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ARTICLE

Rapid identification and quantification of antibiotic susceptibility of lactic acid bacteria using surface enhanced Raman spectroscopy

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Overuse and misuse of antibiotics have resulted in widely antibiotics resistance in bacteria.In this study, surface-enhanced Raman spectroscopy (SERS) was used to rapidly characterize the responses of lactic acid bacteria (LAB) to antibiotics targeting bacterial cell wall. *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) ATCC11842 was used to represent LAB strains that are widely used in food industry. Penicillin G, ampicillin and vancomycin were used to treat *Lb. bulgaricus*ATCC11842 at different toxic levels for 30, 60 and 90 min, respectively. SERS spectra of *Lb. bulgaricus* after each treatment were collected to characterize the responses of *Lb. bulgaricus.* The collected spectra were analyzed by principal components analysis (PCA) and partial least squares regression (PLSR). Results suggested that all three antibiotics induced significant peak changes in LAB, and the spectral changes induced by each antibiotic treatment were significantly different. Moreover, the antibiotic induced spectral changes in *Lb. bulgaricus* had good correlation with its proliferation ability and could be potentially used as a base for rapid quantification of antibiotic susceptibility of bacteria. The developed method could detect thereaction of LAB to antibiotics treatments within 3 h. Therefore, the developed SERS method could be used to rapidly discriminate and quantify the antibiotic susceptibility of LAB.Moreover, such a rapid and direct method could contribute to microbial antibiotic susceptibility detection researches.

Introduction

Antibiotics are major tools used to treat bacterial infections, however, overuse and misuse of antibiotics have resulted in antibiotics resistance in bacteria. When exposed to antibiotics, the susceptible bacterial cells will be inhibited or die, but the resistant bacterial cells will survive and continue to growth. Widely emergence of antibiotic resistant phenotypes makes the antibiotic ineffective and causes serious health and economic problems [1,2]. Rapid identification of resistant strains is predominant for fighting against and controlling the dissemination of antibiotic resistance. However, traditional methods to discriminate antibiotic sensitive and resistant bacteria are relatively time consuming.

Culture based method like disk diffusion, gradient diffusion and agar/broth dilution method are widely used for discriminating antibiotic susceptible and resistant strains [3]. These methods require cultivation of bacterial cells and are especially time consuming for slow-growing bacteria. PCR-based methods are much faster than the traditional methods. PCR-based methods determine the antibiotic susceptibility of bacteria through detecting the corresponding genetic determinants for resistant phenotype in genome or plasmid [4–6]. However, for some newly found resistant phenotypes the related genetic traits are not yet identified, thus making the PCR-based methods useless. Therefore, a robust rapid method for determining the antibiotic susceptibility of bacteria is urgently needed.

Herein, we aimed at developing a SERS method monitoring the chemical changes in bacteria at the early period of antibiotic treatment for direct determination of antibiotic susceptibility. SERS is a novel technique that combines Raman spectroscopy and nanotechnology. Noble metal nanoparticles, such as silver and gold nanoparticles (NPs), interact with bacterial cells and can specifically enhance the Raman scattering signals of biomolecules in the cell to generate “fingerprints” of the bacteria [7–9]. When bacterial cells are interacted with antibiotics, the collected SERS signals that originated from the bacterial cell can be used to reflect the antibiotic induced biochemical changes. Monitoring antibiotic induced biochemical changes in bacteria can help us understand the reaction of bacterial cells to antibiotic treatments as well as determine the susceptibility of bacterial cells [10–15]. SERS offers a great potential solution for rapid identification of antibiotic susceptibility of bacteria [16–18].We have developed a rapid and simple SERS method to characterize antibiotics induced biochemical changes in *Lactococcus lactis* and this method showed the potential to assess the antibiotic susceptibility of bacterial cells [19].

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The objective of this study is to develop a SERS method that can both rapidly discriminate the antibiotic susceptibility of bacteria and predict their proliferation ability after antibiotic treatment. Lactic acid bacteria (LAB) were used as the model bacteria in this study. LAB are a group of Gram-positive bacteria that widely used as probiotics and fermentation starters. Since the resistance genes in pathogenic bacteria and in LAB are similar[20,21], increased attention has given to LAB as reservoirs of antibiotic resistant genes. This caused some safety issues for the application of LAB strains as probiotics and fermentation starters since they might transfer their resistant genes to pathogens in human gastrointestinal [22,23]. And once pathogens acquired the resistance, antibiotics normally used to treat infections in human would be ineffective. Therefore, identification of antibiotic resistance phenotypes in LAB is critically important. In this study, *Lactobacillus bulgaricus*, which was widely used in food fermentation process, was selected to represent the LAB strains used in food industry and to assess the feasibility of the developed methods.

