A novel antimicrobial substance produced by *Lactobacillus rhamnosus* LS8

Lihui Zhang^{1, a}, Lu Wang^{2, a}, Lanhua Yi^{1 a}, Xin Wang^{1a}, Yu Zhang^{1,3}, Junyan Liu⁴, Xing Guo¹, Lian Liu¹, Chungge Shao¹, Xin Lü^{1,*}

(1 College of Food Science and Engineering, Northwest A&F University, Yangling District, Shaanxi, 712100, China. 2 School of Sciences, China Pharmaceutical University, Nanjing, Jiangsu, 211198, China. 3 Beijing Kwinbon Biotechnology CO., LTD. China, Tenth people's hospital of Tongji University, Shanghai, People's Republic of China. 4)

a The authors contributed equally to this work and should be considered as co-first authors.

*Corresponding author. Tel. /Fax: +86-29-8709 2195.

E-mail: xinlu@nwsuaf.edu.cn

Abstract: Superbugs or multi-drug resistant pathogens are not only clinical issue but also highlighted in food safety. However, development of new antibiotics and safe preservatives is declining. A novel workflow was used to purify antimicrobial substance produced by *Lactobacillus rhamnosus* LS-8, in which ethyl acetate extraction, polypeptide column and HPLC were involved in the purification process. Subsequently, a novel antimicrobial substance was purified and identified as 4,6-dimethyl-1,2,5-triazepane-3,7-dione (C₆H₁₁N₃O₂) by mass spectrum, ¹H NMR and ¹³C NMR analysis. Fortunately, it has significant inhibitory effect on both

gram-positive and gram negative strains including multi-drug resistance pathogens. Also it had good heat stability and resistant to proteolytic enzymes, however, it was only active in acidic condition. The results of scanning electron microscopy (SEM) and transmission electron microscope (TEM) showed that LS-8-25 destroyed the ultrastructure of *S. aureus* and *E.coli*. The discovery is adding a new variety of antimicrobial substance produced by lactic acid bacteria.

Key words: *Lactobacillus rhamnosus* LS-8, purification, antimicrobial, multi-drug resistant pathogens

1. Introduction

Emerging superbugs and multi-drug resistance pathogens urgently require new antibiotics/preservatives (Ghafur, 2013; Giles, 2004; Kelland, 2011; P, et al., 2016), however, the discovery of antibiotics/preservatives is declining. Lactic acid bacteria (LAB) are commonly thought to be having health-promoting effects on human (Granato, Branco, Nazzaro, Cruz, & Faria, 2010; Klaenhammer, Kleerebezem, Kopp, & Rescigno, 2012), while they are also potential antibiotic alternatives producers for solving multi-drug resistance pathogens problem(Cotter, Ross, & Hill, 2013). Among these antimicrobial substances, bacteriocin attracts much attention in recent decades and various bacteriocins were purified and characterized aiming to inhibit both Gram

positive and/or Gram negative pathogens(Cavera, Arthur, Kashtanov, & Chikindas, 2015; Lu, Hu, Dang, & Liu, 2014; Lu, Yi, Dang, Dang, & Liu, 2014; Perez, Zendo, & Sonomoto, 2014). However, is there some other antimicrobial substance can be produced by lactic acid bacteria? Here we show a new antimicrobial substance produced by *Lactobacillus rhamnosus* LS-8 isolated from fermented milk of Xinjiang, China. We purified the antimicrobial substance and identified it as 4,6-dimethyl-1,2,5-triazepane-3,7-dione ($C_6H_{11}N_3O_2$) by HRMS and NMR. It was heat-stable, resistant to enzymes and active in acidic condition. Most importantly, it has good inhibitory effect on foodborne multidrug resistant pathogens.

