Screening of lactic acid bacteria with high protease activity from fermented sausages and antioxidant activity assessment of its fermented sausages

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**ABSTRACT**

This study was conducted to screen the lactic acid bacteria (LAB) with admirable technological properties and high protease activity from traditional Chinese fermented sausages, and to investigate the effects of LAB on the antioxidant activity of peptides of fermented sausages. Seven strains were obtained from 133 LAB isolates. They complied with the criteria of meat starter cultures with high proteolytic activity. These strains were analyzed by API and sequence analysis of 16S rRNA to identify the five strains of *Lactobacillus plantarum*, one strain of *L. fermentum*, and one strain of *L. pentosus*. Furthermore, *L. plantarum* CD101 was inoculated with fermented sausages. LC-MS/MS was used to analyze the peptide composition. Compared with control group, using *L. plantarum* CD101 as starter cultures (Group L) led to a significant increase (\(P < 0.05\)) in antioxidant activity. The relative content of most peptides in Group L samples was largely higher than that in control samples.

**Cribado de bacterias del ácido láctico con alta actividad de proteasa presentes en salchichas fermentadas y evaluación de su actividad antioxidante en salchichas fermentadas**

**RESUMEN**

Este estudio se realizó para cribar bacterias del ácido láctico (LAB) con propiedades tecnológicas positivas y alta actividad de proteasa presentes en las salchichas fermentadas chinas tradicionales. Además, se propuso investigar los efectos de las LAB en la actividad antioxidante de los péptidos de las salchichas. Así, se obtuvieron siete cepas de 133 aislamientos de LAB. Estas hidrolizaron las proteínas sarcoplásicas y miofibrilares y cumplieron con los criterios de los cultivos iniciadores de carne. Las siete cepas de LAB seleccionadas se analizaron empleando el API y el análisis de secuencia del rRNA 16S, identificándose cinco cepas de *Lactobacillus plantarum*, una de *L. fermentum* y una de *L. pentosus*. En las salchichas fermentadas se inoculó *L. plantarum* CD101. Para analizar la composición peptídica de las salchichas fermentadas se usó LC-MS/MS. En comparación con el grupo de control (Grupo B), fermentado sin inoculación, el uso de *L. plantarum* CD101 en cultivos iniciadores (Grupo L) aumentó significativamente (\(P < 0.05\)) las actividades de eliminación de radicales DPPH de las salchichas fermentadas. Por otra parte, la actividad de eliminación de radicales ABTS del Grupo L (371.56 \(\mu\)mol/g) fue significativamente mayor (\(P < 0.05\)) que la del Grupo B (306.76 \(\mu\)mol/g). Asimismo, se registrado un valor FRAP del Grupo L (320.31 \(\mu\)mol/g) significativamente más alto (\(P < 0.05\)) que el del Grupo B (246.37 \(\mu\)mol/g). El contenido relativo de la mayoría de los péptidos en las muestras del Grupo L fue mucho más elevado que en las muestras de control.

**1. Introduction**

Under a spontaneous and long-term fermentation, traditional Chinese sausages are produced to have desirable sensory properties including the aroma flavor and texture (Wang, Zhang, Ren, & Zhan, 2018). Previous studies have shown that the microbiota of traditional sausages primarily consists of lactic acid bacteria (LAB), coagulase-negative staphylococci, yeasts, and molds. The microbiota constitute the most active endogenous microorganisms involved in the processes of acidification, denitrification, lipolysis, and proteolysis (Casaburi et al., 2008). LAB can decrease the pH by producing lactic acid, and produce bacteriocins that prevent the growth of pathogenic and spoilage microorganisms, which play an important role in meat fermentation and preservation. LAB exhibit diverse sensory properties; contribute to the development of flavor, color, and texture; and improve the safety, stability, and shelf life of meat products (Frece et al., 2009). An appropriate selection of strains as starter could improve the typical characteristics of fermented sausages (Casaburi, Martino, Ferranti, Picariello, & Villani, 2016). Therefore, study of the technological and biochemical properties of the strains involved in the fermented sausages is fundamental to obtain products of the expected quality.

