



Partial replacement of nitrite with a novel probiotic *Lactobacillus plantarum* on nitrate, color, biogenic amines and gel properties of Chinese fermented sausages

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Myoglobin (Pumbchem CID 102445807)
Tyramine (Pumbchem CID 610)
Histamine (Pumbchem CID 774)
Sodium chloride (Pumbchem CID 5234)
Phenylalanine (Pumbchem CID 6140)
Trichloroacetic acid (Pumbchem CID 6421)
Acetonitrile (Pumbchem CID 6342)
Sodium thiosulfate (Pumbchem CID 24477)

ABSTRACT

This article explored the positive effects of partial replacement of sodium nitrite by *Lactobacillus plantarum* on reducing nitrite and biogenic amine content, improving color and gel structure of Chinese fermented sausages. The results indicated that the strain had nitrate and nitrite reductase activities, which can reduce nitrate and nitrite. And the final reduction products of nitric oxide can react with myoglobin to generate pink nitrosylmyoglobin (Mb(Fe²⁺)-NO), which can improve sausage color. Dynamic rheological test showed that the strain improved viscoelasticity and gel properties of the samples. Low field nuclear magnetic resonance (LF-NMR) measurement illustrated that the strain enhanced immobilised water in myofibrillar network of the sausages. The protein gels were improved by changes of C-H stretching and bending vibrations, reduction of α -helix and increase of β -sheet and random coil in secondary structure, changes of microenvironment in tertiary structure. Analysis of biogenic amines showed that the strain reduced risk from biogenic amines by reducing tyramine content. Overall, our findings demonstrated that combination of *L. plantarum* and low levels of sodium nitrite could be a potential strategy to produce high-quality, healthier sausage by lowering nitrite and biogenic amine levels, improve color and gel properties of Chinese fermented sausages.

1. Introduction

Chinese fermented sausages are typical meat products in China for their unique taste, flavor and relatively long shelf life. At present, most sausages are currently processed by natural fermentation, which is more susceptible to the growth of pathogen (Gao, Li, & Liu, 2014). Therefore, in order to prevent sausage corruption, adding preservatives are considered. Among them, sodium nitrite is the common used additive in meat products. Sodium nitrite can positively improve color, inhibit spoilage of fermented sausages (Aquilani et al., 2018). However, sodium nitrite poses a threat to human body health for it could form carcinogenic nitrosamines (Kim et al., 2017). Many studies focused on nitrite reduction or alternatives (Kim et al., 2017; Sun, Kong, Chen, Han, & Diao, 2017). However, it is difficult to develop alternatives that both have color cured and spoilage inhibition effects. In recent years, studies have shown that some microorganism have color cured and

antiseptic properties. Götterup et al. (2007) reported that *staphylococci* had nitrite reductase activities, which led to the formation of pink nitrosylmyoglobin (Mb(Fe²⁺)-NO) eventually. Li, Luo, Kong, Liu, and Chen (2016) showed that inoculation of lactic acid bacteria and *Staphylococcus xylosus* as nitrite substitution could form red myoglobin derivatives and inhibit spoilage bacteria in raw meat batters. Luo et al. (2013) indicated that nitrate oxide synthase (NOS) and metmyoglobin (Met(Fe³⁺)-Mb) reductase were observed in *Lactobacillus salivarius* H strain, which had a beneficial effect on the color of fresh pork.

The previous studies commonly worked on the impact of nitrite biological substitute on color cured and pathogenic bacteria inhibition in fermented sausages (Gao et al., 2014; Gou, Liu, & Qu, 2019; Slima et al., 2017). Gao et al. (2014) discussed effect of *L. sakei* C2 as starter culture on the microbiological index, lipid oxidation, nitrite, flavor and color of fermented sausages. The results showed that *L. sakei* C2 increased amount of Mb(Fe²⁺)-NO and had potential to be used in

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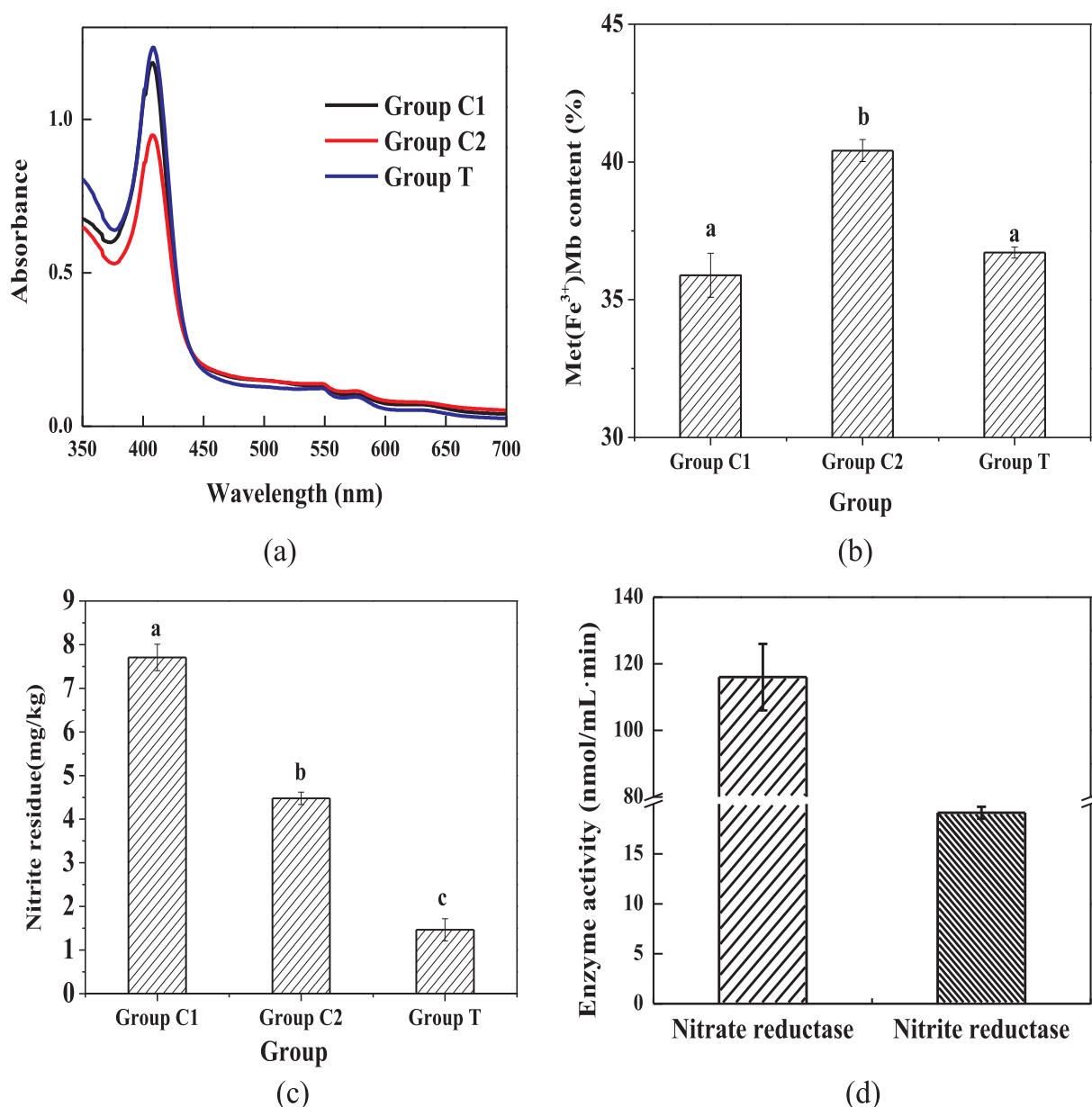