2. Materials and methods

2.1 Isolation and identification of antimicrobial LAB strains

Serial dilution method on MRS plate was used to screen potential LAB isolates from pickled vegetables. The isolated LABs were further screened by double plate method to obtain potential antimicrobial agents producers as described before(Lü, Yi, Dang, Dang, & Liu, 2014). *Escherichia coli* ATCC 25922 (shorten as *E.coli* in latter context) and *Staphylococcus aureus* ATCC 29213 (shorten as *S. aureus* in latter context) were used as indicator strains. The strain with the highest antimicrobial activity was selected and designated as LS-8, after which it was identified based on its 16S rDNA sequence.

2.2 Growth dynamics of LS-8 strain

The LS-8 strain was inoculated into 2 L MRS liquid medium and statically cultured at 30 °C for 72 h. During this process, 10 mL cell culture was taken out every 4 h and cell density was measured at 600 nm by a UV-2102PCS spectrophotometer (UNICO, USA). After removing cells by centrifugation (10000 rpm, 10 min, 4 °C), the supernatant was extracted by ethyl acetate (ethyl acetate: the supernatant=1:1) and concentrated by evaporation as crude antimicrobial substances. Antimicrobial activity of the crude sample was assayed by double plate method as described in 2.1.

2.3 Purification of antimicrobial substance

The LS-8 strain was statically incubated in MRS medium (6 L) at 30 °C for 72h. The cell-free supernatant was obtained by centrifugation (7000 rpm, 15 min, 4°C). After extracted by ethyl acetate, the saturated sodium hydroxide-ethanol solution was used to precipitate the organic acids inside the crude organic extract. Thereafter the organic supernatant was concentrated by rotary evaporation.

The crude ethyl acetate extract was subjected to an AKTA purifier 100 (GE, Sweden) equipped with a polypeptide column (GE superdex peptide 10/300 GL column with 100-7000 separation range) for purification, which was equilibrated with 10 mM citric-phosphate buffer (pH 5.0). 500 µL crude extract was eluted by same buffer at a flow of 0.5 mL/min and the elution process was monitored by UV detector

at 280nm and 254nm. Fractions (1 mL) were automatically collected according to peak, after which antimicrobial activity of fractions was assayed by method described in 2.1.

The fraction with highest antimicrobial activity was further purified by an analytical RP-HPLC (Waters 1525, USA) equipped with a SunFire™ C18 column (150×4.6 mm, 5 μm). Solvent A (water: acetonitrile=99:1) and Solvent B (acetonitrile 100 %) were used for HPLC purification. Linear gradient elution of acetonitrile from 1% to 10% in 10min, then from 10%-100% in next 20min at a flow rate of 0.8 mL/min and monitored at 280 nm and 253 nm. Fractions were collected according to peak. The purity of fraction with maximum antimicrobial activity was identified by the analytical HPLC again, after which the purified antimicrobial substance was designated as LS-8-25.

2.4 Structure analysis

The molecular mass was determined by LC-MS (MS: Bruker, maXis 4G equipped with the standard ESI source in positive ionization mode), in which an ion trap mass spectrometer and C 18 reverse phase column (Waters, USA) were used for analysis. Sample (25 mL) was injected after equilibration by 1% (v/v) acetonitrile. Linear gradient elution of acetonitrile increased from 1% to 10% in 10min, then from 10% to 100% in next 20min at a flow rate of 0.8 mL/min.

The isolated LS-8-25 was examined by NMR. After lyophilization, LS-8-25 dissolved in D₂O was analyzed by a Bruker NMR spectrometer (Avance III 500 MHz,

Switzerland). Proton nuclear magnetic resonance (^1H NMR) spectrum was recorded on 500 MHz spectrometers and carbon nuclear magnetic resonance (^{13}C NMR) spectrum was recorded on 125 MHz spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent signals (D_2O , 4.79 ppm for ^1H NMR). Data are reported as follows: chemical shift (δ), multiplicity (d = doublet, q = quartet, m = multiplet).