Proteolysis, a process catalyzed either by endogenous enzymes present in meat tissues or by those of microbial origin from added starter cultures, is a crucial reaction during fermentation. In fermented meat products, LAB strains help the formation of low molecular weight compounds such as peptides (Simonová et al., 2006). Peptides not only contribute to the
development of a characteristic flavor in fermented products but also exert different bioactivities such as antioxidant, antihypertensive and hypocholesterolemic (Liu, Xing, Fu, Zhou, & Zhang, 2016). Although the endogenous enzymes present in dry-fermented sausages are primarily responsible for protein degradation, the bacterial proteases and peptidases significantly contribute to the initial breakdown of myofibrillar and sarcoplasmic proteins, and the release of small peptides and amino acids (Fernández et al., 2016). Recent studies have confirmed that the peptides extracted from fermented meat products possess antioxidant activity as a substitute of a synthetic antioxidant in the food system (Xing et al., 2018a). Hence, the starter cultures with higher protease activity are very important for producing fermented sausage with merry flavor and high antioxidant activity. However, numerous studies have used LAB with weak or no protease activity isolated from traditional meat products as starter cultures (Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007; Landeta, Curiel, Carrascosa, Muñoz, & De, L. R, 2013). In the context of the relationship between LAB and antioxidant activity, the majority of studies have focused on the antioxidant activity of intact cells, intracellular cell-free extracts, and cell-free supernatants of LAB (Das & Goyal, 2015; Han, Kong, Chen, Sun, & Zhang, 2017). Few studies have reported the effect of LAB on the antioxidant activity of peptides of fermented sausages. Therefore, the main objective of this study was to isolate and identify the LAB with a high protease activity from traditional Chinese fermented sausages, and to investigate the effect of LAB on the antioxidant activity of peptides in fermented sausages. A second objective is to evaluate the technological properties of the isolates.

2. Materials and methods

2.1. Isolation and culture conditions

Twelve samples of traditional Chinese fermented sausages were collected from 10 different regions. For the isolation of LAB, 25 g of each spontaneously fermented sausage sample was collected aseptically, transferred to sterile plastic homogeneous pouches, and homogenized with a 10-fold sterile solution of tryptone (0.3%) and NaCl (0.85%) for 90 s. After 10-fold serial dilution of the samples in the same solution, the appropriate dilution was selected for inoculation on MRS agar (Drosinos et al., 2007). The LAB strains were grown on the MRS agar plates at 37°C in an anaerobic incubator. After an incubation period of 48 h, the colonies with different morphologies were randomly selected and streaked onto the new sterile MRS agar. The pure cultures were characterized according to Gram staining, cell morphology, and catalase production.

2.2. Proteolytic activity

The proteolytic activity of the LAB strains was evaluated as previously described (Zeng et al., 2016). The proteolytic activity was assessed using agar plates supplemented with 1 mg/mL of sterile sarcoplasmic protein and myofibrillar protein extracted according to the method of Drosinos et al. (2007), respectively. The proteolytic activity of each LAB strain was assessed by measuring the diameter of the clear zone (mm).

2.3. Technological properties

The LAB strains must comply with the basic standards of meat starter cultures to be used as starter cultures. Therefore, the above-mentioned LAB strains were selected according to the criteria described below.

Production of lactic acid, salt, and nitrite tolerance ability, biogenic amine formation, nitrate reductase activity, lipolytic activity, gas production ability of LAB strains were evaluated according to the methods of García-Hernández et al. (2016), Todorov et al. (2017), Ruizmoyano et al. (2009), Bonomo, Ricciardi, Zotta, Parente, and Salzano (2008), Martín, Colín, Aranda, Benito, and Córdoba (2007) and Ogunsakin et al. (2017), respectively. The production of hydrogen sulfide was evaluated by using biochemical kit (Hopebio, Qingdao, GB065). The mucus and pigment production ability were determined on the MRS agar medium supplemented with 5% sucrose instead of glucose and 30% sterilized whole milk, respectively.

2.4. Identification of the selected LAB strains

The screened LAB strains were identified using the API50 CH kit (Biomerieux, France) according to the method of Casaburi et al. (2016). Data analysis was conducted using the software API LAB+ (version 5.0).

LAB isolates were cultured overnight in the MRS broth. The DNA of LAB isolates were extracted using a bacterial DNA kit (TIANGEN). DNA samples were then used as a template in subsequent PCR analyses. The 16S rRNA gene was amplified by PCR according to the method described by Angmo, Kumari, Savitri, and Bhalla (2016). DNA sequencing of the amplified fragments was performed by the sequencing service of Sangon Biotech (Shanghai), and the results were compared with GenBank data using the Basic Local Alignment Search Tool from the NCBI.

2.5. Antioxidant activity of peptides of the sausages

2.5.1. Sausage processing and peptides extraction

Fermented sausages were manufactured at the Key Laboratory of Meat Processing and Quality Control (Nanjing, China) according to the method of Chen et al. (2016). The sausages were divided into two treatment groups (5 kg per group): (1) Group B, control without inoculation, (2) Group L, inoculated with Lactobacillus plantarum CD101 (with stronger proteolytic activity across all the 7 LAB strains), and the concentration of the starter culture was 10^7 CFU/g. The peptides in sausages were extracted as previously described (Xing et al., 2016). The sausage samples (25 g) were minced and homogenized (3 × 10 s at 22,000 rpm) with 100 mL of phosphate buffer (0.2 mol/L, pH 7.2) in an ice bath using a homogenizer (IKA T25 digital ultraturrax, Germany). After standing at 4°C for 2 h, the homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. Then, the supernatant was filtered by a filter paper and three volumes of ethanol were added (40%, v/v). The mixture was kept at 4°C for 12 h and then centrifuged again at
12,000 rpm (20 min, 4°C). The resulting supernatant was filtered through 0.45 μm nylon membrane filters, and the pH value of the filtrate was adjusted to 7.2. Finally, the filtrate was dried in a freeze dryer and stored at −20°C.