Three cell wall targeted antibiotics, penicillin G, ampicillin and vancomycin were tested in this study. Penicillin G is the first antibiotic found by human in 1928 and can inhibit the formation of peptidoglycan cross-links in bacterial cell wall. Ampicillin is a member of the amino-penicillin family and differs from penicillin G by the presence of a $–NH_2$ group. The mode of actions for ampicillin and penicillin G are similar [24]. Ampicillin is an inhibitor of transpeptidase, which is needed by bacteria to synthesize their cell walls. Vancomycin also targets the bacterial cell wall but its mode of action is different from penicillin G and ampicillin [25]. It is a glycopeptide antibiotic and can bind on the cell wall to stop N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG) from forming the skeleton of peptidoglycan, which is the main component of the Gram-positive bacteria cell wall. SERS spectra of LAB interacted with the selected antibiotics at different toxic levels for 30, 60 and 90 min were collected and analyzed to assess the antibiotic susceptibility of LAB. SERS spectra could be rapidly obtained using the developed simple SERS method based on commercially available gold NPs colloids. Spectral analysis was done using both principal components analysis (PCA) and partial least squares regression (PLSR). As far as we know, this is the first report of using SERS spectral changes to predict the proliferation ability of LAB after treating with cell wall targeted antibiotics. Moreover, such a rapid and direct method could contribute to microbial antibiotic susceptibility detection researches.

Experimental section

Strain and chemicals

Lactobacillus delbrueckii subsp. *bulgaricus* (*Lb. bulgaricus*) ATCC11842was used in this study. Ampicillin sodium salt (Fisher Scientific, Fair Lawn, NJ, USA), penicillin G (Research Products International Crop, IL, USA) and vancomycin hydrochloride (Fisher Scientific, Fair Lawn, NJ, USA) were purchased through Fisher Scientific. Antibiotic stock solutions (10 mg/mL) were prepared by dissolving antibiotic powders in double distilled water, respectively.

Preparation of bacteria sample

Lb. bulgaricus was revived according to the manufacture's instruction. The growth curve of *Lb. bulgaricus* in MRS broth was depicted based on the optical absorbance at 600 nm ($OD_{600\ nm}$) versus the time of cultivation using UV-vis method (Fig.S1). Minimum inhibitory concentration (MIC) was defined as the lowest concentration of an antibiotic that inhibits the visible growth of microbe after overnight incubation. MICs of ampicillin, pencillin G and vancomycin for *Lb. bulgaricus* were detected using the micro-dilution method, respectively. In preparation for SERS,*Lb. bulgaricus* was first cultivated in MRS broth at 37 °C to reach its log-phase. Different volumes of antibiotics stock solutions were added into 1 mL of *Lb. bulgaricus* culture to reach the concentration of 0.5, 1 and 2 times of their MICs, respectively. After that, the *Lb. bulgaricus* cultures were incubated at 37 °C for 0, 30, 60 and90 min. After incubation, the bacterial cells were collected usingcentrifugation (5000 g, 5 min) and subsequently washed three times with sterile 154 mmol/L NaCl. After washing, bacterial cells were collected and resuspended in 154 mmol/L NaCl. And the bacterial suspension was used to prepare sample for SERS. The proliferation ability of bacterial cells in this suspension was tested using agar dilution method.

SERS spectra collection

SERS spectra of the bacterial samples were collected using 50 nm citrate capped gold nanoparticles (0.05 mg/mL, Nanopartz™) as substrates. 40 µL of the gold nanoparticles was added to 10 µL of the bacteria suspension and vortexed and incubated for 30 min at room temperature (20 ~ 23 °C). After incubation, the mixture was spotted on the gold slide in duplicate and allowed to dry at room temperature. After drying, SERS spectra were collected directly from the samples spots using a DXR Raman Spectro-microscope (Thermo Scientific, Madison, WI). SERS spectra were collected with the following conditions:780 nm excitation wavelength, 1 mW laser power and 50 µm slit width, 50× objective, 2 s integration time. OMNIC™ software version 9.1 was used to control the Raman instrument. 10 spectra were collected for each sample spot within the range of 500–2200 cm^{-1}.

Data analysis

The spectral data were analyzed using TQ Analyst software (version 8.0) developed by Thermo Scientific. The SERS spectra obtained from multiple spots of each sample were averaged. The average spectra of different samples were processed by secondary derivative transformation to remove the baseline and separate overlapped peaks and the second derivative SERS spectra were normalized on the basis of the characteristic peak of citrate-reduced gold NPs appearing between 2160 and 2080 cm^{-1} before comparing against other samples. PCA and PLSR were used to discriminate and quantify the effects of antibiotic treatments on *Lb. bulgaricus*. PLSR is a multivariate method. In this study PLSR was used to assess the performance of using SERS spectra to predict the proliferation abilities of LAB after antibiotics treatments. A total of 150 variables (i.e., Raman intensities in the region of (500–2200 cm^{-1}) were used as the inputs of the model. The SERS spectra have been centred first when PLSR was performed, and leave-one-out correction, which is an internal cross validation, was adopted as validation method to establish PLSR model.