Fig. 1. Visible absorption spectra of extracts (a), Met(Fe³⁺)-Mb content (b), and nitrite residual of the three group sausages (c); activity of nitrate reductase and nitrite reductase of the strains (d).

fermented sausages. Slima et al. (2017) assessed color, physico-chemical and microbiological properties of beef sausages with *L. plantarum* TN8 partial replacing nitrite and indicated that incorporation of strain TN8 in beef sausage was benefit for healthy sausage production by lowering nitrite levels. Gou et al. (2019) studied chromogenic mechanism of LAB and proved that the strain *W. cibaria* X31 had the strongest NO-producing capability, which indicated that *W. cibaria* X31 had potential to substrate nitrite. But few were focused on the effect of the alternatives on capability of nitrate reduction, biogenic amines content and gel properties of the sausages.

The target of this study was to assess the positive effect of *L. plantarum* on nitrate and biogenic amines content reduction, color and gel properties improvement of fermented sausages. Mb(Fe²⁺)-NO formation, nitrate and nitrite reductase activities, nitrite residue, Met(Fe³⁺)-Mb content, dynamic rheological characteristics, water distribution, myofibrillar protein structures and biogenic amines of the fermented sausages substituting 50% sodium nitrite with *L. plantarum* inoculation were surveyed.

2. Materials and methods

2.1. Strains

L. plantarum with chromogenic and pathogenic bacteria inhibition capacity was isolated from Chinese fermented sausages by our team in 2018 and was preserved in China General Microbiological Culture Collection Center (certificate number: CGMCC NO. 161310) and Fermentation Engineering Laboratory of Qingdao Agricultural University.

2.2. Sausage manufacture

The *L. plantarum* cells were cultured in MRS broth at 37 °C for 24 h and centrifuged at 8000g for 10 min, at 4 °C. The collected cells were suspended in phosphate buffer (0.1 M, pH 7) and washed with shaking for 5 min. Then the cells were harvested through centrifugation (8000g, 10 min, 4 °C) and continued to wash twice for later use. The

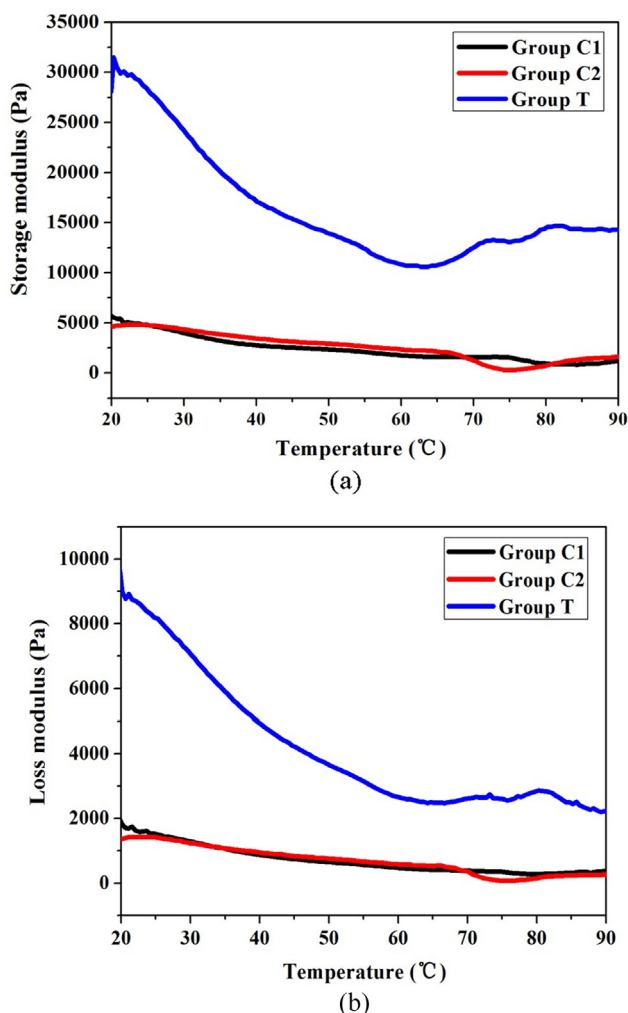


Fig. 2. Storage modulus G' (a) and loss modulus G'' (b) curves of sausages in the dynamic frequency test.

formulation of the three group sausages were as follows: Group C1 presented the control 1 comprised 900 g/kg lean pork, 100 g/kg pork back fat, 23 g/kg sodium chloride and 100 mg/kg NaNO_2 , Group C2 presented the control 2 comprised 900 g/kg lean pork, 100 g/kg pork back fat, 23 g/kg sodium chloride and 50 mg/kg NaNO_2 , Group T presented inoculated group comprised 900 g/kg lean pork, 100 g/kg pork back fat, 23 g/kg sodium chloride, 50 mg/kg NaNO_2 and about $7 \log \text{CFU/g}$ of pork with *L. plantarum* inoculation. After thoroughly mixing, all the raw materials were cured at 4 °C for 24 h and then stuffed into cellulose casings 50 g per casing (about 4 cm in diameter). The followed process involved being fermented at 37 °C about for 3 h until pH dropped below 5.3 with a RH between 90 and 95%, and then ripened at 10 °C with a RH between 70 and 75% for 20 days.