2.5 Antibacterial activity

Antibacterial activity of LS-8-25 was assayed by a modified method (Avonts, Uytven, & Vuyst, 2004; De Vuyst, Vincent, Makras, Leroy, & Pot, 2009). Briefly, equal volumes (50 μL) of two-fold dilutions sample and indicators (10^6 CFU/mL) were mixed in 96 well plate (Costar, Corning, USA) and incubated at 37 $^\circ\text{C}$ for 24 h, *E. coli* and *S. aureus* were used as indicators. The antibacterial activity was expressed in arbitrary units per mL ($\text{AU} \cdot \text{mL}^{-1}$), which was defined as the reciprocal of the highest dilution multiplied by 100.

2.6 Sensitivity to temperature, pH, and enzymes

The effects of temperature, pH and enzymes on LS-8-25 were determined by described method (Lü, et al., 2014). The 100 μL ($320 \text{ AU} \cdot \text{mL}^{-1}$) LS-8-25 was treated at 60 $^\circ\text{C}$, 80 $^\circ\text{C}$, 100 $^\circ\text{C}$, 121 $^\circ\text{C}$ for 30 min, 30 min, 20 min, 20 min respectively. Untreated sample was used as control.

To investigate the effect of pH, 1 mL sample was adjusted to pH 2 to 11 by using

1 M HCl and 1 M NaOH(Gurban oglu Gulahmadov, et al., 2006), the residual antimicrobial activity was assayed by the method described in 2.1. Before determining the residual antimicrobial activity, these different pH sample solutions were incubated at 37 °C for 4 h, then 100 μ L (160 AU $\cdot$ mL⁻¹) sample was taken for antimicrobial activity assay. The same pH buffer without LS-8-25 was used as control.

In order to analyze the enzyme sensitivity of LS-8-25, α - amylase (10 mg $\cdot$ mL⁻¹), pepsin(10 mg $\cdot$ mL⁻¹), papain, (10 mg $\cdot$ mL⁻¹) trypsin(10 mg $\cdot$ mL⁻¹), proteinase K(10 mg $\cdot$ mL⁻¹) (all from Sigma, USA) were used and dissolved in corresponding optimal buffer at 200 μ g $\cdot$ mL⁻¹, pH3.0 sodium-citrate buffer, pH7.0 sodium-phosphate buffer, pH7.0 sodium-phosphate buffer, pH7.0 Tris-HCl buffer, pH7.0 sodium-phosphate buffer respectively. 100 μ L (216 AU $\cdot$ mL⁻¹) LS-8-25 was added into the enzyme solutions. After incubated at 37 °C for 4 h, the mixture was boiled for 5 min to inactivate enzyme. The control as follows: 1 the sample without the enzyme treatment was dissolved in sterile ultrapure water; 2 corresponding enzyme-containing buffer without samples; 3 the sample was dissolved in the corresponding buffer without the enzyme treatment.

The residual anti-microbial activity was evaluated by the method described in 2.1.

2.7 The effect of LS-8-25 on indicator strains

Growth curves of *E. coli* and *S. aureus* were investigated. LS-8-25(192AU $\cdot$ mL⁻¹)

was added into culture when the indicators were cultivated to exponential phase (4 h) .
Bacterium suspensions were taken out each hour for 24 h and absorbance at 600 nm
was measured. Control was indicators growth without the LS-8-25.

2.8 Antimicrobial spectrum

Twenty-two indicator strains including Gram-positive and Gram-negative
food-borne pathogenic bacteria (Table 1) were used to determine the antimicrobial
spectrum of LS-8-25($320 \text{ AU} \cdot \text{mL}^{-1}$). The anti-microbial activity was evaluated by the
method described in 2.1.

2.9 SEM and TEM

The scanning electron microscope (SEM) and transmission electron microscope
(TEM) were used to investigate the effect of LS-8-25 on indicator strains (*E. coli* and
S. aureus).