2.5.2. DPPH radical-scavenging activity
The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity was evaluated according to the method described by Liu, Chen, Huang, Huang, and Zhou (2017). Peptides extracted from fermented sausages were separately adjusted to achieve concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL. Glutathione (GSH) was used as the positive control. The radical-scavenging activity was calculated using the following equation:

\[
\text{DPPH radical} – \text{scavenging activity} \cdot \% = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right) \times 100
\]

where \(A_{\text{sample}}\), \(A_{\text{blank}}\), and \(A_{\text{control}}\) represent the absorbance values of the sample, the blank, and the control groups, respectively.

2.5.3. ABTS radical-scavenging activity
The 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging activity was determined as described by Luo, Li, and Kong (2012). Samples (10 μL) were mixed with 200 μL of fresh ABTS solution. The absorbance was then measured at 734 nm after 6 min. The standard curve for Trolox was provided over a concentration range from 0.15 to 1.5 mM. The trolox equivalent antioxidant capacity (TEAC) of peptides of Chinese fermented sausages was expressed as μmol TE/g sample. The GSH was used as the positive control.

2.5.4. Ferric reducing antioxidant power (FRAP)
The FRAP assay was determined according to Luo et al. (2012). Samples (5 μL) were mixed with 180 μL of FRAP working solution and incubated for 5 min at 37°C. The absorbance of sample was measured at 593 nm. The standard curve of FeSO₄ was prepared over a concentration range from 0.15 to 1.5 mM. The FRAP values of samples were calculated using the standard curve and expressed as micromole Fe (II) equivalent per gram of sample.

2.6. Identification of peptides by LC-MS/MS
After removing salts and impurities using Oasis HLB cartridges (Waters, Massachusetts, USA), peptide samples were identified by LC-MS/MS according to the method provided by Xing et al. (2018b) with a slight modification. The mobile phase contained solutions A (0.1% formic acid in water) and solutions B (0.08% formic acid in 80% acetonitrile). The gradient elution was performed as follows: 0–8 min (98%A, 2%B); 8–12 min, linear gradient B (2–15%); 12–100 min (72%A, 28%B); 100–120 min (45%A, 55%B), 120–144 min (2%A, 98%B) and 144–160 min (98%A, 2%B). The flow rate was set at 350 nL/min. The peptides of fermented sausages of Group B and Group L were analyzed, respectively. The analysis was performed in triplicate. The acquired MS/MS data were further analyzed by PEAKS Studio 8.5 (BSI, Canada) using the database Sus scrofa (http://www.uniprot.org/). In order to evaluate the origin of peptides in parent proteins, the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used, and 100% homology of peptides was identified. The final composition of peptides was identified in samples from both Group B and Group L. The sequence of the peptides from unknown parent protein was not listed.

2.7. Statistical analysis
Data were evaluated using the SAS software (version 9.3, SAS Institute Inc., USA). Duncan’s multiple range tests and one-way analysis of variance (ANOVA) were performed to determine any significant differences (P < 0.05) among treatments.

3. Results and discussion

3.1. Proteolytic activity of LAB strains
A total of 133 Gram-positive and catalase-negative LAB isolates were stored at −80°C in MRS broth supplemented with 30% (v/v) glycerol. Endogenous enzymes in the meat, especially cathepsins, primarily contribute to the initial breakdown of sarcoplasmic and myofibrillar proteins in fermented sausages. Microbial proteases play an important role in the degradation of proteins into small peptides and in the subsequent degradation into amino acids (Fernández et al., 2016). One focus of this study was to screen for a LAB strains with strong protease activity. Therefore, the proteolytic activity of 133 LAB strains was determined.

The result showed that 57.74% of the strains were able to hydrolyze the sarcoplasmic proteins, 63.16% of them were able to hydrolyze the myofibrillar proteins, and 64 isolates (48.12%) were able to hydrolyze both the sarcoplasmic and myofibrillar proteins. This result was similar to that reported by previous study (Casaburi et al., 2008) showing that partial L. casei, L. plantarum, and L. curvatus isolated from sausages actively contributed to the hydrolysis of the sarcoplasmic proteins. The proteolytic activity of these 64 LAB strains is shown in Table 1. The diameter of the clear zone of hydrolyzing sarcoplasmic proteins was from 8.72 mm to 28.51 mm, and hydrolyzing myofibrillar proteins was from 9.03 mm to 28.40 mm. This result also indicated that LAB strains were better at hydrolyzing the myofibrillar proteins than sarcoplasmic proteins, which was in line with previous study (Drosinos et al., 2007). However, the proteolytic diameters of this experiment were generally higher than the study conducted by Ines, Maher, and Mnsasser (2009). This difference may be related to the use of skimmed milk, gelatin, and casein, which are nonmeat substrates, for assessing protease activity (Drosinos et al., 2007). Moreover, the drop in pH may lead to greater proteolytic activity in fermented meat products (Warren et al., 2019).