2.3. $\text{Mb(Fe}^{2+})\text{-NO}$ analysis

The presence of $\text{Mb(Fe}^{2+})\text{-NO}$ was conducted according to the method described by Gao et al. (2014) with slight modifications. The minced sausage sample (10 g) was homogenized in phosphate buffer (pH 6.0, 0.02 M) by a beating homogenizer for 2 min. After being left in dark room at 4 °C for 1 h, the homogenate was centrifuged at 6000g for 10 min and then the supernatant was filtered by passing them through nitrocellulose membrane (0.22 μm). The filtrate was measured from 350 nm to 700 nm with a UV-Vis spectrophotometer (TU-1810, Beijing Purkinj General Instrument Co., LTD., China).

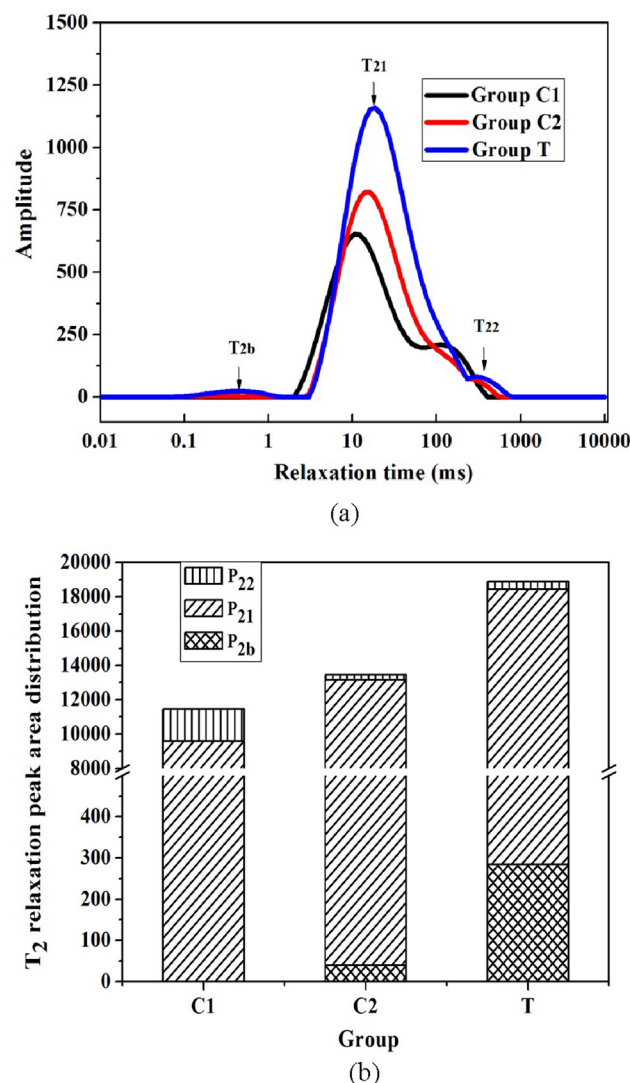


Fig. 3. Distribution of T_2 (a) and water populations of three components (b) in the three group sausages.

2.4. Nitrite residual

The samples (10 g) was minced and mixed with 25 mL of saturated borax solution, then pounded in mortar. Nitrite residual was measured using UV spectrophotometry with hydrochloride naphthodiamide according to previous report (Zhang, Kong, & Xiong, 2007).

2.5. Nitrate and nitrite reductase activity

Nitrate and nitrite reductase activity was measured according to the reports of Götterup et al. (2007) with slight modifications in cell concentration. Nitrate reductase was determined by the accumulation of nitrite in the system. The reaction system is 6 mL, containing 100 mM phosphate buffer (pH 7.0), 1.0 mM benzyl viologen dichloride, 0.4 mM NaNO_3 and 0.1 mL cell suspension ($\text{OD}_{600} = 1.6$). Finally, 4.7 mM sodium thiosulfate was added until full discoloration of the blue reduced benzyl viologen was observed through assay. After centrifugation (8000g, 3 min, 4 °C), the supernatant (0.1 mL) was mixed with Griess reagent (0.9 mL) and reacted for 10 min, and the absorbance at 540 nm was measured through UV-Vis spectrophotometer (TU-1810, Beijing Purkinj General Instrument Co., LTD., China). Nitrate reductase activity was expressed as nmol per min per ml viable cells by calculation of absorbance change.

