, 37 °C), and VRBGA medium was used to culture Enterobacteriaceae (37 °C). These microorganisms were counted after 48 h of culture.

2.6 DNA extraction and sequencing

The TGuide S96 magnetic bead soil/feces genomic DNA extraction kit was used for DNA extraction from horsemeat sausage samples, followed by quality assessment using a micro-UV spectrophotometer. The extracted DNA was required to have a concentration above 20 ng/μL and an $OD_{260\ nm}/OD_{280\ nm}$ value within the range of 1.8−2.0, then stored at –20 °C. Primers targeting the V1–V9 region of the 16S rRNA gene were designed as previously described by Guo et al.[26]. Purification was performed using VAHTSTM DNA Clean Beads magnetic beads before gel cutting and the Monarch DNA gel recovery kit (QIAGEN, Hilden, Germany) after gel cutting, with quantification based on Qubit values for full-length 16S rRNA. The SMRTbell™ template preparation kit (Pacbio, USA) was used for damage repair, end repair, and other processes to create a sequencing library from the mixed product. Subsequently, the library was sequenced on a Sequel II platform (PacBio, USA).

2.7 Determination of free amino acids

The amino acid content of sausages was analyzed using the method outlined by Wang et al.[27]. The samples underwent hydrolysis with 6 mol/L HCl at 110 °C for 24 h. Subsequently, the concentrations of all amino acids in the hydrolyzed solution were determined using Waters liquid chromatography (e2695, USA).

2.8 Determination of putrescine levels

The analysis of putrescine in the sausages was conducted using HPLC, as described by Lu et al.[1]. Sausage samples (5 g) were homogenized in a 20 mL solution of 0.4 mol/L perchloric acid and centrifuged at 4 °C at 5 000 × *g* for 10 min. The supernatant was collected, and the process was repeated thrice. Following filtration, the solution was diluted to 50 mL, and 1 mL of the solution was combined with 200 μL of 2 mol/L NaOH, 300 μL of saturated $NaHCO_3$ buffer, and 2 mL of danoyl chloride (10 mg/mL) for derivatization. The reaction was conducted for 45 s at 40 °C in the dark, after which 100 μL of NH_4OH was added to halt the reaction. The volume was adjusted to 5 mL with acetonitrile. The derivatized solution was filtered using a 0.22 μm filter membrane and transferred to a sample bottle for analysis using the machine.

2.9 Statistical analysis

Statistical Package for the Social Sciences software (version 23, IBM) was used to examine differences between groups, and $P < 0.05$ (ANOVA and Tukey's test) was considered statistically significant. The figures were plotted using Origin 2022 and Adobe Illustrator CC 2015 software.

3. Results and discussion

3.1 Effect of cinnamaldehyde on *O. proteus* growth and putrescine production for 24 h

Fig. 1 illustrated *O. proteus* OP216735 growth and putrescine content alteration throughout 24 h. Cinnamaldehyde inhibited the growth of fertilizer bacteria, as evidenced by the $OD_{600\ nm}$ value of the group containing it being consistently lower ($P < 0.05$) than that of the control group. Moreover, the OD value of *O. proteus* decreased with increasing cinnamaldehyde concentrations. However, cinnamaldehyde did not alter the strain's growth trend. The 5 groups

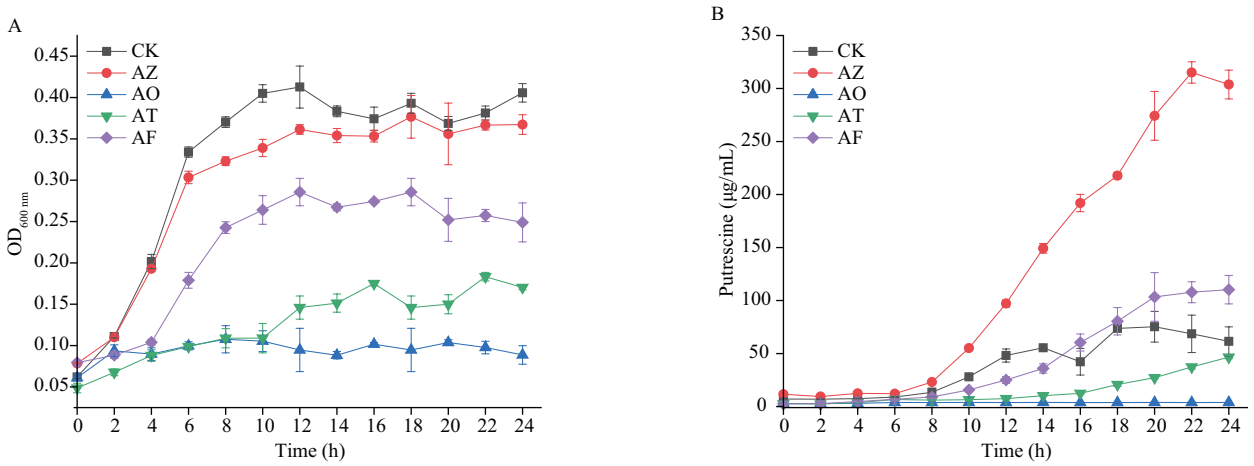

Fig. 1 Effect of cinnamaldehyde on *O. proteus* OP216735 growth (A) and putrescine (B).

underwent 3 phases: lag, logarithmic growth, and stationary. Notably, the bacterial liquid groups (groups AO, AT, and AF) that incorporated cinnamaldehyde differed from the blank and arginine groups (groups CK and AZ) in that their logarithmic growth phase lasted longer, their stationary phase arrived later, and the strain's growth was significantly inhibited. A total of 3 potential antibacterial mechanisms that plant extracts may employ against pathogenic bacteria are thought to be as follows: altering the morphological structures of pathogenic bacteria, like the cell wall, cytoplasm, and membrane; altering the morphological structure of the mycelium to cause mycelium dissolution; and preventing or reducing conidia germination[28-29].