Results and discussion

Determination of MICs

Lb. bulgaricus strains are widely used in yogurt fermentation. *Lb. bulgaricus*ATCC11842 was chosen as representative to develop a rapid SERS method for the determination of antibiotic susceptibility in LAB strains used in food industry. Micro dilution method was used to detect the MICs of penicillin G, ampicillin and vancomycin for *Lb. bulgaricus*ATCC11842, respectively. Results were summarized in Table 1. Based on MIC breakpoints, bacteria could be divided into three categories of susceptibility: susceptible, intermediate or indeterminate, and resistant [26]. In this study, half MIC, which fails to inhibit the growth of bacterial cells, was used to characterize the reaction of *Lb. bulgaricus* to less lethal antibiotic treatment. And twice MIC that definitely inhibits the growth of almost all the bacterial cells after overnight cultivation was used to study symptom of *Lb. bulgaricus* exposed to lethal antibiotic treatment [14].

Table 1 MICs of different antibiotics for *Lb. bulgaricus* ATCC11842.

Antibiotic	Penicillin G	Ampicillin	Vancomycin
MIC (μg/mL)	0.125	0.125	0.250

SERS study of the responses of LAB to antibiotics treatments

Given the selected antibiotics work for bacteria cells in growth and dividing, *Lb. bulgaricus* in mid log-phase (OD_{600}~1.3) was used in this study. SERS spectra of *Lb. bulgaricus* exposed to penicillin G, ampicillin and vancomycin at 0.5, 1 and 2 times of MIC were collected using commercial citrate capped gold NPs as substrates. Our previous results proved this method had good consistency and reproducibility [19]. To further reduce the variation, average spectra were normalized on the basis of the characteristic peak of citrate-reduced gold NPs appearing between 2160 and 2080 cm^{-1} for fair comparisons.

SERS study was started with penicillin G, the first antibiotic found by human. 2nd derivative SERS spectra of *Lb. bulgaricus*ATCC11842 treated with penicillin G at 0.5, 1 and 2 times of MIC for 0 (control), 30, 60 and 90 min were depicted in Fig. 1 (for raw SERS spectra see Fig. S1). Spectrum given in Fig. 1 was the mean of 15 raw spectra. As can be seen from Fig. 1, the intensity of SERS spectra of *Lb. bulgaricus*ATCC11842 treated with penicillin G at half MIC decreased gradually as the time extended. After 90 min of treatment, peaks at 1580 cm^{-1}, 1270 cm^{-1} and 850 cm^{-1} almost disappeared. For*Lb. bulgaricus* treated with penicillin G at MIC a similar trend was observed. It was noticeable that after *Lb. bulgaricus* was treated with penicillin G at MIC for 90 min, intensity of the peak at 735 cm^{-1} decreased to only 19.15% of that in control. However, when bacterial cells were treated with penicillin G at twice MIC, variations were not only observed in peak intensity but also in the pattern of SERS spectrum. Specifically, after 30 min treatment the overall peak intensity decreased to about half of its original intensity, and peaks at 852, 1028, 1165 and 1580 cm^{-1} almost disappeared. After 60 min treatment the peak around 735 cm^{-1} was almost as high as that in the 30 min treatment, which was different from the observations in low concentration treatments. Moreover, peaks at 852, 1028, 1165 and 1580 cm^{-1} appeared again and a new peak at 1630 cm^{-1} was clearly observed, the peak at 1032 cm^{-1} increased, and the peak at 965 cm^{-1} decreased. After 90 min treatment, the pattern of spectrum was similar to that after 60 min treatment, but an increase in the peak at 1028 cm^{-1} was clearly observed.PCA was used to further classify the spectral data. The 3-D PCA score plot for the *Lb. bulgaricus*ATCC11842 treated with penicillin G was shown in Fig. S2 (Supporting information). Fig. S2 shows distance between control and the penicillin G treated samples increases as the treatment time and concentration increase, indicating that the penicillin G induced biochemical changes in bacterial cell wall is a time and dose dependent manner. Data cluster of control were slightly overlapped with the data cluster collected from *Lb. bulgaricus* treated with penicillin G at half MIC for 30 and 60 min and at MIC for 30 min, indicating no significant difference between these samples. However, obvious differences were found between the data cluster of control and the data cluster of *Lb. bulgaricus* treated with penicillin G at twice MIC, namely, the distance between data cluster of control and data cluster of the penicillin G treatment at twice MIC increased with the time extended. This was in a good agreement with the observations in the SERS spectra that after 30 min treatment spectral changes were mainly intensity decrease and after 60 and 90 min treatments obvious differences including both intensity and pattern changes were observed.

