Enzymatic Synthesis of KDN-Containing Sialylated Lactuloses and their Bacteriostatic Activities on *Staphylococcus aureus*

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Summary: Sialic acids are found in various biological tissues and are known to play important roles in many biological processes. However, many sialylated oligosaccharides are not adequately available to study their biological functions and potential uses. Herein, we reported an efficient synthetic approach to obtain sialylated lactulose containing deaminoneuraminc acid (KDN). Both KDNα2,3lactulose and KDNα2,6lactulose were synthesized via one-pot multienzyme sialylation system. The bacteriostatic activities of these two sialylated lactuloses against *Staphylococcus aureus* (*S. aureus*) were analyzed by using approach of optical density OD_{600} measurements. In addition, integrity of cell membrane of *S. aureus* was examined by fluorescence analysis, protein leakage quantification and electron scanning microscope (SEM). The results showed that the maximum inhibition ratios of KDNα2,3lactulose and KDNα2,6lactulose within the first 10 h was up to 34.33% and 32.18%, respectively. KDNα2,3lactulose displayed slightly better inhibition against the growth of *S. aureus* than that of KDNα2,6lactulose. The addition of KDNα2,3lactulose and KDNα2,6lactulose caused a significant decrease ($p<0.05$) in fluorescence intensity compared with control to 47.05±3.06% and 51.16±2.40, respectively, indicated that those two sialylated lactuloses had some extent interference with nucleic acids synthesis or caused decomposition of nucleic acids in *S. aureus* cells. Fluorescence microscopy results showed that *S. aureus* of KDNα2,3lactulose and KDNα2,6lactulose group present obvious loss of viability. The leakage amount of proteins in *S. aureus* treated with KDNα2,3lactulose and KDNα2,6lactulose increased by 223 μg/mL and 205.4 μg/mL, respectively. The morphological alterations on the cells observed by SEM confirmed that KDN-containing sialylated lactuloses possessed antibacterial activities. In a word, KDN-containing sialylated lactuloses have certain effects on inhibiting the growth of *S. aureus*.

Keywords: Deaminoneuraminc Acid; Enzymatic Synthesis; Inhibitory Effects; Sialylated Lactulose; *Staphylococcus aureus*.

Introduction

Sialic acids are widely distributed in various kinds of biological tissues and involved in many physiological and pathological processes, such as cell differentiation, signal transduction, cell recognition, cell immunity, bacterial and viral infection [1-3]. Sialic acids usually exist as the terminal sugars of glycoproteins and glycolipid chains on cell surface [4-5]. There are three basic forms of sialic acids in nature among 50 unique structure forms [6], that is, N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminc acid (KDN). They can be substituted at the C4, C5, C7, C8, and/or C9 hydroxyl group positions [4, 7]. Various sialylated oligosaccharides containing one or more sialic acid molecules were synthesized by such substitutions [4]. KDN is one of the three basic forms of the sialic acids that shares many common features with Neu5Ac, but has a hydroxyl group at C5 instead of the aminoacyl group of Neu5Ac [8]. KDN also exists in various glycoconjugates in vertebrates and Gram-negative bacteria [9]. However, it’s extremely difficult to obtain sufficient KDN-containing oligosaccharides from natural resources or by chemical synthesis to be used for biological and applied research [4, 10].

In recent years, more researchers have become interested in the synthesis of sialyloligosaccharides and their derivatives. A library of 64 structural glycosphingolipid containing different sialic acid forms including Neu5Ac, Neu5Gc and KDN were synthesized by highly effective OPME systems [11]. Other forms of sialyloligosaccharides were also obtained by OPME sialylation system, such as sialylated lacto-N-neotetraose and its sialyl derivatives, lacto-N-tetrasaccharides and sialyl lacto-N-tetrasaccharides, and novel galacto-N-biose derivatives and their sialylated forms [12-14].

Staphylococcusaureus (S. aureus) is a Gram-positive pathogen causing various infections in both humans and animals, and is considered the most relevant species producing virulence factors that facilitate adhesion, bacterial colonization, tissue

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damage, infection, intercellular communication or immune escape [15-18]. *S. aureus* is a well-known foodborne pathogen might also produce enterotoxin that causes foodborne diseases such as nausea and vomiting, diarrhea, or painful abdominal cramps, [19-20]. Food poisoning caused by *S. aureus* is a growing problem in both developing and developed countries [21]. *S. aureus* is also reported to be one of the most common causes of dairy goat mastitis (45.34%). [22]. In China, *S. aureus* and *P. aeruginosa* were the main bacterial isolates found in samples infected with mastitis in different provinces [23]. Moreover, due to the great abuse of antibiotics, antibiotic-resistant strains of *S. aureus* emerged. Therefore, *S. aureus* is widely found in various foods, such as milk, meat, eggs, salads, and cause great harm to human health [24-25]. Therefore, it is very important to control the growth of *S. aureus* in food and in food-processing environments [26].