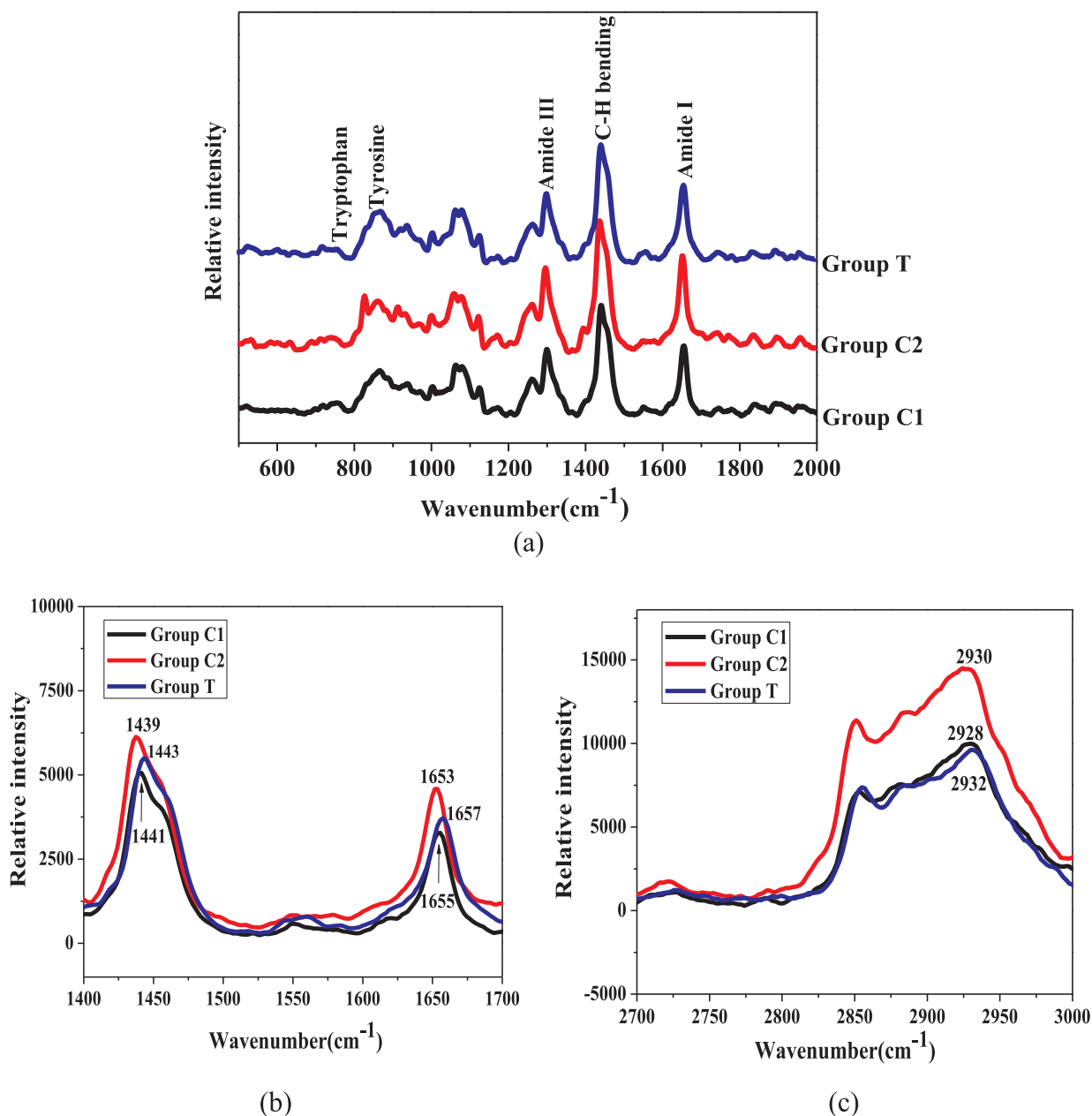


Fig. 4. Raman spectrum of the three groups in the region 500–2000 cm^{-1} (a), in the region 1400–1700 cm^{-1} (b) and in the region 2700–3000 cm^{-1} (c).

Nitrite reductase was detected by nitrite degradation in the reaction system (6 mL) including 100 mM phosphate buffer (pH 7.0), 25 mM glucose, 1.7% Oxyrase (v/v) and 0.1 mL cell suspension ($\text{OD}_{600} = 1.6$). The reaction was initiated by the addition of 0.4 mM NaNO_2 . After reacting with Griess reagent for 20 min, the absorbance at 540 nm was measured through UV-Vis spectrophotometer (TU-1810, Beijing Purkinj General Instrument Co., LTD., China). Nitrite reductase activity was expressed as nmol per min per ml viable cells by calculation of absorbance change.

2.6. $\text{Met(Fe}^{3+}\text{)-Mb}$ analysis

$\text{Met(Fe}^{3+}\text{)-Mb}$ content was measured according to the method of previous report (Luo et al., 2013) with minor modifications. Minced samples (10 g) were dispersed in 80 mL phosphate buffer (pH 6.8, 40 mM) and grinded in a mortar. The homogenate was sealed and placed in dark room at 4 °C for 1 h and then centrifuged at 5000g for

20 min. The supernatant was harvested and filtered through nitrocellulose membrane (0.22 μm). The absorbance of the filtrate was measured at 572, 562, 545 nm and 525 nm with a UV-Vis spectrophotometer (TU-1810, Beijing Purkinj General Instrument Co., LTD., China). The $\text{Met(Fe}^{3+}\text{)-Mb}$ percentage was calculated with the formula below:

$$\% \text{MetMb} = \left\{ -2.51 \left(\frac{A_{572}}{A_{525}} \right) + 0.777 \left(\frac{A_{562}}{A_{525}} \right) + 0.8(v) + 1.098 \right\} \times 100$$

2.7. Dynamic rheological test

Dynamic rheological characteristics were measured with a rotational rheometer (MCR102, Anton Paar, Austria) according to the report (Sun, Chen, Liu, Li, & Yu, 2015) with some changes. The sausage sample with 1 mm thickness was placed between the two plates. The slit distance was 1 mm and sealed with silicon oil. The dynamic rheological