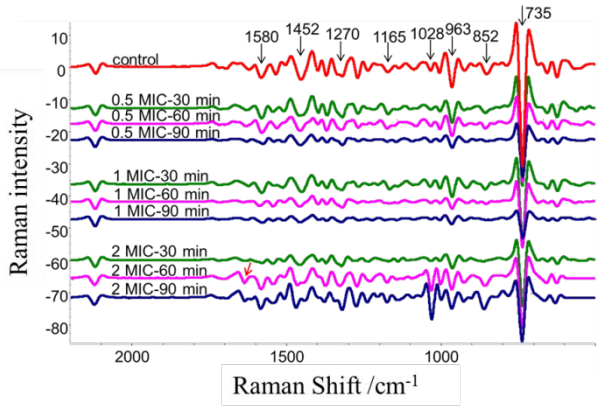

Fig. 1 2nd derivative SERS spectra of *Lb. Bulgaricus* ATCC11842 treated with penicillin G at different concentrations for 30, 60 and 90 min.

Ampicillin was tested after penicillin G. Basically the modes of action for penicillin G and ampicillin are similar. However, the extra $-NH_2$ molecule in ampicillin allows it to penetrate through the cell wall of Gram-negative bacteria, making it can act against Gram-negative bacteria as well as Gram-positive strains. The MIC of ampicillin for *Lb. bulgaricus*ATCC11842 is 0.125 μg/mL. 2nd derivative SERS spectra of *Lb. bulgaricus*ATCC11842 exposed to ampicillin at 0.063, 0.125 and 0.250μg/mL for 0 (control), 30, 60 and 90 minutes were collected and depicted in Fig. 2 (for raw SERS spectra see Fig. S3). Similar to the results observed in penicillin G treatment, when *Lb. bulgaricus*ATCC11842 was treated with ampicillin at half MIC and MIC, the overall peak intensity decreased as treatment time prolonged. However, when treated with

ampicillin at twice MIC for 60 min the overall peak intensity decreased obviously and the intensity of peak at 735 cm^{-1} was only 12.27% of that in control. After 90 min treatment, the spectral pattern was obviously different from control. New peaks at 1640 and 1612 cm^{-1} were observed, intensities of peak at ~1540, 1375, 1270 and 735 cm^{-1} increased and peaks at 1455 cm^{-1} decreased. The PCA plot (Supporting information Fig. S4) indicated that significant changes in *Lb. bulgaricus* could be observed after treating with ampicillin at half MIC for 90 min and at MIC and twice MIC for 60 min.

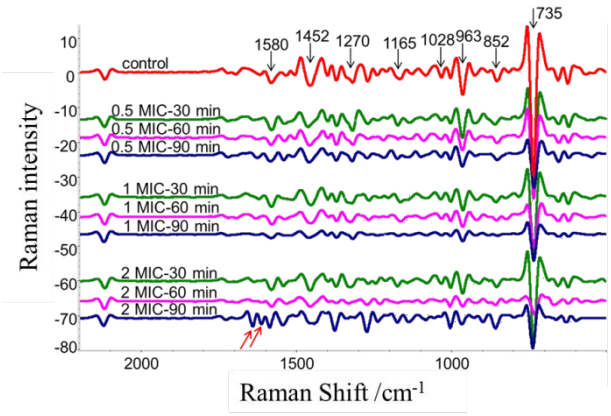

Fig. 2 2nd derivative SERS spectra of Lb. bulgaricus treated with ampicillin sodium salt at different concentrations for 30, 60 and 90 min.

Last but not least, vancomycin was tested and the results were given in Fig. 3 (for raw SERS spectra see Fig. S5). Though the action mode of vancomycin is different from penicillin G and ampicillin, results obtained from vancomycin treatment at half MIC and MIC were similar to that for penicillin G and ampicillin. However, when treated with vancomycin at twice MIC the spectral change was different from that in penicillin G and ampicillin treatments. After treated with vancomycin at twice MIC for 30 min, the overall peak intensity decreased. After 60 min treatment, new peaks at 1643 and 1612 cm^{-1} were observed, peaks at 1399 and 1368 cm^{-1} almost disappeared, and peaks at 1376 and 860 cm^{-1} increased. After 90 min treatment, a new peak at 1141 cm^{-1} was clearly observed, the peak at 1270 cm^{-1} increased obviously and the peak at 735 cm^{-1} decreased slightly. Similar to the results observed in Fig. S2 and Fig. S3, the PCA result for vancomycin treatment (Supporting information Fig. S6) showed minor difference between control and mild treatments. And difference between control and treatments of half MIC for 90 min and MIC and twice MIC for 60 and 90 min was significant.