During the first 6 h, putrescine accumulation was low in all 5 samples (Fig. 1B). After 6 h, the putrescine content in all other groups showed an upward trend, except for the AO group. This may be related to the discovery by Del Rio et al.[30] that the regulatory gene of AGDI and the initiator gene in the operon aguBDAC are activated only during the transition period from microbial growth to the log and stable phases. Putrescine production increased with decreasing cinnamaldehyde concentrations. During the 6–24 h period, as the concentration of cinnamaldehyde increased, a gradual decrease in the putrescine accumulation rate and bacterial growth was observed, revealing a possible correlation between microbial growth and putrescine production.

3.2 *Effect of cinnamaldehyde on cell membrane permeability and cell morphology*

β-Galactosidase is an intramembrane substance that is typically retained within the cell membrane. However, in cases of membrane damage, this intracellular enzyme will leak out of the cell. The enzyme activity of *β*-galactosidase in bacterial fluid serves as an indicator of changes in cell membrane permeability. The cell membrane permeability results showed that when treated with cinnamaldehyde, absorbance values of *o*-nitrophenol (ONP) increased in comparison with the untreated group (Fig. 2), with higher values observed with longer exposure times and higher cinnamaldehyde concentrations. This suggested that cinnamaldehyde disrupts the cell membrane permeability of *O. proteus*, leading to the gradual release of intracellular *β*-galactosidase. Previous studies have also reported similar effects of certain plant extracts on bacterial cell membranes. For instance, origanum compactum essential oil has been shown to impact the permeability of *Escherichia coli* and *Bacillus subtilis* membranes, while emodin can enhance the permeability of *Haemophilus parasuis* cell membranes[21,31].

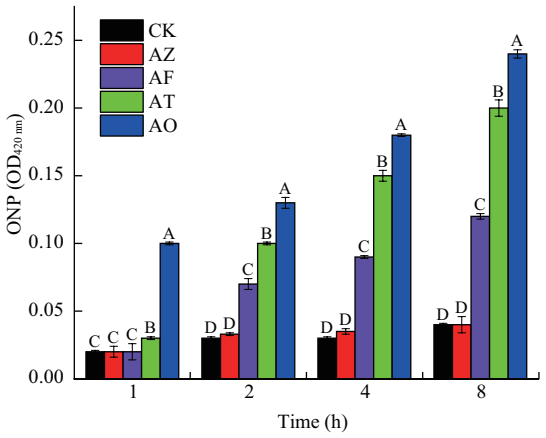

Fig. 2 $OD_{420\ nm}$ values of ONP measured after the action of cinnamaldehyde (mean ± SE). Means followed by different letters in the same time, differ among them at $P < 0.05$.

SEM was used to observe the effects of cinnamaldehyde on cell morphology (Fig. 3). Compared to the control cells with a smooth surface (Fig. 3A), bacteria treated with cinnamaldehyde were damaged (Fig. 3B), with wrinkles on the cell surface, disruption of cell membrane and release of cell contents in *O. proteus*. These results were consistent with Ma et al.[32], who reported that when *E. coli* is exposed to monocaprin, the cytoplasmic disorder is caused by wrinkles on the cell surface, ruptures in the cell membrane, and leakage of the contents of the cell. Using SEM analysis, Zhan et al.[22] found that cinnamaldehyde caused cell shrinkage in *Shigella flexneri*. Ma et al.[32] found that carvacrol did not significantly damage the cell structure of *E. coli* but caused cell membrane blur and cell deformation. This may be due to the different antibacterial mechanisms of plant-active ingredients and different types of microorganisms.

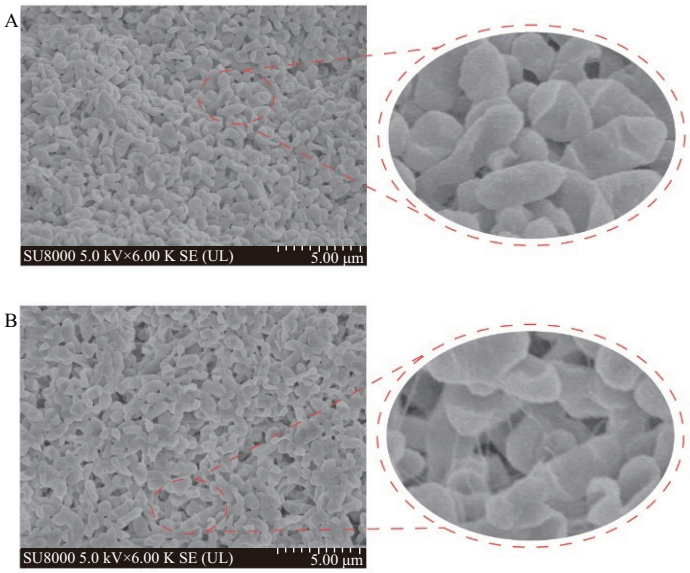

Fig. 3 Effect of cinnamaldehyde on cell morphologies. (A) Untreated bacterial cells; (B) Bacterial cells treated with cinnamaldehyde at MIC.