3.2. Technological properties of LAB
The technological properties and the selection criteria of 64 LAB isolates are represented as the production of lactic acid, salt and nitrite tolerance ability, hydrogen sulfide production, biogenic amine formation, gas production ability, nitrate reductase activity, lipolytic activity, mucus production, and pigment production.

In fermented meat products, low pH can inhibit the growth of spoilage bacteria and promote product color, resulting in the better safety and stability of products. In this study, 64 LAB strains were observed to produce lactic acid, salt and nitrite tolerance ability, hydrogen sulfide production, biogenic amine formation, gas production ability, nitrate reductase activity, lipolytic activity, mucus production, and pigment production.
acid quickly. These 64 LAB strains were good at producing lactic acid with the pH lower than 5.0. This corresponds with previous studies that have shown that *Lactobacillus plantarum* and *L. delbrueckii* produced lactic acid fast (Nediani et al., 2017).

Salt and nitrite are often added to fermented meat products to maintain quality, improve flavor, and prevent food deterioration. Therefore, the LAB strains used as starter cultures must tolerate the appropriate concentrations of salts and nitrates. In this study, all LAB strains were able to grow in MRS broth supplemented with 6.5% salts and 150 mg/kg nitrates. This result was in agreement with previous studies (Landeta et al., 2008). The presence, activity, and specificity of decarboxylases are all different due to the strain-specific property, as reported by Deepika Priyadarshani and Rakshit (2011). In summary, 22 LAB strains were selected for the next test.

The LAB strains used as starter cultures participated in the formation of the typical pink color of meat products through the spontaneous reduction of nitrates to nitric oxide. However, none of the LAB strains analyzed in this experiment exhibited nitrate reductase activity. This result was in agreement with a previous study conducted by Bonomo et al. (2008), who showed that *L. plantarum* and *L. brevis* strains could not reduce nitrate. However, Landeta et al. (2013) reported that a few *L. plantarum* strains could exhibit an intermediate nitrate reductase activity.

Sulfur-containing amino acids may be produced in the metabolic processes of the strains. These amino acids will gradually produce hydrogen sulfide and produce a green color reacting with myoglobin to affect the color and flavor of the products. The results showed that 57 LAB strains did not produce hydrogen sulfide.

Biogenic amines are a class of organic compounds in several fermented foods of animal and vegetable origins. Their existence causes food poisoning. The biogenic amines in fermented meat products primarily include tyramine, cadaverine, putrescine, spermidine, and spermine (Tosukhowong et al., 2012). In this study, it was observed that 56 LAB strains did not decarboxylate arginine, 50 strains did not decarboxylate lysine, forty-eight strains did not decarboxylate tyrosine, and 22 strains did not decarboxylate histidine. The low ability of biogenic amine production for the LAB strains was found in this study. This is in agreement with previous studies (Landeta et al., 2013; Ruizmoyano et al., 2009). The presence, activity, and specificity of decarboxylases are all different due to the strain-specific property, as reported by Deepika Priyadarshani and Rakshit (2011).

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Lipolysis influences the texture and flavor formation of fermented meat products, which is due to a release of the aromatic substances and organic acids by lipase activity from microorganisms. None of the LAB strains in this study showed the activity against pork fat. This result was in accordance with Benito et al. (2007) who reported no or a weak lipolytic activity of LAB in fermented meat products. In addition, seven strains in this study were homofermentative and did not produce mucus and pigment, which was in agreement with the standards of meat starter cultures. The characterization of 64 LAB strains was summarized in supplementary data.