From the results described above, we could also conclude that the spectral changes induced by penicillin G, ampicillin and vancomycin at twice MIC for 90 min were different. To further verify that SERS spectra of *Lb. bulgaricus* treated with these three antibiotics at twice MIC for 90 min were analyzed using principal components analysis (PCA). The PCA result was given in Fig. 4. As can be seen in Fig. 4, SERS spectra of *Lb. bulgaricus* and *Lb. bulgaricus* treated with penicillin G, ampicillin and vancomycin at twice MIC for 90 min were obviously different with each other. In Fig. 4, the first three components explained 85.8% of the difference and PC1, PC2 and PC3 accounted for 77.52%, 5.37% and 2.90%, respectively. This result indicated that the developed SERS method could discriminate the effects of different antibiotics in a short time.

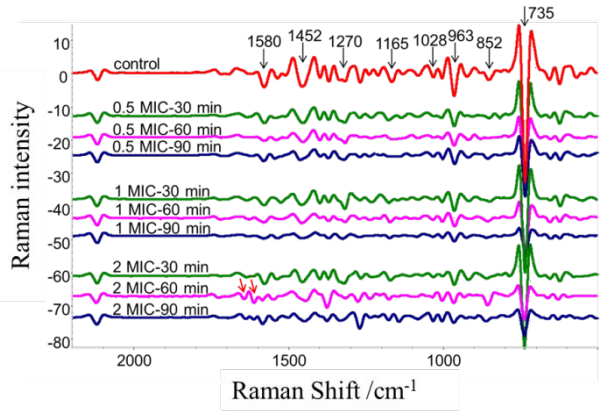

Fig. 3 2nd derivative SERS spectra of *Lb. bulgaricus* treated with vancomycin hydrochloride at different concentrations for 30, 60 and 90 min.

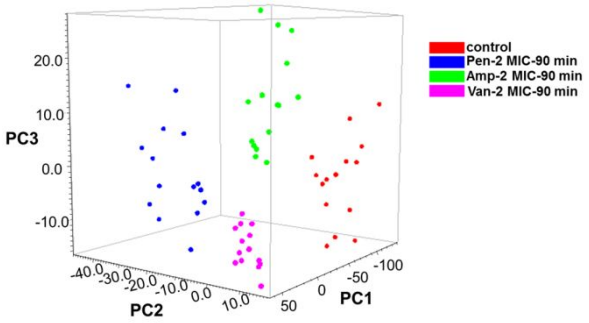

Fig. 4 3-D PCA plot of *Lb. bulgaricus treated* with penicillin G, ampicillin and vancomycin for 90 min.

Interpretation of the SERS spectra

To better understand the SERS spectra of *Lb. bulgaricus* ATCC11842 subjected to antibiotics targeting the bacterial cell wall, varied peaks were carefully assigned to the related biochemical components (Table 2) [27–35]. The highest peak in the spectrum of *Lb. bulgaricus* around 735 cm^{-1} mainly originated fromadenine and nucleic acids [34,35]. Peaks at 963, 1270, 1370 and 1580 cm^{-1} probably originated from proteins. When *Lb. bulgaricus* was treated with penicillin G, ampicillin and vancomycin at half MIC and MIC, nucleic acids (735 cm^{-1}) and proteins (963, 1270, and 1580 cm^{-1}) in the bacteria decreased as the time extended. Since no new spectral feature was detected, bacteria cells possibly still maintained a high integrity at this time. And the detected signal at 735 cm^{-1} probably originated from nucleic acids in the S-layer instead of DNA in the bacterial cell [35]. S-layer is a monomolecular layer composed of identical proteins or glycoproteins, and is incorporated onto the bacterial cell envelope. The presence of DNA in the S-layer has been reported [36–38]. Functions of proteins in gram-positive bacteria cell envelop are remarkably diverse [39]. Alterations in the structure of proteins might results in decreased metabolic activity [40]. Decrease of amide II (1580 cm^{-1}) and III (1270 cm^{-1}) in proteins might caused by self-defense actions in living bacterial cells.