3.3 Related gene expression

Putrescine is produced by 2 main pathways: 1) AGDI and 2) ODC pathways. The gene tract responsible for this pathway includes the regulatory gene *aguR*, followed by the catabolic genes *aguB*, *aguD*, *aguA*, and *aguC*, which are co-transcribed into multigene mRNA[23]. However, our study exhibited that putrescine in *O. proteus* is mainly produced by the arginine and proline metabolic pathways under the amino acid metabolic pathway (Fig. 4). A total of 9 genes are involved in this pathway, among which *adiA* and *speF* genes are responsible for putrescine production and have the most direct impact on the process. The ODC pathway is the main pathway involved in putrescine production. The transcription of the *speF* and *adiA* genes was significantly ($P < 0.05$) inhibited by increasing concentrations of cinnamaldehyde. The greater the concentration of cinnamaldehyde, the stronger the inhibitory effect. The maximum transcription activity of the *speF* gene was observed in 0% cinnamaldehyde (16.68 times the expression level of MIC cinnamaldehyde group). Similarly, the maximum transcription activity of the *adiA* gene was also observed in 0% cinnamaldehyde (3.54 times the expression level of MIC cinnamaldehyde group).

3.4 Microbial analysis during the fermentation of sausages

The changes in the bacterial counts in each group of sausages during fermentation are presented in Table 2. On day 0, the number of GGC+ cells in group O was lower than that in the other sausage groups, including CK, R, and OR groups ($P < 0.05$). This difference may be attributed to the inoculation of Enterobacteriaceae in group O. Throughout the fermentation, the GGC+ content of sausage depicted an initial increase, followed by a decrease, reaching its peak on the third day. However, on day 28, there was a non-significant difference in the number of GGC+ between groups. The growth trend of LAB during fermentation was similar to that of the GGC+ bacteria. LAB rapidly increased in number and became the dominant microorganism in the early stages of fermentation. It is known that LAB can accelerate the decline in pH, ensuring the safe production of sausages and contributing to flavor formation. At the end of fermentation, there was no significant difference between the 4 groups of sausage samples ($P > 0.05$), indicating that cinnamic aldehyde did not inhibit the growth of LAB. Similar results were reported by Lu et al.[1], Li et al.[33], and Sun et al.[34]. Enterobacteriaceae have high amino acid decarboxylase activity and are prone to produce BAs, like histamine, tyramine, and putrescine[1,10,35]. Throughout the fermentation, the number of Enterobacteriaceae in the sausages of the OR group was lower than that of the O group, indicating that cinnamic aldehyde can effectively inhibit the growth of putrescine-producing microorganisms, including *O. proteus*. Additionally, the number of Enterobacteriaceae in the R group was consistently the lowest among all groups, suggesting that cinnamic aldehyde can impede the reproduction of Enterobacteriaceae and improve fermentation quality and microbial safety during the fermentation of sausage. Sun et al.[34] demonstrated that cinnamon, star anise, and cloves exhibit inhibitory effects on Enterobacteriaceae. Furthermore, Amalaradjou et al.[12] and Guan et al.[14] revealed that cinnamic aldehyde effectively inhibited the growth of *E. coli* and *Listeria*. It has been reported that cinnamaldehyde could induce cell deformation and leakage of cellular content by affecting the cell membrane and bacterial peptidoglycan layer[17,22].

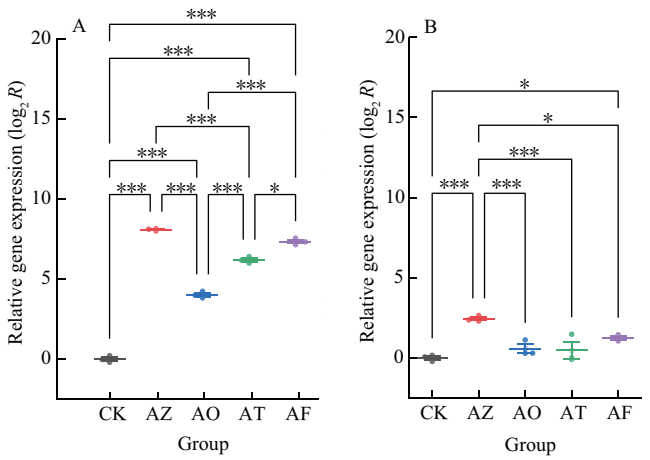

Fig. 4 Effect of cinnamaldehyde on the relative expression level of related genes in *O. proteus*. (A) *speF*, (B) *adiA*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.5 Bacterial community shifts of sausage.

The Shannon diversity index dilution curve (Fig. 5A) reflects the changes in the species diversity of samples as the number of sequencing times increases. The Shannon index was positively correlated with the species type and richness. A steep curve indicates insufficient sequencing time and an undiscovered species. A flat curve suggests no new species in the sample and sufficient data for subsequent analysis. Fig. 5 shows that the Shannon index curves of all samples have stabilized, indicating that most bacterial species information in the samples was detected. The current sequencing data volume and depth met the conditions for bioinformatics analysis.