The indicator strains (10^6 CFU/mL) were re-suspended in fresh LS media and
treated by LS-8-25 ($160 \text{ AU} \cdot \text{mL}^{-1}$) at 37 °C for 30 min and 2 h respectively. After
treatment, the indicators were collected by centrifugation and washed using PBS (0.1
M, pH 7.2), then fixed with 2.5% glutaraldehyde overnight at 4 °C. The cells were
washed by PBS again and post-fixed in 1% osmium tetroxide at 4 °C for 2 h. After
washing, the cells were dehydrated in a series of ethanol solutions and permeated in
white resin. Embedding was done at 60°C for 48 h. The ultrathin sections were cut
and observed by a transmission electron micro-scope (JEOL, Japan).

For SEM observation, the indicator strains were treated same as described in TEM analysis until post fixation by osmium tetroxide, thereafter it was treated progressively by 50%, 70%, 80%, and 90% ethanol for 10 min. 100% ethanol for 20 min and 100% acetone for 20 min. Isoamyl acetate was used to replace ethanol for 10 min twice at last. Then cells were dried by CO₂ for 4 h and coated with gold. The field emission scanning electron microscope S-4800 (Hitachi, Japan) was used to observe cell ultrastructure.

2.10 Statistical analysis

All experiments were carried out in triplicate. Results were statistically analyzed by ANOVA and followed by Duncan's multiple range test using Origin Pro7.5. In all difference analysis, $p < 0.05$ was considered as significant level.

3. Results and discussion

3.1 Isolation and identification of LS-8 strain

LS-8 with highest antibacterial activity was selected and was identified as *Lactobacillus rhamnous* according to 16S rDNA sequence (1482bp, Genbank accession No. KJ152776) after BLAST searching in NCBI (identity $\geq$ 99%, E value=0) .

3.2 Growth dynamics of LS-8 and the effect of LS-8-25 on indicator strains

Fig 1

Fig 2

Fig.1 indicates that LS-8 strain reaches the stationary phase after 40 h, the antimicrobial activity starts from the exponential growth period and the maximum antimicrobial activity is observed in the stationary phase (45 h-60 h). Based on the growth curve and antimicrobial activity, the optimal growth time for LS-8 strain to produce antimicrobial substance in MRS at 30 °C was 72 h. Additionally, LS-8-25 inhibited the growth of both *E. coli* and *S. aureus* in early logarithmic-phase obviously as shown in Fig. 2.

3.3 Purification of LS-8-25

Fig. 3

The LS-8 strain was incubated in MRS medium at 30 °C for 72h, after centrifuged, the supernatant was extracted by using ethyl acetate. The crude organic extract was concentrated by rotary evaporation at 50 °C. The concentrated crude sample was purified by AKTA purifier system equipped with polypeptide column. As is shown in Fig.3, there are four peaks isolated, among which the second peak (F2) has the maximum antimicrobial activity. The fraction (F2) was further purified by RP-HPLC, by which seven peaks were observed and collected (Fig.3). Peak F₂₅ with highest antimicrobial activity was further checked by HPLC to verify its purity, after which it was found to be in pure form and designated as LS-8-25.

3.4 Molecular mass and nuclear magnetic resonance analysis

The molecular mass of LS-8-25 was 157.09 Da determined by high resolution MS (Fig.4). Combined with NMR results (Fig.5), the structure of LS-8-25 was established as follow,

4,6-dimethyl-1,2,5-triazepane-3,7-dione: $^1\text{H-NMR}$ (500MHz, D_2O), $\delta(\text{ppm})$:1.33-1.35(d,6H, $J=7\text{Hz}$,-CH $_3$),4.27-4.31(q,2H, $J=7\text{Hz}$,-CH-),4.7-5.0(m,3H,-NH - and D_2O solvent residual signals); $^{13}\text{C-NMR}$ (125 MHz, D_2O) $\delta(\text{ppm})$:19.32, 66.56, 178.86 ; MS(ES-API) m/z 158.0925(M^+) .

The molecular formula of LS-8-25 was $\text{C}_6\text{H}_{11}\text{N}_3\text{O}_2$. It was deduced that it had symmetric structure and enol interconvert because the two peaks in HPLC have same mass spectrum. According to the structure, this is the novel antimicrobial substance produced by *Lactobacillus rhamnosus*.