The acidic oligosaccharides modified with monomer sialic acid play an important role in preventing pathogenic bacteria from adhering to epithelial surfaces [27-29]. The glycan chain of IgA showed antipathogenic properties. It was reported that the sIgA glycans had important roles on the interaction with Gram-positive commensal gut bacteria. Distinct residues on the sIgA glacan chain mediate bacterial - host interactions of different pathogens [30]. The glycans rich in mannose can inhibit the binding of *E. coli* and colonic HT29 cell lines, which are mediated by type I fimbriae, and type I fimbriae is a widespread toxic factor among *E. coli* pathotypes which could interact with host cell surface molecules through mannose residues [31-32].

Recently, we reported the synthesis of sialylated lactulose containing Neu5Ac via OPTE system from lactulose and examined their antibacterial activity on *Staphylococcus aureus* (*S. aureus*) [33]. Herein, we reported the synthesis of two novel sialylated lactulose containing KDN via OPME sialylation systems. In addition, the antibacterial activities and potential mechanism of sialylated lactulose containing KDN against *S. aureus* were evaluated. More over, SEM, fluorescence microscopy, DAPI fluorescence staining and protein leakage quantification were also used for the investigation of the antibacterial mechanisms.

Experimental

Materials

Chemicals were purchased and used without further purification. Lactulose was purchased from Carbosynth Ltd. (Berkshire, UK). Cytosine triphosphate sodium (CTP) was purchased from the Hangzhou Meiya Pharmaceutical Co., Ltd (Hangzhou, China). Mannose was purchased from Yiyan Research Biological Technology Co., Ltd. Sialic acid aldolase from *Pasteurella multocida* (Pmaldolase), CMP-sialic acid synthetase from *Neisseria meningitidis* (NmCSS), multifunctional α2-3-sialyltransferase from *Pasteurella multocida* 1 M144D mutant (PmST1 M144D), and α-2,6-sialyltransferase from a recombinant *Photobacterium damselae* (Pd2-6ST) [11-12] were kindly provided by the National Glycoengineering Research Center (NGRC) at Shandong University, Jinan, China. Sodium pyruvate, magnesium chloride ($MgCl_2$), p-anisaldehyde, acetoxyacetyl chloride, ethyl acetate (EtOAc), methyl alcohol (MeOH), normal propyl alcohol (n-propanol), ammonia, ethyl alcohol (EtOH), acetic acid (HOAc), sodium chloride, glucose and sodium hydrogen sulfite were of analytical grade (AR). Lysogeny broth (LB) and agar powder were of biotech grade (BR) and purchased from the Beijing Aoboxing Bio-Tech Co., Ltd. (Beijing, China).

Preparation of *p*-anisaldehyde sugar stain: 25 mL of p-anisaldehyde was added into 425 mL of ice-cold methanol. With vigorous stirring and without splashing, cautiously add 50 mL of concentrated H_2SO_4 dropwise during a 60-minute period to the methanol solution cooled in an ice bath. Store the prepared light yellow staining solution at -20 °C before use.

Bacterial strains and culture conditions

S. aureus was kindly provided by a biological lab at the School of Food Science, Henan Institute of Science and Technology, China. *S. aureus* was routinely cultured in LB medium.

LB Medium (liquid): LB powder (25 g) was dissolved in 1000 mL of distilled water, which was then sterilized at 121 °C for 15 min.

LB Medium (solid): 1.8 g of agar powder was added to LB liquid medium, which was then sterilized at 121 °C for 15 min and cooled to room temperature.