The cumulative species relative abundance plots (Fig. 5B) illustrate the relationship between the number of samples and annotated species. Each red box represents the total number of species in a sample. As the number of sampled samples increased, all red boxes formed an accumulation curve, indicating the rate at which new species appeared with increasing sample size. This trend was consistent with the changing pattern observed in the Shannon curve. Fig. 5B displays the species accumulation curve, demonstrating that as the number of sequencing samples increased, the red and green boxes of the curve gradually flattened, indicating that species abundance and shared species in the samples tended to reach saturation. This suggests that subsequent community structure analysis can be performed.

The Venn diagram (Fig. 5C) displays that the eight groups of sausage samples shared only 37 operational taxonomic units (OTUs). On day 0, the ZO group had 25 OTUs, which was different from the other groups, possibly because of inoculation with *Proteobacterium*. This resulted in an increase in miscellaneous bacteria in the horsemeat sausage samples. The ZOR group had significantly fewer OTUs than the ZO group, indicating a potential inhibitory effect of cinnamic aldehyde on certain microorganisms like Enterobacteriaceae. Our study is consistent with the findings of Yu et al.[36] and Wang et al.[37], who reported the inhibitory effects of plant extracts like green tea polyphenols and thyme essential oil on microorganisms. On the 28th day, the number of OTUs in all groups was significantly lower than that on day 0, possibly because of the decrease in moisture content during fermentation. To analyze the distribution of bacterial communities at 0 and 28 days, β-diversity assessment was performed based on the abundance of OTUs (Fig. 5D). The different groups of sausages at the 2 fermentation times (0 and 28 days) were divided into 8 groups. The bacterial communities in each group during the various periods showed relatively little similarity.

Table 2
Microbial counts (lg (CFU/g)) during ripening of sausage.

Bacteria	Batches	Time (day)				
		0	3	7	14	28
GGC+	CK	4.72 ± 0.91^{Bd}	6.06 ± 0.29^{Ba}	5.61 ± 0.33^{Ab}	5.08 ± 0.04^{Ac}	4.96 ± 0.13^{Acd}
	R	4.80 ± 0.05^{Bbc}	5.37 ± 0.34^{Ca}	5.31 ± 0.08^{Ca}	5.11 ± 0.29^{Aab}	5.02 ± 0.01^{Ab}
	O	4.49 ± 1.03^{Cd}	6.11 ± 0.36^{Ba}	5.46 ± 0.51^{Bb}	4.48 ± 0.31^{Bd}	4.55 ± 0.02^{Ac}
	OR	5.10 ± 0.88^{Ad}	6.62 ± 0.58^{Aa}	5.59 ± 0.30^{Ab}	4.85 ± 0.20^{Ae}	5.07 ± 0.27^{Ad}
LAB	CK	3.88 ± 0.12^{Bc}	4.70 ± 0.01^{Ca}	3.74 ± 0.14^{ABc}	3.99 ± 0.01^{Abc}	4.26 ± 0.24^{Ab}
	R	3.68 ± 0.05^{Bc}	5.55 ± 0.02^{Aa}	3.50 ± 0.03^{Bd}	3.66 ± 0.38^{Ac}	4.33 ± 0.03^{Ab}
	O	4.40 ± 0.28^{Aa}	5.01 ± 0.16^{Ba}	4.36 ± 0.55^{Aa}	3.20 ± 0.36^{Bb}	4.48 ± 0.05^{Aa}
	OR	4.71 ± 0.02^{Ab}	5.18 ± 0.05^{Ba}	4.04 ± 0.03^{ABd}	3.79 ± 0.03^{Ae}	4.56 ± 0.01^{Ac}
Enterobacteriaceae	CK	3.34 ± 0.04^{Aa}	3.01 ± 0.04^{Cb}	2.83 ± 0.48^{Bc}	2.20 ± 0.17^{Bd}	1.57 ± 0.04^{Be}
	R	3.16 ± 0.13^{Aa}	2.98 ± 0.07^{Cb}	2.72 ± 0.40^{Bb}	2.08 ± 0.34^{Cc}	1.56 ± 0.07^{Bd}
	O	3.73 ± 0.39^{Aa}	4.18 ± 0.28^{Ab}	3.77 ± 0.22^{Aa}	2.87 ± 0.15^{Ac}	2.65 ± 0.38^{Ad}
	OR	3.48 ± 0.36^{Aa}	3.47 ± 1.26^{Ba}	3.20 ± 0.03^{Bb}	2.10 ± 0.32^{Bc}	2.51 ± 0.12^{Ab}

Note: Means followed by different lowercase letters in the same sausage group, differ among them at $P < 0.05$. Means followed by different uppercase letters in the same storage time, differ among them at $P < 0.05$. The results were expressed as mean ± SE.