### Table 1. Proteolytic activities of LAB strains.

| Strains         | Myofibrillar | Strains         | Myofibrillar |
|-----------------|--------------|-----------------|--------------|
| HN104           | 12.25 ± 0.29 | GZ107           | 22.55 ± 0.92 |
| HN106           | 14.12 ± 0.37 | GZ114           | 14.88 ± 0.68 |
| HN108           | 8.72 ± 0.52  | GZ116           | 26.64 ± 0.76 |
| HN110           | 9.65 ± 0.50  | NJ107           | 17.63 ± 0.72 |
| HN111           | 15.41 ± 0.84 | CQ01-101        | 12.27 ± 0.15 |
| HN112           | 8.75 ± 0.33  | CQ01-107        | 25.01 ± 0.74 |
| HN115           | 10.48 ± 0.48 | CQ01-117        | 22.19 ± 0.17 |
| HN116           | 24.87 ± 1.08 | CQ01-121        | 12.99 ± 0.69 |
| HN117           | 16.68 ± 1.04 | CQ01-122        | 17.85 ± 0.04 |
| HN118           | 28.17 ± 0.34 | CQ01-123        | 10.72 ± 0.17 |
| HN119           | 15.16 ± 1.17 | CQ02-106        | 11.29 ± 0.13 |
| HN120           | 28.51 ± 1.49 | CQ02-108        | 12.63 ± 0.26 |
| HN121           | 15.72 ± 1.18 | CQ02-114        | 12.30 ± 0.21 |
| HN123           | 23.75 ± 0.39 | CQ02-115        | 10.12 ± 0.44 |
| HN125           | 15.83 ± 0.65 | CQ02-116        | 11.39 ± 0.11 |
| HN126           | 10.21 ± 0.24 | CQ02-117        | 10.84 ± 0.37 |
| HN127           | 10.27 ± 0.27 | CQ02-118        | 11.65 ± 0.45 |
| HN128           | 25.27 ± 1.25 | CQ02-119        | 11.48 ± 0.56 |
| HN129           | 9.70 ± 0.40  | CQ02-120        | 10.98 ± 0.41 |
| HN130           | 28.35 ± 0.50 | CD101           | 23.71 ± 1.58 |
| HN131           | 9.88 ± 0.32  | CD103           | 17.93 ± 0.58 |
| HN133           | 17.09 ± 0.91 | CD105           | 16.56 ± 0.10 |
| KM105           | 16.45 ± 0.53 | CD106           | 15.00 ± 0.25 |
| KM109           | 15.43 ± 0.20 | CD109           | 16.81 ± 0.05 |
| KM112           | 18.97 ± 0.90 | CD110           | 20.79 ± 0.73 |
| KM113           | 15.79 ± 0.69 | CD113           | 19.44 ± 0.15 |
| KM119           | 21.67 ± 0.45 | CD118           | 20.03 ± 1.00 |
| KM120           | 14.47 ± 0.24 | CD119           | 16.32 ± 0.24 |
| KM123           | 14.43 ± 0.05 | CD122           | 18.07 ± 0.62 |
| KM124           | 21.12 ± 0.12 | CD125           | 17.04 ± 0.11 |
| KM125           | 16.57 ± 0.92 | CD126           | 20.62 ± 0.38 |
| GZ105           | 21.78 ± 1.30 | CD127           | 11.20 ± 0.09 |

**Table 1. Actividades proteolíticas de las cepas LAB.**

A Diameter of clear zone on sarcoplasmic/myofibrillar proteins (mm).

Data are shown as the mean ± standard deviation (n = 3).

**Diámetro de la zona clara en proteínas sarcoplásmicas/miofibrilares (mm).**

Los datos indican la media ± desviación estándar (n = 3).
3.3. Identification of selected LAB strains

The seven LAB isolates were subjected to biochemical analysis using API50 CH identification kits, and the results are shown in Table 2. CQ01-122 was identified as L. pentosus, GZ114 was identified as L. fermentum, and the other isolates were identified as L. plantarum. The biochemical tests resulted in high identity levels (identification with ID in the range of 93.6–99.9%). The results of molecular analysis and NCBI accession are shown in Table 2. These results were basically similar to the identification provided by the API. The result indicated the species most frequently identified were L. plantarum among the LAB strains isolated from Chinese fermented sausages. It has been reported by Landeta et al. (2013) that the species most frequently identified were L. sakei among the LAB strains isolated from Spanish dry-cured sausages, and L. plantarum were also found.

3.4. Antioxidant activity of peptides of the sausages

The DPPH radical has a maximum absorbance at 517 nm. The radical will be scavenged when it encounters an anti-radical compound such as an antioxidant. The DPPH radical-scavenging activities (RSA) of peptide samples are shown in Figure 1. For the Group B and Group L samples, peptides have different DPPH RSA at different protein concentration (P < 0.05). When the protein concentration of two groups was at 0.5 mg/mL. The RSA of Group L was 27.89%, whereas that of the Group B was only 17.55%. The RSA of group L increased to 76.25% at the protein concentration of 2.5 mg/mL, which was significantly higher (P < 0.05) than that of the Group B (62.44%). This result indicated that using L. plantarum CD101 as the starter cultures led to a significant increase in the antioxidant activity of peptides in fermented sausages. This result was in agreement with Mejri, Vásquez-Villanueva, Hassouna, Marina, and García (2017) who has reported that the ripening process of the dry fermented camel sausages inoculated with different starter bacteria such as S. xylosus, L. plantarum, and L. pentosus (107 CFU/g) resulted in an increase of RSA. During the ripening period of fermented sausage, proteins are degraded into small peptides and free amino acids, which may show antioxidant properties including inhibiting oxidation of fatty acids and scavenging radical activity (Feng et al., 2014).