3.5 Antimicrobial spectrum and sensitivity to heat, pH and enzymes

As is shown in Table 1 that LS-8-25 exhibits a broad anti-microbial spectrum against the indicator strains, including both Gram-negative and Gram-positive

← Fig. 4

Fig 5

← Table 1

bacteria. More important, LS-8-25 has good antimicrobial activity on these multidrug resistance pathogens, which make it a potent antibiotic to defeat the superbugs.

← **Table 2**

LS-8-25 showed good heat stability even treated at 121°C for 20 min, there was no significant differences between different temperatures ($p > 0.05$). However, LS-8-25 only has activity under acidic conditions (Table 2).

← **Table 3**

It is shown in Table 3 that LS-8-25 is not sensitive to pepsin, papain, trypsin, proteinase K and α - amylase, which is different with other antibacterial substances previously reported(Lash, Mysliwiec, & Gourama, 2005).

3.6 SEM and TEM

← **Fig 6**

The cell shape of *E. coli* and *S. aureus* was destroyed after incubation with LS-8-25 (Fig. 6). After incubation with LS-8-25 for 30 min, membrane integrity appeared to be destroyed, the cells began to shrink (arrow1 and arrow 3 in Fig.6). After incubated for 2 h, severe damage can be seen on the surface of target cells (arrow 2 and arrow 4 in Fig.6). The leakage of cell content becomes more serious with the increase of treatment time (arrow 5, arrow 6, arrow 7 and arrow 8 in Fig.6). Antibacterial substances can inhibit or kill target bugs by various mechanism(Brogden, 2005; Derouaux, et al., 2011; Yang, et al., 2013), among which pore formation is thought to be the key mode of action(Elias Fatta1, 1994). The accurate inhibition mechanism needs further investigation in the future.

4. Conclusions

This is first time to purify a novel variety of antimicrobial substance with cyclic structure from lactic acid bacteria, which can inhibit both gram positive and gram negative pathogens including some multi-drug resistance pathogens. It can be deduced from the discovery that common bacteria can possibly be new antibiotics and preservatives producers. Moreover, these strains can possibly reduce utilization of antibiotics through changing gut microbiota balance.

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333 Captions of Figures and Tables

334 **Figure 1** Production of active substance during the growth of LS-8 strain and
335 antimicrobial activity against *E. coli* and *S. aureus*. Growth curve of LS-8 (■); effect
336 of active substance on *S. aureus* (▣); effect of crude bacteriocin on *E. coli* (▤)

337

338 **Figure 2** The effect of crude sample on the growth of *E. coli* (■, control; □, addition
339 of crude sample) and *S. aureus* (●, control; ○, addition of crude sample)

340

341 **Figure3** Purification of antimicrobial substance produced by LS-8. A: purification by
342 AKTA purifier system equipped with polypeptide column (fraction F1 to F4 were
343 collected to assay the antimicrobial activity); B: purification by RP-HPLC (fraction
344 F21 to F27 were collected to assay the antimicrobial activity)

345

346 **Figure4** Analytical HPLC chromatography of LS-8-25 (A) and its high resolution
347 mass spectrum (B)

348

349 **Figure 5** NMR (HMBC spectra) of LS-8-25 (The group of $\text{CH}_3 \times 2$ with δ values of
350 ^1H -NMR and ^{13}C -NMR was 1.34 and 19.32 ppm, relating with CH and CO with δ values of
351 ^{13}C -NMR was 66.83 and 179.55 ppm, respectively. These results were showed in **Fig 5** as points
352 (1.345, 66.830), (1.336, 66.944), (1.346, 179.552) and (1.337, 179.336). The group of $\text{CH} \times 2$ with
353 δ values of ^1H -NMR and ^{13}C -NMR was 4.29 and 66.56 ppm, relating with CH_3 and CO with δ

values of ^{13}C -NMR was 19.40 and 179.09 ppm, respectively. These results were showed in **Fig 5** as points (4.299, 19.405), (4.299, 173.503) and (4.99, 179.089).The hydrogen atoms of D_2O covered $\text{NH}\times 3$ leading to the display of messy points with ^1H -NMR δ values around 4.79 ppm)