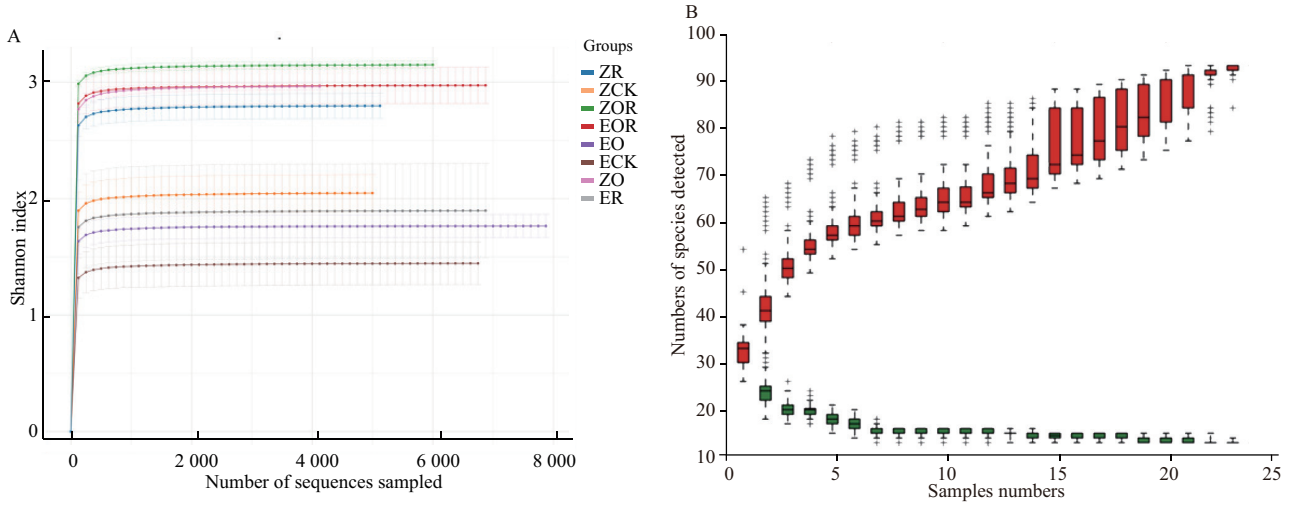

Fig. 5 Shannon index curve (A), species accumulation curve (B), Venn diagram (C), and principal coordinate analysis (D) of bacterial composition in sausage at 0 (Z) and 28 (E) days.

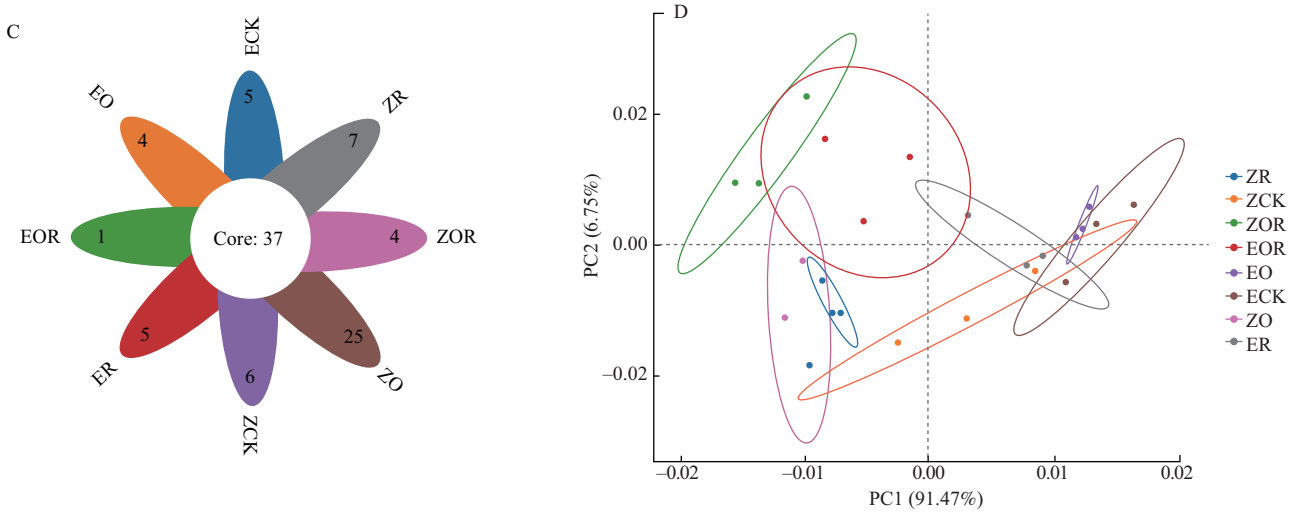

Fig. 5 (*Continued*)

3.6 Compositional analysis of bacterial communities in sausages

Throughout the sausage fermentation period (Fig. 6), Firmicutes and Proteobacteria were the most dominant microbial groups. The relative abundance of Firmicutes gradually increased as fermentation progressed, whereas the relative abundance of Proteobacteria decreased with an increase in Firmicutes. In the ZO group, a small amount of Bacteroidota was observed, which aligns with the findings of the Venn diagram. This observation could be attributed to infection by other bacteria during inoculation with Proteobacterium. Other phyla were rare or not observed in the other 3 groups.