Table 2 Tentative assignments of peaks varied when *Lb. bulgaricus* ATCC11842 was exposed to penicillin G, ampicillin and vancomycin. Variation was based on control and 90 min of antibiotic treatment. 参考文献格式需要修改

Raman shift/cm^{-1}	Pen G	Amp	Van	Assignment	Reference
735	↓	↓	↓	Adenine (nucleic acids)	Fan et al.[29], Kahraman et al.[30]
~850	↑	↑	↓	C–C stretching in 1,4-glycosidic link	Sundaram et al.[30]
898	—	↑	—	COC stretching	Maquelin et al.[28]
963	↓	↓	↓	C–C–C or C–C–N stretching in proteins	Kahraman et al.[25]
1002	↓	↑	—	C–C aromatic ring stretching (phenylalanine)	Kahraman et al.[25]
1028	↑	↓	↑	C–C stretching in lipid layer components	Sundaram et al.[30]
1136	↑	↑	↑	=C–C= of unsaturated fatty acids in lipids	Efrima and Zeiri[22]
1270	↓	↑	↑	Amide III	Jarvis et al.[24]
1320, ~1370	↑, ↓	↓, ↑	↓, ↓	C-H bend in protein	Kahraman et al.[25]
1452	↓	↓	↓	CH_2 bend in protein or lipid	Kahraman et al.[25]
~1540, 1580	↓	↑, ↑	↑, ↓	NH deformation and CN stretching in amide II	Prucek et al.[27]
1612	—	↑	↑	Tyrosine	Maquelin et al.[26]
~1635	↑	↑	↑	Amide I	Efrima and Zeiri[22]

When *Lb. bulgaricus* was treated with antibiotics at twice MIC the biochemical changes were more complicated. For penicillin G treatments, the contents of 1,4-glycosidic link (~850 cm^{-1}), aromatic protein (1002 cm^{-1}), C–C stretching in lipid (1028 cm^{-1}), amide III (1270 cm^{-1}), and amide II (1580 cm^{-1}) first decreased then increased gradually. The new peak observed around 1635 cm^{-1} after 60 min treatment was assigned to amide I [27]. Amide I was used to study the secondary structure of protein [41]. Observation of new amide I signal and variation of amide II and amide III signals probably indicated changes in the structure of proteins and might indicated an impaired activity in bacteria. Though the modes of action for ampicillin and penicillin G are similar, the observed biochemical changes in ampicillin treatments at twice MIC were different with that in penicillin G treatments. Specifically, increases in 1,4-glycosidic link (850 cm^{-1}), aromatic protein (1002 cm^{-1}), un-saturated fatty acids (1136 cm^{-1}), amide III (1270 cm^{-1}), and C-H bend in protein (1375 cm^{-1}) were observed after 90 min treatment. The peak at 735 cm^{-1} was decreased sharply after 60 min treatment and increased obviously after 90 min treatment. The interpretation for this phenomenon was that in the first 60 min treatment the peak at 735 cm^{-1} mainly represented nucleic acids in the S-layer and adenine; and after 90 min treatment this peak could also originated from DNA inside the bacterial cell since the cell wall of some bacteria started to rupture [19]. In addition to the Amide I (1635 cm^{-1}) signal, tyrosine (1612 cm^{-1}) signal was also detected in the ampicillin treatment. Presence of tyrosine in bacterial cell membrane might related to a narrower pore size of integral protein [42,43]. Observation of tyrosine signal as well as increased protein signals that mainly originated from the cell membrane possibly indicated damage of the bacterial cell wall. For vancomycin treatments, the detail biochemical changes in *Lb. bulgaricus* at twice MIC were different with penicillin G and ampicillin, but signals of amide I (1635 cm^{-1}) and tyrosine (1612 cm^{-1}) were also detected after 60 min treatment. It's noticeable that contents of 1,4-glycosidic link (850 cm^{-1}) and C-H bend in protein (1375 cm^{-1}) increased after 60 min but almost disappeared after 90 min when *Lb. bulgaricus* was exposed to vancomycin at twice MIC. Decrease in the peak of 1,4-glycosidic link possibly reflected that the effects of vancomycin could be detected within 90 min since the action mode of vancomycin was to inhibit the formation of 1,4-glycosidic linkthat connects NAM and NAG to form the skeleton of peptidoglycan.

Based on the observations in the SERS spectra of *Lb. bulgaricus* treated with antibiotics targeting the bacterial cell wall, we speculated that decrease in SERS intensity at the early time (~90 min) of interaction might related to self-defense action in *Lb. bulgaricus*, revealing that bacterial cells are still alive but was possibly inhibited. And peaks at 735, 1270 and 1580 cm^{-1} might be used as biomarkers to reflect the inhibitory effects of cell wall targeted antibiotics. New features observed in the SERS spectra of *Lb. bulgaricus* exposing to antibiotics for 90 min might cause by the damage of bacterial cell wall and peaks at 1612 and 1635 cm^{-1} might be used as indicators of bacteria subjecting to antibiotics at

lethal level. To further investigate the relation between spectral changes and the growth state of bacterial cells, proliferation abilities of bacterial cells after each antibiotic treatment were detected using agar dilution method.