Figure 6 SEM (A, B) and TEM (C, D) of *S. aureus* (B, D) and *E. coli* (A, C). Untreated (A1, B1, C1, D1), treated withLS-8-25 for 30 min (A2, B2, C2, D2) and treated with LS-8-25for 2 h (A3, B3, C3, D3). White arrows (Arrow 1 to Arrow 8) indicate the destroyed sites after treated by LS-8-25

Table1 Antimicrobial spectrum of LS-8-25

Table2 Effect of heat, pH and enzymes on LS-8-25

Table 3 Effect of enzymes on LS-8-25

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1

Indicator strain	Inhibitory diameter/mm	Resistant antibiotics*
<i>Salmonella</i> ATCC51005	33.6 ± 0.2	-
<i>Staphylococcus aureus</i> ATCC29213	34.5 ± 0.1	-
<i>Staphylococcus aureus</i> ATCC25923	34.9 ± 0.3	-
<i>Sakazakii</i> ATCC29544	32.0 ± 0.1	-
<i>Enterococcus faecalis</i> ATCC51299	38.0 ± 0.2	-
<i>Escherichia coli</i> ATCC25922	36.5 ± 0.3	-
<i>Listeria monocytogenes</i>	38.0 ± 0.4	-
<i>Salmonella</i> 1006D	29.5 ± 0.2	AM,AMX,C,GM,K,NA,SXT,TC
<i>Salmonella</i> 36T	31.5 ± 0.1	AM,AMX,AN,C,CIP,CFP,CR O,GAT,GM,K,LVX,NA,SXT,TC
<i>Salmonella</i> 557D	30.0 ± 0.1	AM,AMX,AN,C,FOX,GAT,GM,K,LVX,NA,SXT,TC
<i>Salmonella</i> 798D	31.0 ± 0.2	AM,AMX,AN,C,CIP,CF,CR O,FOX,GAT,GM,K,LVX,NA,S,SXT,TC
<i>Salmonella</i> 87T4	31.5 ± 0.1	AM,AMX,AN,C,CIP,CF,CFP,CRO,GAT,GM,K,LVX,NA,S,SXT,TC
<i>S. aureus</i> 1	29.5 ± 0.2	CF,CIP,CLR,CXM,E,FOX,GM,LVX,NN,OFX,OX,PIP,VA
<i>S. aureus</i> 2	30.0 ± 0.3	CIP,FOX,GM,LVX,MI,NN,OFX,P,PIP,VA
<i>S. aureus</i> 3	27.5 ± 0.2	CIP,FOX,GM,LVX,MI,NN,OFX,VA
<i>S. aureus</i> 4	30.0 ± 0.1	CIP,FOX,GM,LVX,MI,NN,OFX,VA
<i>S. aureus</i> 5	28.0 ± 0.1	CIP,FOX,GM,LVX,MI,NN,OFX,OX,VA
<i>Sakazakii</i> 6-12 (1)	30.0 ± 0.3	FOX,RA
<i>Sakazakii</i> 6-12 (2)	30.5 ± 0.2	C,RA,TC
<i>Sakazakii</i> 11-18 (2)	32.0 ± 0.1	CIP,RA,S
<i>Sakazakii</i> 14-18 (2)	33.0 ± 0.3	CIP,RA,S
<i>Sakazakii</i> 18-15 (2)	30.5 ± 0.2	AM,AMX,C,NA,RA,S,TC

*AM = ampicillin, AMX = amoxicillin, AN = amikacin, C = chloramphenicol, CIP = ciprofloxacin, CF = cephalothin, CFP = cefoperazone, CLR = clarithromycin, CRO = ceftriaxome, CXM = cefuroxime, FOX = ceftiofur, GAT = gatifloxacin, GM = gentamicin, K = kanamycin, LVX = levofloxacin, MI = minocycline, NA = nalidixic acid, NN = tobramycin, OFX = ofloxacin, OX = oxacillin, P = penicillin, PIP = piperacillin, RA = rifampin, S = streptomycin, S = streptomycin, SXT = trimethoprim-sulfamethoxazole, TC = tetracycline, VA = vancomycin.