At the species level (Fig. 6B), bacterial species, such as *Staphylococcus xylosus*, *Pseudomonas psychrophila*, *Lactococcus garvieae*, *L. lactis*, *Pseudomonas putida*, *Weissella hellenica*, *Macrococcus caseolyticus*, *Psychrobacter alimentarius* and *O. proteus* were identified (relative abundances were > 5% of total bacteria). On day 0, the dominant bacterial genus in the sausage was *Pseudomonas*, which included *Pseudomonas psychrophilic* and *P. putida*. These bacteria are known as the main psychrophilic spoilage bacteria in meat, which are stored aerobically and originate from the meat processing environment, like soil or water[38]. Moreover, *Pseudomonas* can gain an ecological advantage by penetrating meat through proteolytic activity, allowing it to utilize newly available resources and establish new ecological niches[39]. The abundance of *Lactococcus*, including *L. lactis* and *L. garvieae*, revealed a decreasing trend from day 0 to day 28, consistent with the results of Hu et al.[40]. Bacteriocins produced by *Lactococcus* can inhibit many Gram-positive foodborne pathogens in meat products, which may help maintain the stability of bacterial communities[41]. On day 28, *S. xylosus* was the dominant strain in the sausage. *Staphylococcus* was also found to be the main dominant strain in salami, accounting for 97.45% of the total abundance[42]. *S. xylosus* can promote product color formation and stability through its nitrate reductase activity and product flavor formation through protease and lipase activities[43]. The abundance of *W. hellenica* presented an increasing trend from day 0 to day 28, consistent with the findings of Huang et al.[44]. The flavor-promoting effect of *Weissella* is reflected in its ability to hydrolyze proteins in the matrix and produce small-molecule flavor compounds[45]. *O. proteus* is a strain with amino acid decarboxylase activity. The proportion of *O. proteus* in the ZO group was higher than in the other groups (ZR, ZCK, and ZOR), which aligns with the results of microbial counting. Additionally, on day 28, the abundance of *S. xylosus* and *W. hellenica* in the groups with added cinnamaldehyde (ER and EOR) was higher than that in the 2 groups that did not contain cinnamaldehyde (EO and ECK). These 2 bacteria are crucial for flavor production, indicating that cinnamic aldehyde promotes flavor development in fermented meat products to some extent. Furthermore, the groups (ER and EOR) that were supplemented with cinnamaldehyde had lower levels of *Pseudomonas psychrotrophies*, *P. putida*, and *O. proteus*, suggesting that cinnamaldehyde has an inhibitory effect on the growth of these bacteria. Huang et al.[44] also discovered that thyme essential oil can significantly reduce the number of Enterobacteriaceae bacteria through high-throughput sequencing.

3.7 Analysis of free amino acids

Table 3 provides a detailed account of the fluctuations in free amino acid levels during sausage maturation. Initially, there were no significant differences in amino acid content among the 4 groups. However, by the 28th day, a notable distinction in amino acid content was observed ($P < 0.05$) across the groups. The CK batch exhibited the highest total free amino acid content, while the O batch had the lowest. Aspartic acid, glutamic acid, glycine, alanine, and leucine emerged as the most abundant amino acids in sausages, playing a crucial role in augmenting the overall amino acid content. The increase in amino acid content during the ripening process may be attributed to microbial enzyme activity in the later stages of sausage processing, leading to substantial amino acid synthesis[46]. Ruiz et al.[47] suggested that fluctuations in free amino acid concentrations are primarily influenced by chemical and enzymatic reactions, particularly the amino acid decarboxylase activity of surviving microorganisms. In the EO group, a decrease in arginine content and an increase in

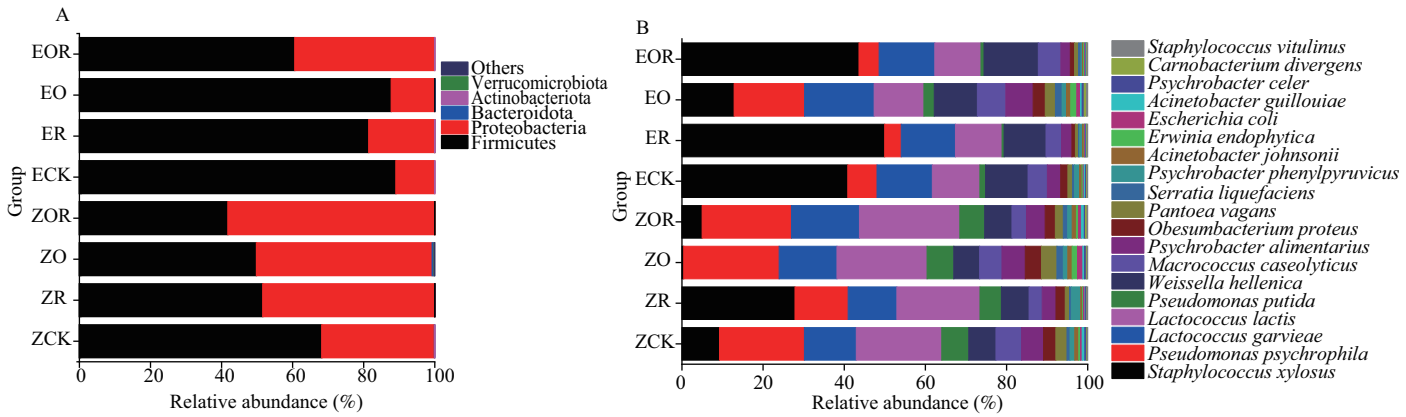

Fig. 6 Relative abundance of bacterial compositions at the phylum level (A) and species levels (B) in sausage at 0 and 28 days.

Table 3
Changes in free amino acids (mg/100 g) of sausage at 0 and 28 days.