Proliferation abilities of LAB after antibiotics treatments

Proliferation abilities of bacterial cells after treating with the selected antibiotics at half, one and two times of MIC for 30, 60 and 90 min were given in Fig. 5. As can be seen from Fig. 5(A), when *Lb. bulgaricus* was exposed to penicillin G at half MIC, no significant difference was observed in the proliferation ability of *Lb. bulgaricus* ability decreased slightly, but after 90 min exposure only 52.5±2.5% bacterial cells can proliferate. Moreover, the proliferation ability for exposed to penicillin G for different time. When *Lb. bulgaricus* was subjected to penicillin G at MIC, in the first 60 min the proliferation

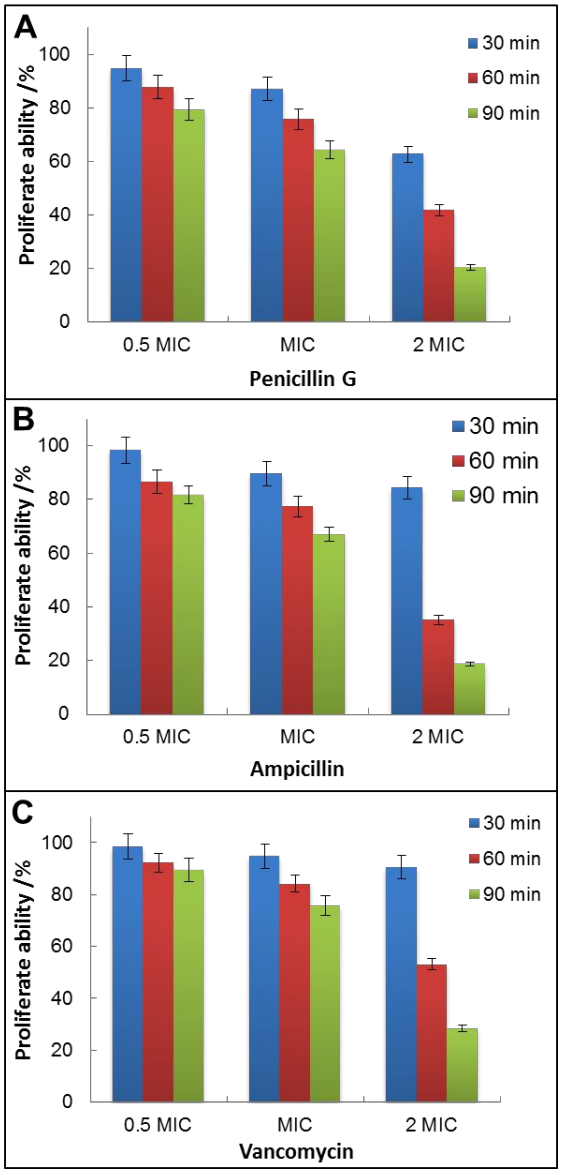

Fig. 5 Proliferation abilities of *Lb. Bulgaricus* ATCC11842 treated with (A) penicillin G, (B) ampicillin and (C) vancomycin at 0.5, 1 and 2 times of MIC for 30, 60 and 90 min.

Lb. bulgaricus decreased sharply when treated with penicillin G at twice MIC. After 30 min, the proliferation ability was 62.6±3.1%, which was significantly different from treating at half and one time of MIC for the same time. The proliferation ability for 60 min treatment was much lower than that for 30 min treatment and after 90 min treatment the proliferation ability was only 16.5±1.5%. Similar results were observed for ampicillin (Fig. 5(B)) and vancomycin (Fig. 5(C)) treatments. PLSR was then used to analyze the correlation of SERS spectral changes and proliferation abilities of LAB.