S. aureus was aerobically grown at 37 °C in LB liquid medium with shaking at 190 revolutions per minute (rpm).

Synthesis and purification of KDN-containing sialylated lactuloses

The enzymatic synthesis of KDN-containing sialylated lactuloses were carried out using an efficient OPME approach as previously reported with some modifications [11, 34]. All reactions were carried out in a 50 mL tube in 10 mL of Tris-HCl buffer (100 mM, pH 8.5) containing lactulose, mannose (1.5 equiv.), sodium pyruvate (5 equiv.), CTP (1.5 equiv.), and $MgCl_2$ (20 mM). The appropriate amount of aldolase, NmCSS, and PmST1 or Pd2,6ST were added. The reaction mixtures were incubated at 37°C for 3 hr with agitating at 110 rmp. The reactions were monitored by TLC using EtOAc/MeOH/H_2O(5:2:1) by volume and *p*-anisaldehyde stain solution. The reaction mixtures were quenched with equal volume of 95% EtOH and centrifuged to remove insoluble precipitates. The supernatants were concentrated in vacuo by a YRE-52AA rotary evaporator (Yuhua Instrument co. Ltd., Gongyi, China) and filtered through a silica gel column with gradient eluting EtOAc:MeOH:H_2O (6 : 2 : 1 then 5: 2 : 1) to obtain the desired sialylated lactulose products. The sialylated lactulose products were dried by an Alpha 1-4LSC vacuum freeze drying instrument (Marin Christ, Osterode, Germany) and then characterized by ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy.

Characterization

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance Ⅲ HD 600 spectrometer (Bruker BioSpin, Billerica, MA, USA) as 600MHz. Mass spectrum analyses were carried out on a tandem quadrupole detector (TQD) liquid chromatography mass spectrometry (LC/MS) system (Waters, USA) and ion-trap mass spectrometer with an electrospray ionization (ESI) interface. Scan ranges were from 150−800 m/z.

KDNα2,3lactulose **(3a)**

Yield, 75%; white solid. ^{1}H-NMR (600 MHz, D_2O) δ 4.54 (d, *J* = 7.8 Hz, 0.6H), 4.44 (d, *J* = 8.4 Hz, 0.4H), 4.20– 3.43 (m, 21H), 2.62 (dd, *J* = 12.0 and 4.8 Hz, 1H), 1.66 (t, *J* = 12.0 Hz, 1H). ^{13}C-NMR (151 MHz, D_2O) δ 173.96, 100.32, 99.74, 98.03, 77.23, 75.60, 75.14, 73.86, 72.06, 70.21, 69.71, 69.18, 67.67, 67.42, 66.55, 66.03, 63.88, 62.91, 62.59, 61.09.

KDNα2,6lactulose **(3b)**

Yield, 75%; white solid.^{1}H-NMR (600 MHz, D_2O) δ 4.43 (d, *J* = 8.4 Hz, 0.6H), 4.44 (d, *J* = 7.8 Hz, 0.4H), 4.20– 3.42 (m, 21H), 2.57 (dd, *J* = 12.0 and 4.8 Hz, 1H), 1.59 (t, *J* = 12.0 Hz, 1H); ^{13}C-NMR (151 MHz, D_2O) δ 173.62, 101.23, 100.30, 98.06, 78.24, 73.63, 73.52, 72.40, 71.98, 70.62, 70.12, 69.86, 68.63, 67.99, 67.06, 66.25, 63.86, 63.67, 62.96, 62.56.

Antibacterial assays

Microplate laser nephelometry was conducted in accordance to OD measurements with some modifications [35]. Single colonies of *S. aureus* were added to LB liquid medium and shock cultured over night, and then diluted OD value to 0.05 with Sterilized LB liquid medium. 100 μl of *S. aureus* cell suspension were put into the liquid medium in respective wells of the 96-well microplate (Costar 3599, Corning Incorporated, USA) with a concentration of 8 mg/mL KDNα2,3lactulose and KDNα2,6lactulose. Microplates were covered with a clear lid and there are some gaps at the right brim of the well to allow gas exchange. Microplates were then placed in the biochemical incubator and incubated for 24 h at 37 °C. During incubation, microplates were gently shaken in the incubator except for the duration of every two hours measurement of OD value at 600 nm by a microplate reader (Infinite F50, Tecan, Switzerland). Control experiments (ck), 8 mg/mL lactulose and 8 μg/mL antibiotic kanamycin were conducted at the same time without sialylated lactuloses. The inhibition ratio can be described as the ratio of the decreased OD value of *S. aureus* in the experimental group to that of the control group at a certain time. The inhibition ratio was calculated using following formula:

$$\text{Inhibition ratio (\%)} = (OD_{ck} - OD_t) / OD_{ck}$$

OD_{ck} means the OD value of the control group at some point; OD_t means the OD value of experimental group at the same incubation time.