Free amino acids	ZCK	ZR	ZO	ZOR	ECK	ER	EO	EOR
Asp	32.72 ± 0.03^{Ab}	33.59 ± 0.06^{Ab}	35.02 ± 0.81^{Ab}	33.31 ± 0.12^{Ab}	42.54 ± 0.75^{Ca}	48.96 ± 0.37^{Aa}	39.60 ± 0.18^{Da}	44.76 ± 0.91^{Ba}
Glu	51.46 ± 0.02^{Ab}	52.50 ± 0.07^{Ab}	51.93 ± 0.14^{Ab}	53.38 ± 0.06^{Ab}	69.77 ± 0.53^{Ba}	79.04 ± 0.28^{Aa}	64.77 ± 0.29^{Ca}	74.73 ± 0.88^{Aa}
Ser	16.71 ± 0.23^{Ab}	15.48 ± 0.02^{Ab}	17.84 ± 0.21^{Ab}	17.74 ± 0.05^{Ab}	30.21 ± 0.61^{ABa}	32.08 ± 0.32^{Aa}	27.16 ± 0.40^{BCa}	30.81 ± 0.24^{Aa}
Gly	26.92 ± 0.35^{Ab}	26.01 ± 0.05^{Ab}	27.18 ± 0.21^{Ab}	27.67 ± 0.03^{Ab}	57.40 ± 0.42^{ABa}	53.83 ± 0.27^{BCa}	44.15 ± 0.12^{Da}	52.20 ± 0.06^{Ca}
His	14.21 ± 0.27^{Ab}	14.15 ± 0.03^{Ab}	15.18 ± 0.02^{Ab}	15.99 ± 0.03^{Ab}	22.57 ± 0.31^{ABa}	24.43 ± 0.02^{Aa}	20.21 ± 0.10^{BCa}	23.44 ± 0.15^{Aa}
Arg	18.91 ± 0.12^{Ab}	19.61 ± 0.02^{Ab}	20.41 ± 0.22^{Ab}	19.61 ± 0.01^{Ab}	29.90 ± 0.22^{ABa}	33.16 ± 0.10^{Aa}	26.94 ± 0.18^{BCa}	30.17 ± 0.27^{ABa}
Ala	27.21 ± 0.11^{Ab}	26.63 ± 0.02^{Ab}	28.92 ± 0.26^{Ab}	28.93 ± 0.02^{Ab}	52.32 ± 0.53^{Aa}	55.64 ± 0.10^{Aa}	45.36 ± 0.47^{Ba}	52.06 ± 0.47^{Aa}
Pro	19.23 ± 0.22^{Ab}	19.21 ± 0.32^{Ab}	20.69 ± 0.21^{Ab}	22.81 ± 0.04^{Ab}	35.95 ± 0.97^{Aa}	32.25 ± 0.97^{ABa}	27.20 ± 0.08^{Ca}	32.00 ± 0.71^{BDa}
Tyr	10.17 ± 0.28^{Ab}	10.76 ± 0.03^{Ab}	11.41 ± 0.12^{Ab}	10.73 ± 0.01^{Ab}	17.52 ± 0.29^{Aa}	18.64 ± 0.21^{Aa}	14.90 ± 0.09^{BCa}	16.55 ± 0.27^{ABa}
Val	16.29 ± 0.17^{Ab}	16.67 ± 0.01^{Ab}	18.48 ± 0.13^{Ab}	18.30 ± 0.04^{Ab}	31.50 ± 0.57^{BCa}	36.30 ± 0.61^{Aa}	28.55 ± 0.16^{CDa}	30.41 ± 0.52^{BCa}
Met	5.34 ± 0.26^{Ab}	5.25 ± 0.04^{Ab}	5.66 ± 0.02^{Ab}	5.53 ± 0.01^{Ab}	12.45 ± 0.35^{Ba}	16.61 ± 0.01^{Aa}	9.18 ± 0.04^{CDa}	11.64 ± 0.09^{BCa}
Cys	4.88 ± 0.19^{ABb}	4.61 ± 0.09^{Ba}	5.07 ± 0.02^{Aa}	5.01 ± 0.01^{Aab}	6.78 ± 0.18^{Aa}	3.95 ± 0.01^{Db}	5.05 ± 0.01^{Ca}	5.92 ± 0.02^{Ba}
Ile	16.17 ± 0.04^{Ab}	16.42 ± 0.09^{Ab}	17.66 ± 0.21^{Ab}	17.57 ± 0.06^{Ab}	32.89 ± 0.07^{Ba}	35.24 ± 0.75^{Aa}	28.72 ± 0.11^{BCa}	32.13 ± 0.72^{ABa}
Leu	28.31 ± 0.05^{Ab}	27.73 ± 0.10^{Ab}	29.78 ± 0.36^{Ab}	30.62 ± 0.04^{Ab}	57.81 ± 0.41^{Ba}	59.27 ± 2.80^{Aa}	49.84 ± 0.11^{BCa}	56.75 ± 0.29^{ABa}
Thr	41.24 ± 0.03^{Ab}	39.46 ± 0.05^{Ab}	39.49 ± 0.29^{Ab}	36.91 ± 0.02^{Aab}	38.66 ± 0.97^{Ba}	46.02 ± 0.03^{Aa}	46.84 ± 0.20^{Aa}	39.19 ± 0.55^{Ba}
Phe	11.21 ± 0.03^{Ab}	10.77 ± 0.06^{Ab}	11.92 ± 0.15^{Ab}	11.99 ± 0.01^{Ab}	20.96 ± 0.12^{BCa}	22.82 ± 0.14^{ABa}	18.48 ± 0.15^{DEa}	20.43 ± 0.09^{CDa}
Lys	18.15 ± 0.23^{Ab}	19.49 ± 0.02^{Aa}	20.16 ± 0.15^{Aa}	20.06 ± 0.05^{Aa}	21.74 ± 0.31^{Aa}	18.69 ± 0.01^{Bb}	ND	21.83 ± 0.29^{Aa}