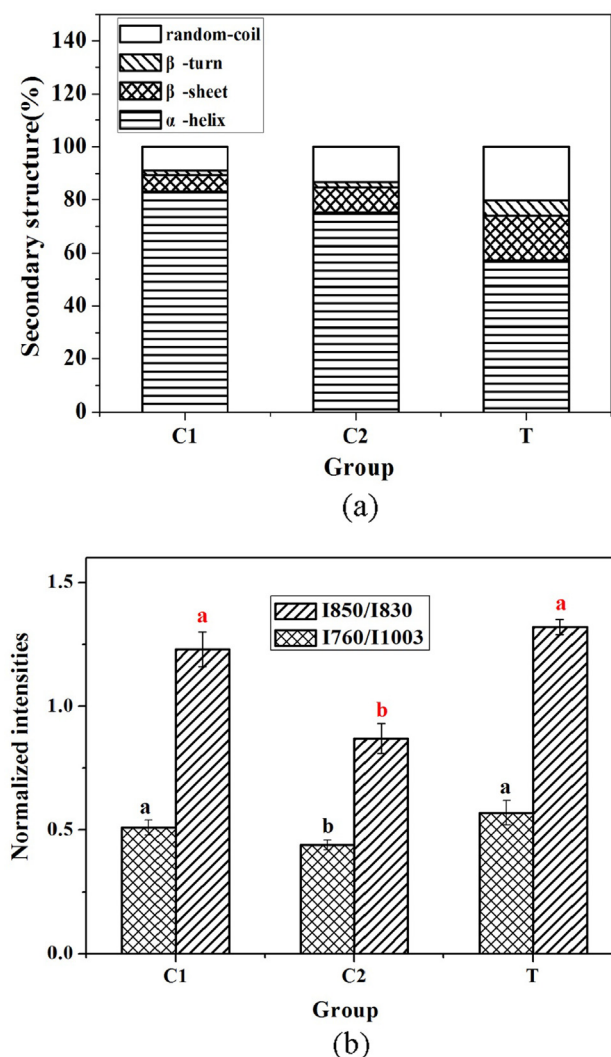


Fig. 5. Secondary structure components of gel proteins calculated from the amide I band (a) and normalized intensities of tryptophan and tyrosine residues (b).

characteristics were measured from the initial temperature of 20 °C up to 90 °C at a heating rate of 5 °C · min⁻¹ and frequency was set to 1 Hz. The corresponding storage modulus (G') and the loss modulus (G'') were recorded respectively.

2.8. Low field nuclear magnetic resonance (LF-NMR) measurement

LF-NMR measurement was according to Guo Liping et al. (2019) and cylindrical sausage with diameter of 2 cm and thickness of 3 cm cut from each sample was placed in a test tube (Volume 40 mL). Then the tube was inserted in a NMR analyzer (NM120-040V-1, Niumag Analytical Instrument Corporation, Suzhou, China). The setting parameters were: resonance frequency was 22 MHz, a series of echoes were 3000 ms and repetition accumulations were 16 scans. Three relaxation times (T_{2b} , T_{21} and T_{22}) and water populations (P_{2b} , P_{21} and P_{22}) were recorded.

2.9. Raman spectroscopic analysis

The Raman spectrum was measured on a Raman Spectrometer (DXR2xi, Thermo Fisher Scientific, Madison, America). The sausage slice with 0.5 mm in thickness was placed on a concave slide and then placed in a Raman Spectrometer. A 785 laser transmitter was used and

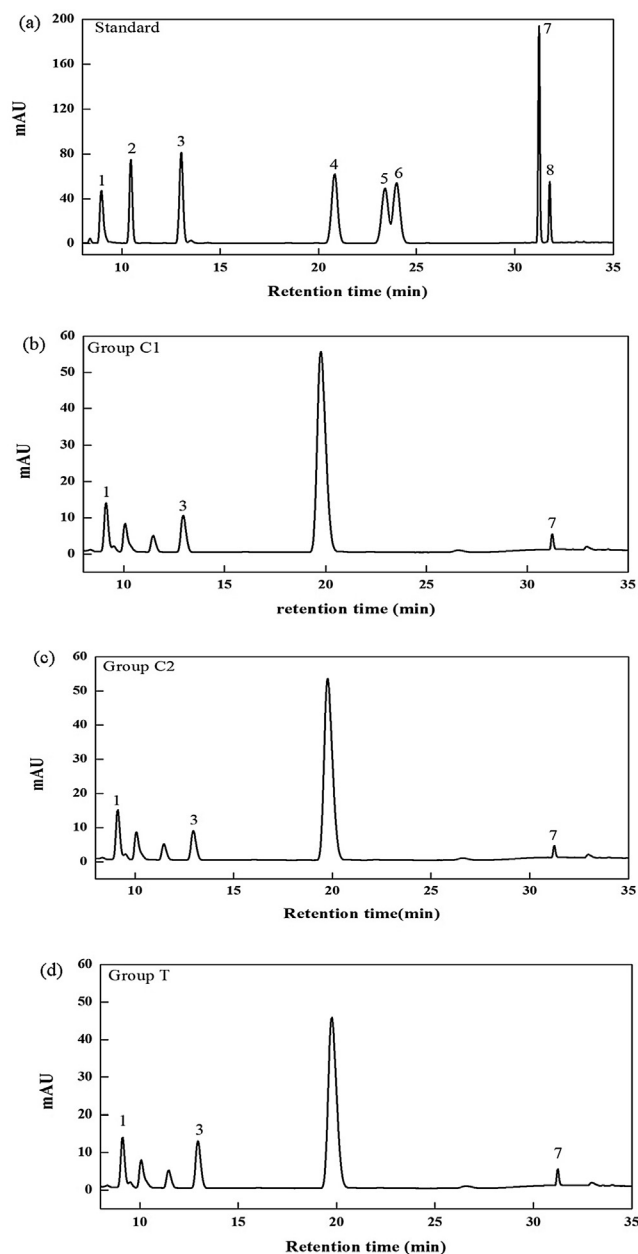


Fig. 6. HPLC chromatograms of standard mixture (a), sample of group C1 (b), sample of group C2 (c), sample of group T (d). Eight kinds of biogenic amines have been marked in Fig. 6a. and the Numbers from 1 to 8 represented Tyramine, Putrescine, Cadaverine, Spermidine, Tryptamine, Phenylethylamine, Spermine and histamine respectively.

the measurement parameters were set as follows: laser power 21.5 mW, exposure time 1.0000 sec, and scanning times 10,000. Baseline correction and normalization of the spectrum to phenylalanine band about 1003 cm⁻¹ were implemented. The secondary structures of muscle proteins were investigated with percentages of α -helix, β -sheet, β -turn and random coil according to Nawrocka et al. (2017).

2.10. Analysis of biogenic amines by high performance liquid chromatography (HPLC) analysis

The extraction and followed derivatization of biogenic amines were carried out as described by Nie, Zhang, and Lin (2014) with minor changes. Minced sausage sample (1 g) was homogenized with 10 mL of 5% (v/v) trichloroacetic acid solution and extracted by ultrasonic

Table 1

Content of biogenic amine in sausages, the superscripted letter a b c denotes significantly different ($P < 0.05$) in the same line, and A B C denotes significantly different ($P < 0.05$) in the same column, and ND means this kind of biogenic amine has been undetected.