DNA content of S. aureus

A 90 μl of *S. aureus* cell suspensions (overnight cultures diluted to OD = 0.05) was put into each well of the 96-well microplate, followed by adding 10 μL 8 mg/mL KDNα2,3lactulose or KDNα2,6lactulose into the wells of the 96-well microplate and incubated at 37 °C for 8 h. Then 100 μL of an *S. aureus* cell suspension was collected, and its OD_{600} was adjusted to approximately 0.6. Then added three times of DAPI (5 μg/mL), and the samples stood for 15 min in dark. After two washes with phosphate-buffered saline (PBS), the fluorescence of the samples was measured at an emission wavelength of 454 nm using a Fluorescence spectrophotometer (Cary Eclipse G9800A, Agilent Technologies, America) and excitation at 364 nm. *S. aureus* without sialyllactulose was used as the control. 10 μL 8

mg/mL lactulose and 8 μg/mL antibiotic kanamycin were conducted with the same measurement in order to compare with KDNα2,3lactulose and KDNα2,6lactulose group.

Fluorescence microscopy

Single colonies of *S. aureus* were added to LB liquid medium and shock cultured for 8 h; a fraction of this culture was then added to LB liquid medium until the OD_{600} was 0.05. Next, 90 μL of this *S. aureus* cell suspension was placed in a 96-well microplate, and 10 μL of 8 mg/mL KDNα2,3lactulose or KDNα2,6lactulose was added to the wells. The microplate was then put into a thermoshaker (MB100, Hangzhou, China) and incubated at 37 °C for 8 h at 500 rpm. The same volume of 5 μg/mL 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) was then added, and the samples was mixed uniformly and remained for 10 min in a dark room. The fluorescence was observed using an inverted fluorescence microscope with a Zeiss-Series 120Q light source (America), and the sample was visually inspected for color changes. A 100-μL sample of OD_{600} 0.05 *S. aureus* without sialyllactulose was used as a control. 10 μL 8 mg/mL lactulose or 8 μg/mL antibiotic kanamycin replaced KDNα2,3lactulose or KDNα2,6lactulose and were conducted with the same measurement in order to compare with KDNα2,3lactulose and KDNα2,6lactulose group.

Protein leakage in cell membranes

S. aureus cell suspensions treated with different sialyllactuloses were incubated at 37 °C for 8 h and centrifuged for 5 min at 8000 rpm using a Thermo Scientific refrigerated centrifuge (Multifuge X1R, Heraeus, Germany). A 0.1 mL aliquot of supernatant was transferred by pipette to a test tube, and 1 mL of G-250 Coomassie brilliant blue solution was added. The solution was blended and allowed to stand for 5 min after which 0.1 mL of mixture was absorbed to 96-well plates. Protein leakage was determined by measuring the optical density of the cell supernatants at 595 nm using a microplate reader (Infinite F50, Tecan, Switzerland). A standard curve ($y = 17.106x + 0.2698$. y, absorbance; x, content of protein) was used to calculate the amount of protein leakage content.

Scanning electron microscope (SEM)

450 μl of *S. aureus* cell suspension (overnight cultures diluted to OD=0.05) was put into each well of the 96-well microplate, then added 50 μL 8 mg/mL KDNα2,3lactulose, KDNα2,6lactulose, lactulose or 8 ug/mL kanamycin, respectively. 500 μL of *S. aureus (*OD = 0.05*)* was used as control group. Microplates were then placed in the biochemical incubator and incubated at 37 °C for 6 h with agitation at 220 rpm. Then the cells were washed twice using sterile water and fixed overnight with 2.5% glutaraldehyde at 4 °C. After removing the residual glutaraldehyde by washing in sterile water, followed by dehydration with a series of ethanol solutions (35, 50, 70, 80, and 100%; v/v) at 4 °C for 10~15 min. The dehydrated cell sample was dispersed on a 1 cm × 1 cm piece of aluminum foil paper and natural drying at room temperature. Then, the samples were sputter coated with a thin layer of gold using an E-1010 ion-sputtering apparatus (Hitachi, Ltd., HQ, Japan) prior to scanning electron microscopy (SEM) observation. Digital images were acquired using a Quanta 200 environmental scanning electron microscope (FEI Company, Hillsboro, Oregon, USA) at an instrumental magnification of 15,000 or 16 000.

Statistical analysis

Data obtained from the experiment were analyzed by Microsoft Excel 2007 to determine statistical significances. Differences were considered statistically significant when $P < 0.05$.