Based on the total antioxidant capacity assay with ABTS method and FRAP method, the antioxidant activity of fermented sausages was determined (Table 3). The result of ABTS radical-scavenging activity indicated that the activity of Group L (371.56 µmol/g) was significantly higher than Group B (306.76 µmol/g) (P < 0.05) while both activities were lower than GSH. This antioxidant activity was better than Xuanwei hams (148.97 µmol/g) which was reported by Xing et al. (2018b). Similar tendency was also observed in the FRAP values. Liu et al. (2017) reported the FRAP values of duck meat sample after 3 days post-mortem were about 300 µmol/g, which was similar to that of fermented sausages inoculated with the L. plantarum CD101 (320.31 µmol/g). During the sausage fermentation, the microbial population and fermentation conditions significantly influenced the low molecular weight nitrogen profile and antioxidant activities (Fernández et al., 2016). This is primarily due to an increase in free amino acids, amines such as lysine, methionine, tyramine and small peptides (Fernández et al., 2016). The sequences of peptide played an important role in scavenging free radicals. The small peptides below 5 kDa had better scavenging effects on free radicals than large molecular peptides (Shi, Kovacs-Nolan, Jiang, Tsao, & Mine, 2014).

Figure 1. The DPPH radical-scavenging activity of the peptides extracts from Chinese fermented sausages and difference among Group B and Group L.

The antioxidant activity of peptides of the sausages.

3.5. Identification of peptides by LC-MS/MS

The degraded products of proteins from the Group B and Group L were further identified and quantified using LC-MS

Table 3. The antioxidant activity of ABTS and FRAP values of fermented sausages.

| Group   | ABTS (µmol/g) | FRAP (µmol/g) |
|---------|---------------|---------------|
| Group B | 306.76 ± 31.64a | 246.37 ± 20.93b |
| Group L | 371.56 ± 24.21b | 320.31 ± 21.02b |
| GSH     | 1238.21 ± 26.59a | 1230.71 ± 33.05a |

a The data are shown as the mean ± standard deviation (n = 3).

b Within the same column, values with different lowercase letters differ significantly (P < 0.05).

The synthetic glutathione (GSH) was used as positive control.

a Los datos indican la media ± desviación estándar (n = 3).

b Los valores con diferentes letras minúsculas en la misma columna difieren significativamente (P < 0.05).

El glutatión sintético (GSH) se utilizó como control positivo.
/MS and PEAKS software. Total ion chromatogram (TIC) spectra of peptide samples are shown in Figure 2. TIC spectra showed great rise in the intensity of detected ions from the Group B to Group L samples. This is probably because bacterial proteases and peptidases contribute to a release of small peptides and amino acids (Fernández et al., 2016) in Group L which were inoculated with *Lactobacillus plantarum* CD101. Fifty-three sequences of the peptides were identified in both Group B and Group L samples, shown in Table 4. The main protein origins of these peptides were zinc finger proteins, alpha skeletal muscle, myosin light chain 1/3, skeletal muscle isoform, myosin-1, and troponin T fast skeletal muscle. The peptides which were identified were less than 3228.6 Da, and had amino acid sequences between 7 and 28 amino acids in length. The ratio of the peptide content in samples of Group L to that in control samples (Group B) is shown in Table 4. The relative content of all eight small peptides (peptides with 7 to 9 amino acids) in the L group samples was higher than that in the B group. Moreover, the relative content of some peptides in Group L samples, such as AVFPSIVGRPR, FAGDDAPRAVFPS, DDAPRAVFPSIVGRPRH, DAPRAVFPSIVGRPRHQG, FAGDDAPRAVFPSIVGRPRHQ, etc., was largely higher than that in control samples. The short-chain peptides with 2 to 20 amino acids have higher antioxidant activity than their parent proteins and polypeptides, which was confirmed by Zhu et al. (2013). Previous studies (Majumder & Wu, 2010; Samaranayaka & Li-Chan, 2011) have reported that peptides with the presence of hydrophobic amino acids, such as Val, Phe, Ala, Pro, Leu, Met, Trp, Ile, and Lys, were indicated to have stronger ability to scavenge free radicals. Hydrophobic amino acids were more accessible to hydrophobic free radicals, because they enhance the solubility of peptides in lipids. Moreover, Glu, Asp and His were also usually found in antioxidant peptides (Sarmadi & Ismail, 2010). In this study, the presence of histidine in identified peptides was consistent with Mejri et al. (2017) who identified antioxidant peptides of sausages like EDHTKAHV. Furthermore, RGLVVPVI, KKVAKPN, GRVISHAI, AVFPSIVGRPR, FAGDDAPRAVFPS, APKIPEGEKVDFDIQKR, etc., those identified peptides were abundant in Leu, Val, Pro, Ile, Lys, Ala, Phe and Asp. The content of hydrophobic amino acids of each peptide was more than 50%. This result was similar to the study of Zarei et al. (2014). As shown in Table 4, the peptides which hydrophobic amino acids located at N-terminal included GGGRTDDPGRVISHAIIDRGLVPVIID ILEFGLKDFAVPSIVGRPRDFAGDDAPRAVFPS, etc. The result indicated that the relative content of them in Group L was higher than that in the Group B. It has been reported by Liu et al. (2017) that hydrophobic amino acids located at N-terminal played an important role in affecting the antioxidant properties of peptides. Therefore, this result can explain that the antioxidant activity of peptides in fermented sausages inoculated *L. plantarum* CD101 was significantly higher than that in the control samples.