Table 2

Treatment	Residual antimicrobial activity/mm	
	<i>E. coli</i>	<i>S. aureus</i>
	ATCC25922	ATCC25923
Temperature		
control	28.5 ± 0.1 ^b	29.0 ± 0.2 ^{bc}
60℃	28.5 ± 0.3 ^b	31.5 ± 0.1 ^a
80℃	28.5 ± 0.1 ^b	29.5 ± 0.2 ^{bc}
100℃	29.0 ± 0.2 ^b	30.0 ± 0.3 ^b
120℃	30.5 ± 0.3 ^a	28.5 ± 0.2 ^c
pH		
2 (control 10.5 ± 0.2)	30.0 ± 0.4 ^a	32.5 ± 0.1 ^a
3 (control 0.0 ± 0.0)	31.5 ± 0.2 ^a	31.0 ± 0.3 ^b
4 (control 0.0 ± 0.0)	30.5 ± 0.2 ^a	29.5 ± 0.2 ^b
5 (control 0.0 ± 0.0)	21.5 ± 0.1 ^b	22.0 ± 0.2 ^c
6 (control 0.0 ± 0.0)	15.0 ± 0.0 ^c	15.5 ± 0.2 ^d
7-11 (control 0.0 ± 0.0)	0.0 ± 0.0 ^d	0.0 ± 0.0 ^e

Duncan test , $p<0.05$

Table 3

Treatment	Residual antimicrobial activity/mm	
	<i>E. coli</i> ATCC25922	<i>S. aureus</i> ATCC25923
Pepsin	21.5 ± 0.2 ^{ab}	21.0 ± 0.2 ^a
Pe-1	21.5 ± 0.3 ^a	21.5 ± 0.1 ^a
Pe-2	0.0 ± 0.0 ^c	0.0 ± 0.0 ^b
Pe-3	21.0 ± 0.2 ^b	21.0 ± 0.1 ^a
Trypsin	22.0 ± 0.2 ^b	21.5 ± 0.3 ^a
Try-1	22.0 ± 0.1 ^b	21.5 ± 0.2 ^a
Try-2	0.0 ± 0.0 ^c	0.0 ± 0.0 ^b
Try-3	23.5 ± 0.1 ^a	21.5 ± 0.2 ^a
Papain	20.0 ± 0.1 ^b	20.5 ± 0.1 ^a
Pa-1	22.5 ± 0.2 ^a	20.8 ± 0.1 ^a
Pa-2	0.0 ± 0.0 ^c	0.0 ± 0.0 ^b
Pa-3	22.0 ± 0.1 ^a	20.5 ± 0.1 ^a
Proteinase K	19.5 ± 0.1 ^a	30.5 ± 0.1 ^a
PK-1	22.5 ± 0.1 ^b	30.5 ± 0.1 ^a
PK-2	0.0 ± 0.0 ^d	0.0 ± 0.0 ^c
PK-3	20.5 ± 0.1 ^c	20.0 ± 0.1 ^b
α -amylase	22.5 ± 0.3 ^a	20.7 ± 0.1 ^b
α-1	23.1 ± 0.2 ^a	22.5 ± 0.1 ^a
α-2	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c
α-3	21.0 ± 0.1 ^b	20.3 ± 0.1 ^b

1 the sample without the enzyme treatment was dissolved in sterile ultrapure;

2 corresponding enzyme-containing buffer without samples;

3 the sample was dissolved in the corresponding buffer without the enzyme treatment.

Duncan test, $p < 0.05$