Result and Discussion

Synthesis, purification and characteristics of KDN-containing sialylated lactulose

Sialylated lactulose were synthesized using an efficient OPME approach starting from mannose (**1**) and lactulose (**2**). The enzyme reactions were conducted according to previous articles [11, 34]. The reactions were monitored by TLC using EtOAc/MeOH/H_2O (6:2:1) by volume (Fig. 1). Then the products were purified by silica gel flash column chromatography with gradient eluting using solvents of EtOAc: MeOH:H_2O (6 : 2 : 1 then 5: 2 : 1) to offer the desired sialylated lactulose products (**3a and 3b**) in 75% yields. ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy were used to characterize the obtained pure sialylated lactulose. The antibacterial activities of these sialylated lactuloses were evaluated against *S. aureus*.

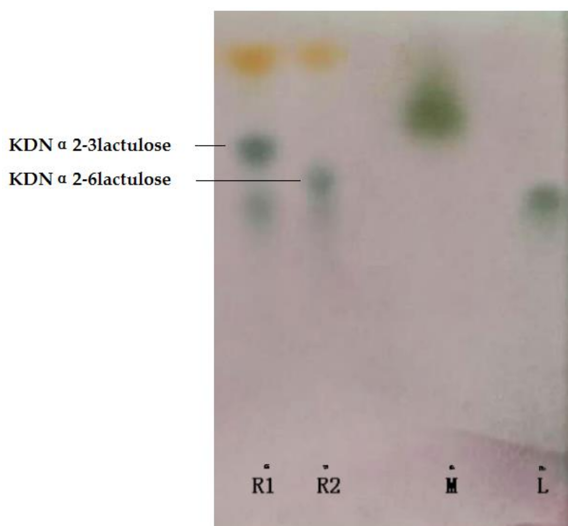

Fig. 1: R1, reaction of KDNα2,3lactulose; R2, reaction of KDNα2,6lactulose; M, mannose; L, Lactulose.

Bacteriostatic activities

In this paper, we successfully synthesized two sialylated lactuloses containing KDN by OPME sialylation system. In order to assay the antibacterial activities of sialylated lactulose containing KDN against *S. aureus,* the standard approach of optical density (OD) measurements were used for characterizing *S. aureus* concentrations in culture media [36]. In industrial and microbiological experiments, OD measurement is a preferred method because it is simple and quick to obtain answers [37]. As has been reported, the OD_{600} measurement is very reliable and repeatable [38]. Herein, we verified the correlation of OD_{600} and live cell number and a significant linear correlation (R^2 =0.9842) was obtained (Fig. 2). The OD measurements of *S. aureus* were carried out and the correlation of OD_{600} and live cell number is shown in Fig. 2**.** The OD value and live cell number of *S. aureus* showed a significant linear correlation and the correlation coefficient or R^2 is above 0.9842. We further evaluated the activities of obtained sialylated lactuloses on the growth of *S. aureus* by OD measurements. The growth of *S. aureus* on LB medium supplemented with 8 mg/mL KDNα2,3lactulose and KDNα2,6lactulose were assessed by OD_{600} measurement for 24 hours under aerobic conditions. As shown in Fig. 3, KDNα2,3lactulose and KDNα2,6lactulose were found to inhibit the growth of *S. aureus*. The growths of *S. aureus* after 4 h were slower when lactulose, KDNα2,3lactulose and KDNα2,6lactulose were added compare to the control (ck). Additionally, the inhibition of KDNα2,3lactulose on *S. aureus* was better than that of lactulose and KDNα2,6lactulose. In addition, the effect of kanamycin on the growth of *S. aureus* was used to compare with sialylated lactuloses. The maximum inhibition reached was after 4 h and decreased over time. This decrease might be due to the light sensitivity of Kanamycin or its decomposition, while KDNα2,3lactulose and KDNα2,6lactulose still retained a stable inhibition during the assay time.

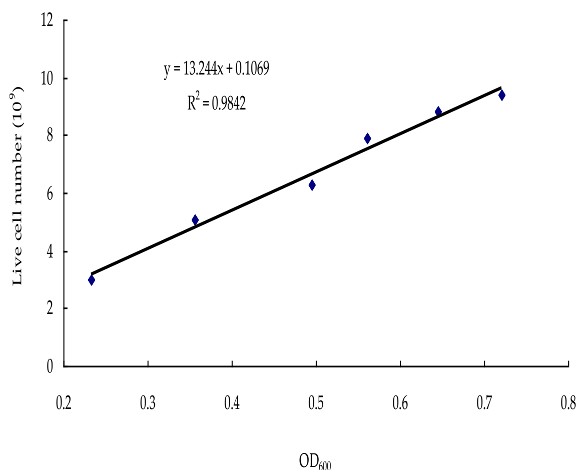

Fig. 2: Correlation between OD600 and live cell number of S. aureus.