Biogenic amine (mg/kg)	Sausage		
	Group C1	Group C2	Group T
Tyramine	13.38 ± 0.34 ^{ba}	14.39 ± 0.17 ^{aA}	13.26 ± 0.11 ^{b A}
Putrescine	ND	ND	ND
Cadaverine	8.26 ± 0.79 ^{cb}	6.94 ± 0.26 ^{bb}	10.26 ± 0.56 ^{ab}
Spermidine	ND	ND	ND
Tryptamine	ND	ND	ND
Phenylethylamine	ND	ND	ND
Spermine	1.47 ± 0.06 ^{ac}	1.19 ± 0.04 ^{bc}	1.50 ± 0.03 ^{ac}
histamine	ND	ND	ND
Total	23.11 ± 1.00 ^b	22.41 ± 0.21 ^b	25.02 ± 0.48 ^a

extraction for 30 min, followed by centrifugation at 5000g for 10 min at 4 °C. Ultrasonic extraction was performed twice and the supernatants were then combined. The extract (1 mL) was mixed 0.2 mL of 2 M NaOH and 100 µL of benzoyl chloride. After vortexing for 20 s, it was kept at 40 °C for 30 min. Then the reaction was terminated with methanol and the mixture was filtered through membrane (0.22 µm) for HPLC.

HPLC analysis was conducted according to Hazar, Kaban, and Kaya (2017). Synchronis C18 column was equipped in HPLC system and the injection volume was 20 µL. The column temperature was 35 °C, the mobile phase included solvent A (90% acetonitrile, 10% 0.01 M ammonium acetate solution) and solvent B (10% acetonitrile, 90% 0.01 M ammonium acetate solution). The gradient elution procedure was started at 70% A and 30% B with flow rate of 0.8 mL/min, then solvent B was raised gradually to 70% within 38 min and maintained for 4 min. The detection analysis was implemented at 254 nm and the biogenic amine content was calculated by comparison with the standard.

2.11. Statistical analysis

Three replicas were for each sample and all measurements of the replicas were performed in triplicate respectively. Statistical analyses were implemented with SPSS Version 18.0 (IBM, New York, NY, USA) and the experiments data was conducted with single factor analysis of variance (one-way ANOVA) and multiple comparisons with Duncan

method. A probability value ($P < 0.05$) was accepted for the level of significance through Duncan's multiple range tests.

3. Results and discussion

3.1. Mb(Fe²⁺)-NO and Met(Fe³⁺)-Mb analysis

In meat products, myoglobin exists in four forms derivatives consisting of oxy-myoglobin (Oxy(Fe³⁺)-Mb), deoxy-myoglobin (Deoxy(Fe²⁺)-Mb), Met(Fe³⁺)-Mb and Mb(Fe²⁺)-NO. The characteristic pink red color of meat products is caused by Mb(Fe²⁺)-NO, which has typical absorption peaks (at 421, 540 and 579 nm) different from other derivatives (Zhang et al., 2007). In Fig. 1a, the absorbance spectra of the three groups all showed three typical absorption peaks (at 418, 540 and 576 nm), which basically consistent with those of Mb(Fe²⁺)-NO. So Mb(Fe²⁺)-NO was considered to exist in the pigments extracted from the three groups. However, the magnitude of absorption peaks of the group T (50 mg/kg of nitrite addition and 7 log cfu/g of *L. plantarum* inoculation) was similar with the control C1 (100 mg/kg of nitrite addition), which indicated that *L. plantarum* inoculation (7 log cfu/g) might promote the formation of Mb(Fe²⁺)-NO and had the potential to substitute 50% sodium nitrite. The result was similar with the previous reports that *L. sakei* C2 inoculated in the fermented sausages could increase the amount of Mb(Fe²⁺)-NO (Gao et al., 2014). Me(Fe³⁺)-Mb was the oxidation products of myoglobin which had negative effect on sausage color (Howes, Milazzo, Droghetti, Nocentini, & Smulevich, 2019). It could be seen from Fig. 1b that the Met(Fe³⁺)-Mb content of group T was significantly lower ($P < 0.05$) than group C2, and there was no significant difference ($P < 0.05$) with group C1. This illustrated that *L. plantarum* could inhibit Met(Fe³⁺)-Mb oxidation, and low concentration of sodium nitrite combination with *L. plantarum* achieved antioxidant effect the same as high concentration of sodium nitrite.

3.2. Nitrate reductase, nitrite reductase activity and nitrate residual

The nitrate reductase and nitrite reductase activity were 120 nmol/mL·min and 19.12 nmol/mL·min respectively (Fig. 1d). Hammes, Bantleon, and Min (1990) indicated nitrate and nitrite reductase activities of the strain might ensure the required low content of nitrate in final products. The nitrate residual of group T was much lower than those of the two control groups (Fig. 1c), which further confirmed that the strain can reduce nitrite residual. Since nitrite was common used

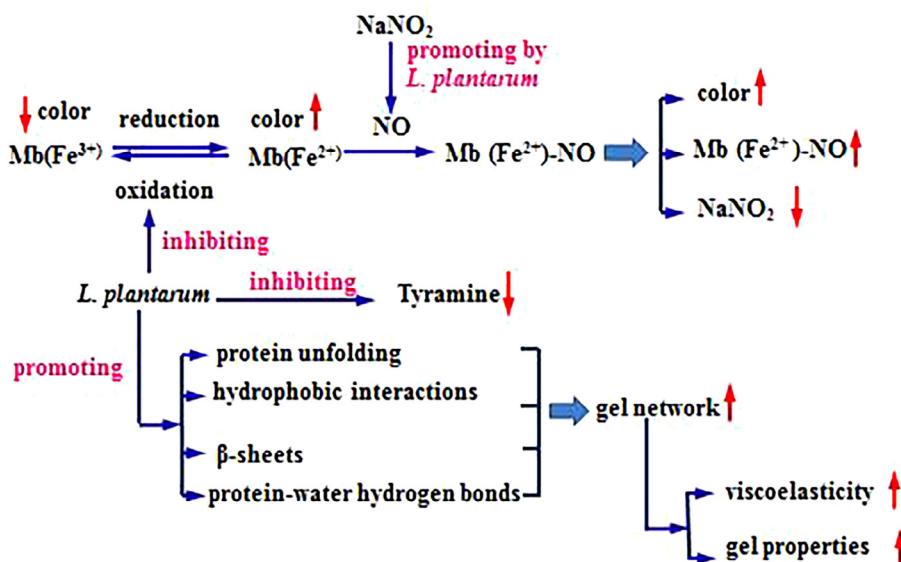


Fig. 7. Tentative mechanism that *L. plantarum* combination with low levels NaNO₂ improved quality and safety of fermented sausage.