Correlation between the spectral changes and the proliferation abilities of LAB

Calibration models of the spectral changes and their corresponding proliferation abilities were set up using PLSR and the results were given in Fig. 6. The circles in the figure stand for calibration standards, the crosses stand for validation standards, and the input spectra were grouped using the Suggest Standards founction of TQ analyst (version 8.0). Actual PA in X axis is the standard values, namely the values tested using plate count colony method. Calculated PA in Y axis is the fitted values, namely the calculated values of the model using SERS spectra. As can be seen in Fig. 6, the correlation coefficients of the calibration models of penicillin G, ampicillin and vancomycin treatments were 0.9541, 0.9830 and 0.8823, respectively. Therefore, the spectral changes in the SERS spectra of bacterial cells had great correlations with their proliferation abilities and these calibration models could be potentially used to predict the proliferation ability of *Lb. bulgaricus* using its SERS spectrum. Two spectra were randomly picked from each group and used to validate the developed calibration models and PLSR models based on both calibration and validation data were given in Fig. S7 (Supporting information). The correlation coefficients of the PLSR validation models were 0.9534, 0.9582 and 0.8811, respectively. Though the correlation coefficients of the validation models were slightly decreased, they also indicated that SERS spectra of bacterial cells had good correlations with their proliferation abilities. The root mean squared errors of the calibration models of penicillin G, ampicillin and vancomycin treatments were 7.37, 4.12 and 10.7, respectively. The root mean squared errors of the validation models of penicillin G, ampicillin and vancomycin treatments were 7.41, 6.71 and 10.7 respectively, which were quite close to that of the corresponding calibration models, indicating the developed calibration models are robust for predicting the proliferation ability of bacteria. Spectra of the first factors used to build PLSR models were given in Fig. S6 (Supporting information). The first factors explained most of the spectral features used to develop the PLSR models. Fig. S8 shows that peaks at 1580, 1452, 963 and 735 cm^{-1}, which were decreased under the treatment of antibiotics, are the main peaks used in building PLSR models. Peak at 1612 cm^{-1}, which represented pattern change, was also considered in PLSR models. Therefore, the developed PLSR models considered both intensity changes and pattern change and had a good reasonableness.

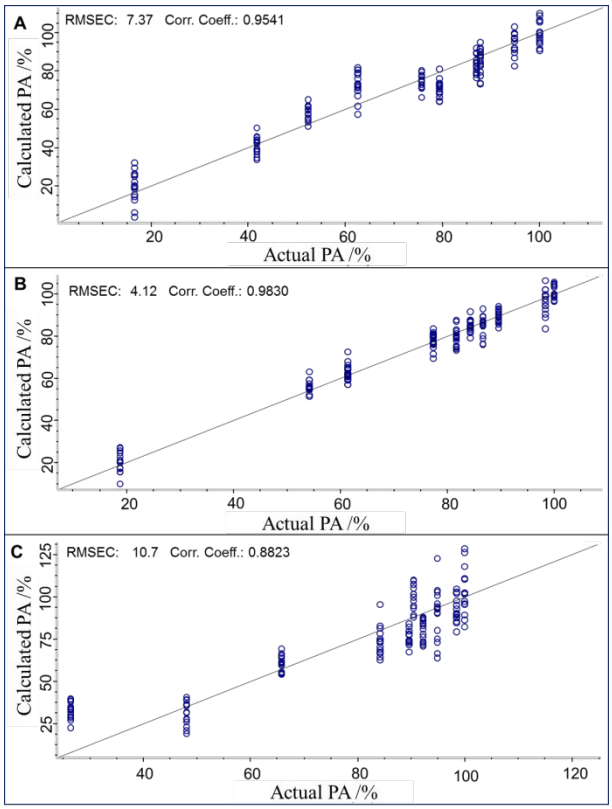

Fig. 6 PLSR plot of SERS spectra and the corresponding proliferation abilities, (A) penicillin G, (B) ampicillin, and (C) vancomycin (PA stands for proliferation ability).

Conclusions

In this study, a simple SERS method based on commercially available Au NPs was developed to directly determine the antibiotic susceptibility of LAB at the early period of antibiotic treatments (90 min). SERS spectra of *Lb. bulgaricus* exposed to antibiotic at half MIC, MIC and twice MIC for 90 min were significantly different. When *Lb. bulgaricus* was treated with antibiotics at half MIC and MIC, the overall peak intensity decreased and the corresponding proliferation ability were relatively high (above 50%). When treated at twice MIC, new peaks around 1612 and 1640 cm^{-1} were observed. These new peaks were assigned to amide I and tyrosine, respectively. At this time, low proliferation abilities were observed and these two peaks might indicate damage in bacteria cell wall caused by antibiotic treatments at lethal level. PCA analysis indicated that the spectral changes induced by different antibiotics were obviously different. PLSR analysis showed that spectral changes induced by antibiotics treatments in *Lb. bulgaricus* had great correlations with their proliferation abilities. Therefore, the developed SERS method showed great potential to discriminate the sensitive and resistant reactions of LAB and the effects of different antibiotics at the early period of treatment. Moreover, the proliferation ability of bacterial cells after antibiotic treatment could be quantified using the developed PLSR model. The detection took only 3 h in total, making the developed SERS method an incomparable rapid method for antibiotic susceptibility detection and quantification.

Conflicts of interest

There are no conflicts to declare.

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SERS was used to discriminate and quantify the antibiotic susceptibility of lactic acid bacteria at the early period of treatment.