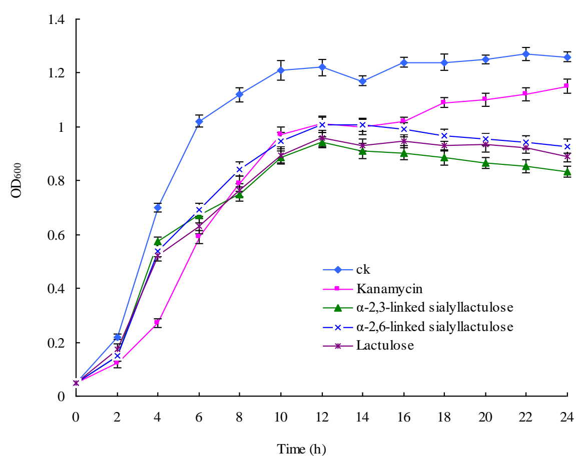

Fig. 3: The effects of KDNα2,3lactulose and KDNα2,6lactulose on the growth of S. aureus.

The inhibition ratios of KDNα2,3lactulose and KDNα2,6lactulose on *S. aureus* are shown in Fig. 4. Addition of 8μg/mL kanamycin showed inhibited the growth of *S. aureus* significantly from 2 h to 8 h in comparison to that of lactulose and sialyllactulose. However, after 10 h the inhibition ratios of kanamycin gradually reduced and were lower than that of

lactulose and sialyllactulose. The maximum inhibition ratios of KDNα2,3 lactulose and KDNα2,6lactulose within the first 10 h was up to 34.33% and 32.18%, respectively. The inhibition ratio of KDNα2,3 lactulose was higher than that of KDNα2,6lactulose and lactulose from 10 h to 24 h, and that the inhibition ratio of lactulose was higher than that of KDNα2,6lactulose.

We regard our products as functional oligosaccharides rather than antibiotics and the KDN-containing sialylated lactuloses do not achieve the inhibitory effect of antibiotics. However, we wanted to know what level of inhibition they had compared to antibiotics kanamycin. The results indicated that the antibacterial effect of 8 mg/mL KDNα2,3lactulose or KDNα2,6lactulose was slightly better than 8μg/mL kanamycin.

DNA content of S. aureus

The potential mechanism of sialylated lactulose containing KDN against *S. aureus* was conducted by determination of membrane integrity of *S. aureus*. It is well known that some chemicals cause severe membrane damage by disrupting membrane integrity [22]. Membrane integrity plays an vital role in maintaining optimal internal conditions for metabolism and energy transduction [39]. Even relatively minor damages to the structural integrity of cell membrane can impair cell metabolism, lead to cell growth inhibition and even death [40]. Hence, membrane integrity can be used as a parameter to investigate inhibition mechanism of KDN-containing sialylated lactuloses. Live cell or dead cell staining method could rapidly evaluate the integrity of cell membranes [41]. DAPI is often used as a fluorescent dye that binds DNA from bacterial strains and detects cell survival. DAPI can go through a full membrane, so DAPI can rapidly enter living cells and bind DNA. This process can be used for staining living and fixed cells. The higher the amount of nucleic acid, the stronger the blue fluorescence, indicating cell survival, on the contrary, the lower the fluorescence intensity, or no fluorescence, indicating cell growth was inhibited [23]. Therefore, the viable and nonviable cells can be distinguished under the fluorescence microscope [42].

Changes in the DNA content of *S. aureus* with or without KDNα2,3lactulose and KDNα2,6lactulose were measured by the fluorescent intensity scanning from 400 nm to 600 nm, as shown in Fig. 5. The control was placed as 100% line. The fluorescent intensity of *S. aureus* of lactulose group were detected to be 87.59±2.78%. The addition of KDNα2,3lactulose and KDNα2,6lactulose gave rise to a significant decrease (p<0.05) in fluorescence intensity compared with control to 47.05±3.06% and 51.16±2.40, respectively; and significant decrease (p<0.05) compared with lactulose group from 87.59±2.78% to 47.05±3.06% and 51.16±2.40%, respectively. The *S. aureus* treated with 8 μg/mL kanamycin showed that the fluorescent intensity was 67.35±4.07%. Therefore, KDN-containing sialylated lactuloses had some extent interference with nucleic acids synthesis or caused decomposition of nucleic acids in *S. aureus* cells. These results indicated that KDN-containing sialylated lactuloses gave rise to a minor damage on the membrane integrity of *S. aureus.*