as main additives in meat products, nitrite reductase was supposed to be a critical player to reduce the risk of nitrite. Nitrite could be degraded by nitrite reductase and the reduction products of NO played a critical role to color development of the meat products. For NO is one of the reactants in generation of Mb(Fe²⁺)-NO (Götterup et al., 2007). Hammes et al. (1990) also indicated that *L. sakei* and *L. farcinis* could reduce nitrite to NO, which could participate in the formation of Mb(Fe²⁺)-NO in fermented sausages. So the *L. plantarum* had great potential to partially replace nitrite because it promoted the production of Mb(Fe²⁺)-NO, reduced sodium nitrite residual and inhibited Mb(Fe²⁺) oxidation simultaneously.

3.3. Dynamic rheological character

Dynamic rheological character can be used to assess formation ability and kinetics characteristics of protein gel (Wang et al., 2017). The moduli of G' and G'' represents the elastic and viscous behaviors of the gel, respectively (Oh, Lee, Lee, & Lee, 2019). In Fig. 2 G' and G'' character of group T showed a downward trend from 20 °C to 60 °C and then increased slowly from 60 °C to 72 °C during heat treatment. As the temperature continued to rise above 72 °C, G' and G'' began to decrease again. At 75 °C they decreased to the lowest point and then increased again. The decrease of G' and G'' might be attributed to the unfolding and denaturation of protein molecules under heating, which resulted in weakening of ionic and hydrogen bonds (Wang et al., 2017). The rise of G' and G'' indicated that the proteins began to glue together to form gel and a cross-linked protein gel network was formed when reaching to the peak, so viscoelasticity reached a high point (Xue et al., 2017). Since denaturation process could make protein aggregation and cross-linking through active groups, the formation of stronger gel network increased (Hu, Pereira, Xing, Zhou, & Zhang, 2017). However, the two controls didn't show obvious gel properties. Besides, The G' and G'' of group T were higher than the two controls from beginning to end, indicating that *L. plantarum* improved rheological character and viscoelasticity of the sausages (Sun et al., 2015). In addition, the above results illustrated that *L. plantarum* enhanced gel properties due to higher G' and G'' (Kouzounis, Lazaridou, & Katsanidis, 2017).

3.4. LF-NMR

LF-NMR can reflect the changes of mobility and distribution of the moisture inside the myofibrillar gel of sausages. Shao, Deng, Jia, Li, Cao, Liu, and Li (2016) found that the relaxation time (T) and its corresponding intensity can significantly reflect the distribution of different water groups in meat products. In Fig. 3a, three peaks represented three types of moisture in sausages. According to the previous report, the T_{2b} has been assigned to hydration water tightly combined together with macromolecular, the T₂₁ refers to immobilised water, which is situated in the myofibrillar network and the T₂₂ was on behalf of free water outside the myofibrillar lattice (Guo Liping et al., 2019). T_{2b}, T₂₁ and T₂₂ of group T moved to high relaxation time, which indicated that water hydrations of the two control groups were tighter than group T (Shao et al., 2016). Fig. 3b showed that P₂₁ of the group T was significantly higher than the two controls, so the group T had much more immobilised water in myofibrillar network. Water holding capacity of the products is closely related to moisture content in myofibrillar network (Shao et al. 2016). P₂₂ of group T was less than the control C1, which illustrated that group T contained relatively little free water. Higher levels of free water might lead to food spoilage easily (Andrews & Harris, 2000).

3.5. Protein structure based on Raman spectroscopy analysis

Fig. 4a showed a characteristic Raman spectrum (500–2000 cm⁻¹) of sausages and the main bands had been marked according to the reports by Liu, Zhao, Xie, and Xiong (2011). Raman spectral data were

selectively analyzed in two wavenumber scopes of 1400–1700 cm⁻¹ (in Fig. 4b) and 2700–3000 cm⁻¹ (in Fig. 4c) to study protein structure.

3.5.1. Changes of C–H vibrations and amide I

The C–H fraction is a basic component of the aliphatic residues, so C–H stretching and bending vibrations were indicator of the aliphatic residues, which can offer value information of protein conformations near the aliphatic residues (Xue et al., 2017). The C–H stretching and bending vibrations are in the scope of 2800–3000 cm⁻¹ and 1440–1465 cm⁻¹, respectively according to the reports (Liu et al., 2011). Compared to the group C1, C–H stretching vibrations of group C2 and group T shifted from 2928 cm⁻¹ to 2930 and 2932 cm⁻¹ respectively (Fig. 4c), and C–H bending vibrations shifted from 1441 cm⁻¹ to 1439 and 1443 cm⁻¹ respectively (Fig. 4b). The shifts might attribute that the inoculated strains caused protein unfolding and exposure of aliphatic residues (Liu et al., 2011). The intensity of C–H stretching and bending vibrations of group T significantly decreased compared to group C2. The reduction might be attributed to the strength of hydrophobic interactions around the aliphatic side chains, which were beneficial to formation of good gel network structure (Sun, Li, Xu, & Zhou, 2011). The data indicated that partially substituting sodium nitrite with *L. plantarum* caused protein unfolding and hydrophobic interactions around the aliphatic residues strengthen, which resulted in the formation of good gel network.