Note: Means followed by different lowercase letters in the same sausage group, differ among them at $P < 0.05$. Means followed by different uppercase letters in the same storage time, differ among them at $P < 0.05$. ND, not detected. The results were expressed as mean ± SE.

putrescine content were observed, with putrescine production linked to the arginine and proline metabolism pathways within the amino acid metabolism pathway. This outcome may be due to inoculation with *O. proteus*, which displays the highest amino acid decarboxylase activity to convert arginine to putrescine[48]. In addition, it is worth noting that the amino acid content of the group with added cinnamaldehyde was generally higher than that of the group without added cinnamaldehyde, indicating that cinnamaldehyde partially enhanced protein hydrolysis and promoted the release of amino acids.

3.8 Putrescine analysis

Putrescine, a compound found in fermented meat products, is primarily produced through microbial decarboxylation. During storage and sales, the putrescine content of these products tends to increase[49]. Excessive consumption of putrescine can lead to symptoms like arrhythmia and hypotension. Additionally, putrescine can enhance the toxic effects of histamine and tyramine[50]. Studies conducted in mouse models have demonstrated that a high dietary intake of putrescine can increase the malignancy of adenomas. Furthermore, elevated concentrations of putrescine have been observed in gastric cancer cases associated with *Helicobacter pylori* infection[51]. The putrescine contents of the four sausage groups are shown in Fig. 7. Initially, on day 0, lower concentrations of putrescine were observed among the 4 groups, and non-significant differences were found between the groups. However, on the third day, the group (O and OR) inoculated with *O. proteus* revealed a rapid increase in putrescine content. By the 14th day, the putrescine content in each group had reached its maximum value, and there was a significant difference between the groups. The accumulation of putrescine continued to increase as the fermentation progressed. On the 28th day, the content of putrescine in horsemeat sausage with cinnamaldehyde (batch R) was markedly lower ($P < 0.05$) (28.49 mg/kg) than in other batches (CK, O, and OR). This indicated that cinnamaldehyde as a plant extract in batch R significantly ($P < 0.05$) reduced putrescine accumulation in batch O by 44.15%. The above situation can be

attributed to cinnamaldehyde's effective inhibition of putrescine-producing microorganisms, particularly *O. proteus*. Our findings align with previous studies, demonstrating plant extracts notable impact in reducing BAs in fermented products[1,52].

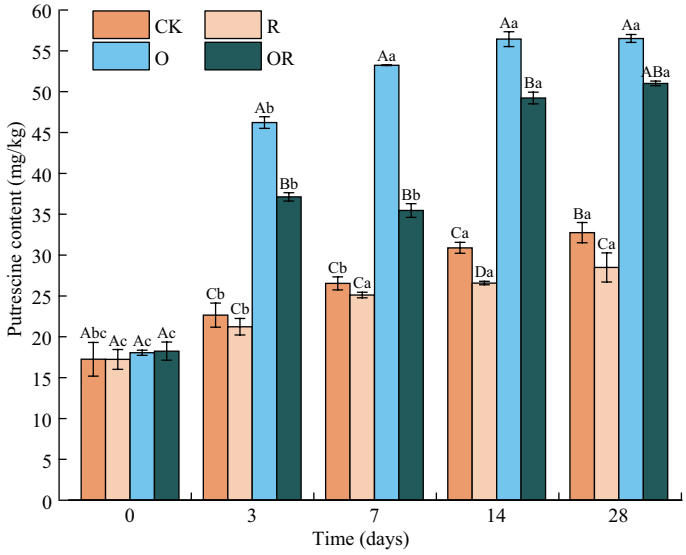

Fig. 7 Effect of cinnamaldehyde on putrescine accumulation in sausage during fermentation and ripening (mean ± SE). Means followed by different lowercase letters in the same sausage group, differ among them at $P < 0.05$. Means followed by different uppercase letters in the same storage time, differ among them at $P < 0.05$.

4. Conclusions

This study demonstrated that cinnamaldehyde could reduce putrescine accumulation by leaking the cell contents, changing membrane permeability of *O. proteus* and inhibiting its growth. Putrescine accumulation is linked to putrescine-generating microbes and associated with the expression of *speF* and *adiA* genes of the arginine and proline metabolic pathways under the amino acid metabolic pathway. Cinnamaldehyde significantly ($P < 0.05$) inhibited the expression of *speF* and *adiA* genes, and the greater the cinnamaldehyde concentration, the more significant the inhibitory effect. The amount was reduced by 83% and 44.15% in the pure culture and sausage, respectively. Cinnamaldehyde reduced putrescine accumulation in sausages by inhibiting the growth of strains (Enterobacteriaceae) and the relative abundances of Enterobacteriaceae and *Pseudomonas*. Furthermore, cinnamaldehyde also promoted the release of free amino acids. Consequently, adding a certain amount of cinnamaldehyde to sausages can significantly improve product safety.

Declaration of competing interest

The authors declare no conflict of interest.

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