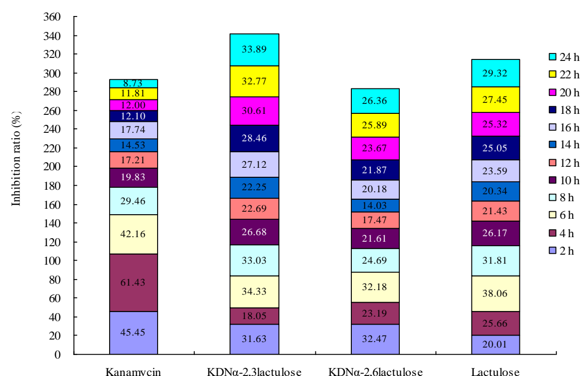

Fig. 4: The inhibition ratios (%) of KDNα2,3 lactulose and KDNα2,6lactulose on S. aureus.

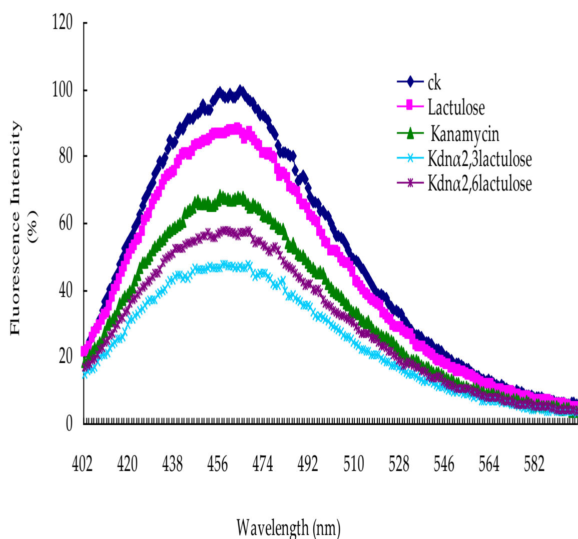

Fig. 5: Changes of DNA content of S. aureus with KDNα2,3lactulose and KDNα2,6lactulose

Fluorescence microscopy

Fi. 6 showed the staining determination of *S. aureus* with fluorescence and without fluorescence. The microscopic images showed that *S. aureus* cells of control group produced intensely blue fluorescence (Fig. 6 a,b). The fluorescence intensity of *S. aureus* with KDNα2,3lactulose (Fig. 6 g,h) and KDNα2,6lactulose (Fig. 6 i,j) were relatively weak compared with that of control and lactulose groups (Fig. 6 c,d). The the fluorescent intensity of treated *S. aureus* cells with 8 µg/mL kanamycin (Fig. 6 e,f) was weak similar to that of the samples with KDNα2,3lactulose and KDNα2,6lactulose. Staining-based methods had shown that *S. aureus* of KDNα2,3lactulose and KDNα2,6lactulose group present a obvious loss of viability, which further demonstrated the inhibition effects of KDNα2,3lactulose and KDNα2,6lactulose against the *S. aureus*.

Our results suggested that bacteria cells of KDNα2,3 lactulose and KDNα2,6lactulose group may lose their membrane integrity during the process of culture. Staining-based methods had shown that *S. aureus* of KDNα2,3 lactulose and KDNα2,6lactulose group present a obvious loss of viability, which further demonstrated the inhibition effects of KDNα2,3lactulose and KDNα2,6lactulose against the *S. aureus*. The exposure of *S. aureus* to KDN-containing sialylated lactuloses decreased the fluorescent intensity.