4. Conclusion
This study reported that seven LAB strains with desirable technological characteristics, including *L. plantarum* HN108, KM119, NJ107, CQ01-107, and CD101 and *L. pentosus* CQ01-122 and *L. fermentum* GZ114, were isolated from traditional Chinese sausages. These seven strains exhibited high proteolytic activities and complied with the criteria of meat starter cultures. Therefore, these strains are recommended to be applied in the industrial manufacturing of meat products. Compared with naturally fermented sausages, the antioxidant activity of sausages inoculated with *L. plantarum* CD101 was significantly increased (*P* < 0.05). Analysis of peptides by LC-MS/MS, the small peptides were dominated across the identified peptides in Chinese fermented sausages. The relative content of most peptides in sausages inoculated *L. plantarum* CD101 was much higher than that in the control samples. Therefore, the inoculation of well-adapted starter cultures with a high proteolytic activity can increase the content of peptides of fermented sausages. This may be an important factor in increasing the

Figure 2. (a) Total ion chromatogram (TIC) obtained by LC-MS/MS with peptides in fermented sausages inoculated with starter cultures *L. plantarum* CD101 (Group L). (b) Total ion chromatogram (TIC) obtained by LC-MS/MS with peptides in control group (Group B).

Figure 2. (a) Cromatograma de iones totales (TIC) obtenido por LC-MS/MS de péptidos de salchichas fermentadas inoculadas con cultivos iniciadores *L. plantarum* CD101 (Grupo L). (b) Cromatograma de iones totales (TIC) obtenido por LC-MS/MS de péptidos en el grupo de control (Grupo B).
### Table 4. The composition of crude peptides in fermented sausages.