3.5.2. Secondary structure

Amide I bands are commonly used to study the secondary structure of proteins. The amide I bands consist of overlapping bands in 1650–1660 cm⁻¹, 1665–1680 cm⁻¹, 1680 cm⁻¹ and 1660–1665 cm⁻¹ ranges, which correspond to α -helices, β -sheets, β -turn and random coil structures, respectively (Sheng, Wang, Huang, Xu, & Ma, 2016). From Fig. 4a, compared to the two controls, amide I of group T inoculated with *L. plantarum* shifted to higher wavenumbers (1657 cm⁻¹), implying α -helices unfolding and increase in β -sheet and/or random coil according to the previous report (Kang, Chen, & Ma, 2016). Quantitative information about protein secondary structure estimated from the amide I bands was shown in Fig. 5a. It could be observed that the inoculation of *L. plantarum* had significant effects ($P < 0.05$) on secondary structure in fermented sausages, mainly decreased α -helices structures accompanied by increase in β -sheets, β -turn and random coil structures. The result was in agreement with the amide I spectral shifts (Fig. 4a). The β -sheets is the base and key factor for gel formation, so an increase in β -sheets percentage could improve gel structure of protein (Liu et al., 2011).

3.5.3. Tertiary structure

Local microenvironments can provide tertiary structure information of proteins, and some Raman bands including tryptophan residues and tyrosine residues mainly indicates hydrophobic interactions among protein molecular (Zhu, Kang, Ma, Xu, & Zhou, 2018). The tyrosine doublet ratio (I850/I830) is usually used to determine the micro-environment nearby tyrosine residues (Zhang, Yang, Tang, Chen, & You, 2015). When the ratio was below 0.9, the tyrosine residues are located in the hydrophobic environment. When the ratio is in the range from 0.90 to 2.5, the tyrosine residues are exposed to hydrophilic environment (Liu et al., 2011). In Fig. 5b, A higher I850/830 ratio of group T than group C2 ($P < 0.05$) indicated that the tyrosine residues of group T were exposed to hydrophilic environment and previous research had the similar result in the preparation of frankfurters with pre-emulsified soy oil (Kang et al., 2016). The result indicated that more hydroxyl groups provided by tyrosine residues were exposed to hydrophilic environment (Yi et al., 2020) and resulted in generation of moderate protein-water hydrogen bonds and decrease of protein-protein hydrogen bonds of the gel structure (Zhang, Yang, Tang, Chen, & You, 2016).

The Raman band near 760 cm⁻¹ represents tryptophan residues,

which demonstrates the stretching vibration and hydrophobicity of ring in tryptophan residues (Song, Pan, Wu, & Ren, 2016). They could further provide the hydrophobic interactions information of protein gel (Wang et al., 2018). Fig. 5b showed an increase of normalized intensity of the band in group T compared to group C2. According to Nawrocka et al. (2017), an increase of the band intensity suggested burial of the tryptophan residues in a hydrophobic environment and the tryptophan residues are related to the structure of the compound. This illustrated that more tryptophan residues were gathered in the hydrophobic environment with *L. plantarum* inoculation and resulted in formation of more complex protein gel network (Nawrocka et al. 2017).

3.6. Analysis of biogenic amines by HPLC analysis

The results of the biogenic amine analysis are shown in Fig. 6 and Table 1. According to Ezzat, Zare, Karim, and Ghazali (2015), the most common biogenic amine intoxication is usually caused by histamine and tyramine in fermented products. The content of histamine in food should not exceed 100 mg/kg according 91-493-EEC of the regulation of European Community (Li et al., 2019). And the maximum content of tyramine in food should not exceed 800 mg/kg according to Ercan, Soysal, and Bozkurt (2019). Fig. 6 clearly showed that only tyramine, cadaverine and spermine were detected and no histamine was detected in the three groups of sausages. It could be seen from Table 1 that the tyramine contents of the three groups were all far below the specified limit (800 mg/kg). An increase ($P < 0.05$) in total amount of biogenic amine in group T was observed compared to the two controls, mainly due to the increase of Cadaverine. However, there was no significant difference ($P < 0.05$) in tyramine content between group T and group C1, and they were both lower than group C2. From the point of safety, partially substituting sodium nitrite with *L. plantarum* will reduce the risk of tyramine caused by nitrite reduction (see Fig. 7).

3.7. Tentative mechanism of quality improvement of fermented sausage

Tentative mechanism that *L. plantarum* combination of low levels of NaNO_2 could improve quality and safety of fermented sausage was proposed as follows (Fig. 7): *L. plantarum* combination of low levels of NaNO_2 synergistically improved the color of sausage by inhibiting $\text{Mb(Fe}^{2+})$ oxidation and promoting $\text{Mb(Fe}^{2+})$ -NO formation. $\text{Mb(Fe}^{2+})$ oxidation inhibition was inferred from $\text{Met(Fe}^{3+})$ -Mb content reduction. $\text{Mb(Fe}^{2+})$ -NO formation promotion was concluded from Fig. 1a that typical absorption peak of $\text{Mb(Fe}^{2+})$ -NO increased, which might attribute to the nitrate and nitrite reductase activity of the strain and resulted in NO formation. On the other hand, *L. plantarum* reduced risk from biogenic amines by reducing tyramine content. Furthermore, *L. plantarum* promoted the formation of gel network of the sausage by increasing protein unfolding, hydrophobic interactions, β -sheets and protein-water hydrogen bonds, which resulted in viscoelasticity and gel properties increase.

4. Conclusion

In summary, *L. plantarum* partially substituting sodium nitrite in Chinese fermented sausages could produce almost as much $\text{Mb(Fe}^{2+})$ -NO as the control might due to the activity of nitrite reductase in the strain, which can reduce nitrite and the final products of nitric oxide can react with myoglobin to produce pink $\text{Mb(Fe}^{2+})$ -NO. More importantly, the strain inoculation improved gel properties and high viscoelasticity and these improvements might attribute to the changes of C-H stretching and bending vibrations, reduction of α -helix and increase of β -sheet and random coil in secondary structure, changes of microenvironment in tertiary structure. In addition, the inoculation reduced risk from biogenic amines by reducing tyramine content. So low levels of sodium nitrite combination with *L. plantarum* might have great potential to reduce the risk of nitrite and biogenic amines,

improve color and gel properties of Chinese fermented sausages.

CRediT authorship contribution statement

Yinglian Zhu: Conceptualization, Methodology, Software, Data curation, Formal analysis, Funding acquisition, Writing - original draft.
Liping Guo: Resources, Visualization, Investigation, Supervision.
Qingli Yang: Project administration, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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