| Peptide | Mass  | Length | m/z  | Ratioa | Parent protein |
|---------|-------|--------|------|--------|----------------|
| GGGRTDP | 773.33| 8      | 387.68| 2.04   | Zinc finger protein 281 |
| KVRKPN | 783.50| 7      | 392.76| 1.64   | Coiled-coil domain containing 136 |
| GRVISHN | 851.56| 8      | 426.74| 1.25   | Carbamoyl-phosphate synthase synthase 1 |
| RGLVVP | 851.56| 12     | 426.74| 3.70   | Dihydrolipoyllysine-residue succinyltransferase 1 |
| EVDSPLK | 899.50| 9      | 450.78| 1.32   | mitochondrial dynamin like GTPase |
| ILEFFFLK | 965.56| 5      | 483.80| 7.14   | Protein dslfide-isomerase |
| MQARLAK | 972.57| 7      | 487.30| 1.75   | Zinc finger protein 704 |
| AGAQPPGPGPRP | 975.45| 12 | 488.78| 0.80   | Alpha 1 chain of type I collagen |
| KDILPTE | 986.53| 7      | 494.25| 5.88   | Microtubule-associated protein |
| YRGGVNSVK | 1091.61| 10 | 546.84| 4.35   | Rho GTPase activating protein 42 |
| AVFSPVSRPR | 1159.70| 11 | 400.25| 33.33  | alpha skeletal muscle |
| FAGGDPARVPPS | 1348.64| 13 | 675.32| 50.00  | alpha skeletal muscle |
| GGGLVVKAFGGGAPRA | 1587.80| 17 | 794.91| 6.25   | alpha skeletal muscle |
| NAAKLAPDTEVCLCAPPT | 1696.83| 17 | 849.41| 5.00   | Myosin light chain 1/3, skeletal muscle isoform |
| DNGSGLVKAAGGDADPR | 1745.83| 18 | 879.33| 9.09   | alpha skeletal muscle |
| DNGSGLVKAAGGDADPRA | 1816.87| 17 | 909.46| 14.29  | alpha skeletal muscle |
| AGDGDPARSPVSPRPR | 1800.00| 18 | 627.66| 3.85   | alpha skeletal muscle |
| DDAGDPARSPVSPRPRH | 1889.00| 17 | 630.67| 25.00  | alpha skeletal muscle |
| VCGDNSGLVKAAGGDADPR | 1947.91| 20 | 650.30| 1.82   | alpha skeletal muscle |
| DAPRVSPPSVRPHQG | 1959.05| 18 | 490.77| 33.33  | alpha skeletal muscle |
| APAPAPAAPAPAPAPAPAPAPAP | 1974.06| 21 | 659.03| 4.76   | Myosin light chain 1/3, skeletal muscle isoform |
| VCGDNSGLVKAAGGDADPR | 2018.95| 21 | 673.99| 2.22   | alpha skeletal muscle |
| SSDQEMAGAIEAPYLRK | 2035.98| 18 | 1028.00| 0.83  | Myosin-1 |
| APKIEPEGVFDFOQIKQ | 2056.08| 20 | 866.38| 1.47   | Troponin T fast skeletal muscle type |
| LRDVEDEGNVMTVMGARLHR | 2128.08| 19 | 533.03| 0.41   | Myosin light chain 1/3, skeletal muscle isoform |
| SSDQEMAGAIEAPYLRK | 2141.81| 18 | 1071.51| 2.63  | Myosin-1 |
| WITROEDEAPSIVHRK | 2156.10| 18 | 540.03| 2.86   | alpha skeletal muscle |
| AGDGDPARSPVSPRPHQG | 2202.14| 21 | 441.44| 5.26   | alpha skeletal muscle |
| IFGEAAPYLRKSEKREIA | 2206.17| 20 | 442.24| 0.35   | Myosin-1 |
| SSDQEMAGAIEAPYLRKSE | 2270.05| 20 | 1136.04| 5.88  | Myosin-1 |
| FADGDPARSPVRPPHRQ | 2292.19| 21 | 459.45| 50.00  | alpha skeletal muscle |
| APKIEPEGVFDFOQIKQ | 2340.23| 20 | 469.07| 10.00  | Troponin T fast skeletal muscle type |
| APKIEPEGVFDFOQIKKQ | 2340.24| 20 | 469.07| 4.17   | Troponin T fast skeletal muscle type |
| FADGDPARSPVSPRPHQG | 2349.21| 21 | 784.04| 25.00  | alpha skeletal muscle |
| APKIEPEGVFDFOQIKKQ | 2454.29| 21 | 491.86| 4.00   | Troponin T fast skeletal muscle type |
| DALKPTKRFPRLGDEETVRK | 2458.30| 21 | 492.68| 3.09   | Phosphoglycerate mutase |
| SSDQEMAGAIEAPYLRKSEQE | 2527.19| 22 | 1264.61| 1.33  | Myosin-1 |
| DALKPTKRFPRLGDEETVRK | 2529.34| 22 | 506.88| 6.25   | Phosphoglycerate mutase |
| SSDQEMAGAIEAPYLRKSEQER | 2683.29| 23 | 895.46| 1.19   | Myosin-1 |
| APAPAPAAPAPAPAPAPAPPEKI | 2714.50| 27 | 679.63| 1.92   | Myosin light chain 1/3, skeletal muscle isoform |
| PAEVEHEEHVEEHVEPKEPR | 2738.34| 23 | 548.67| 6.25   | Troponin T fast skeletal muscle type |
| APKIEPEGVFDFOQIKKQ | 2810.49| 24 | 703.64| 0.22   | Troponin T fast skeletal muscle type |
| RVAPEEHPTLTEAPLNKANEKEM | 2840.50| 25 | 569.11| 1.59   | alpha skeletal muscle |
| DEDETALLVCDNSGLVKAAGGDADPR | 2864.28| 28 | 1433.18| 0.50  | alpha skeletal muscle |
| KPRPLTAPKIEPEGVFDFOQIKQ | 2876.61| 25 | 480.45| 0.35   | Troponin T fast skeletal muscle type |
| PAPAEVEHEEHVEHEPEEKP | 2912.41| 25 | 587.49| 3.70   | Troponin T fast skeletal muscle type |
| SSDQEMAGAIEAPYLRKSEQERIA | 2996.45| 26 | 999.84| 3.03   | Myosin-1 |
| APPPPAPEVEHEEHVEHEPEEKP | 3034.26| 26 | 601.70| 2.38   | Troponin T fast skeletal muscle type |
| KPRPLTAPKIEPEGVFDFOQIKQR | 3037.71| 26 | 434.25| 4.17   | Troponin T fast skeletal muscle type |
| APKIEPEGVFDFOQIKKQ | 3039.64| 26 | 628.93| 1.82   | Troponin T fast skeletal muscle type |
| SDOEVHEVEIEEEACAPAAPAEVEHVEV | 3090.32| 27 | 1031.12| 0.14  | Troponin T fast skeletal muscle type |
| APPPPAPEVEHEEHVEHEPEEKP | 3100.50| 27 | 776.13| 5.56   | Troponin T fast skeletal muscle type |
| APPPPAPEVEHEEHVEHEEKP | 3228.60| 28 | 462.24| 0.29   | Troponin T fast skeletal muscle type |

a Ratio: The ratio of the relative content of the peptides in the Group L to the Group B.

b Relación entre el contenido relativo de péptidos en los grupos L y B.

The next step will be studied whether there are other factors.

### Disclosure statement
No potential conflict of interest was reported by the authors.

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