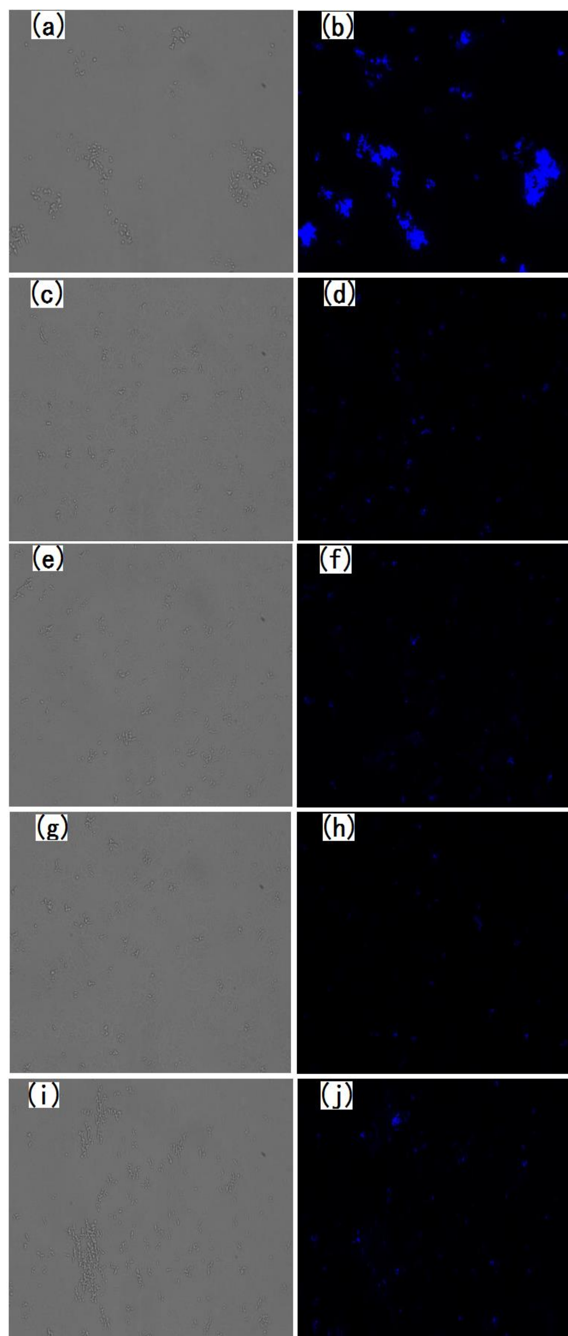

Fig. 6: (a), no fluorescence of ck; (b), fluorescence of ck; (c), no fluorescence of lactulose group; (d), fluorescence of lactulose group; (e), no fluorescence of Kanamycin group; (f), fluorescence of Kanamycin group; (g), no fluorescence of KDNα2,3lactulose group; (h), fluorescence of KDNα2,3 lactulose group; (i), no fluorescence of KDNα2,6lactulose group; (j), fluorescence of KDNα2,6lactulose group.

Protein leakage

Protein leakage caused by membrane damage of *S. aureus* cell was measured in this work. The leakage amount of proteins in *S. aureus* groups exposed to KDNα2,3 lactulose and KDNα2,6lactulose were 916.2 μg/mL and 898.6 μg/mL, respectively, which were found to be higher than that of control, lactulose and kanamycin treated samples (693.2 μg/mL, 669.3 μg/mL and 685.4 μg/mL, respectively). The protein contents in *S. aureus* treated with KDNα2,3 lactulose and KDNα2,6lactulose increased by 223 μg/mL and 205.4 μg/mL, respectively. These results were consistent with above results of microplate assay, fluorescence microscopic image and fluorescence intensity, further confirmed the inhibition effect of KDN-containing sialylated lactuloses against the *S. aureus*.

The release of intracellular proteins is an indication of membrane deterioration [43]. The effects of sialyllactulose on *S. aureus* cell structures and membranes could be used to investigate the antimicrobial mode. The gram-positive *S. aureus* might have lower antibacteria sensitivity than that of the gram-negative bacteria, such as *P. aeruginosa*, *E. coli*, *Escherichia fergusoni* [27, 44-45]. Altogether, our results showed protein leakages in *S. aureus* cells treated by KDNα2,3 lactulose and KDNα2,6lactulose were slightly increased, indicating that the KDN-containing sialylated lactuloses could impair the membrane integrity, leading to the leakage of intracellular proteins [46-47]. Integral and peripheral membrane proteins are important parts of the cytoplasm membrane; they provide a variety of cellular functions, including nutrition transportation, enzyme activity and cell information transfer. Hence, the interaction between sialic acid oligosaccharides containing KDN and membrane proteins may enable us to further understand the mechanism of action of antibacteria.

Scanning electron microscope

SEM analyses was used to observe the the cell morphology of *S. aureus*. In general, exponential growth of cells up to 6h is considered mature and is often used as observed cells. The SEM results of bacterial cells with KDN-containing sialylated lactuloses were shown in Fig. **7**.

As shown in the SEM observation, The cells in the control group were regular and typical. The normal cells were full and smooth with uniform size and distribution (Fig. **7** a). In contrast, some cells that treated with 8 mg/mL lactulose, KDNα2,3lactulose and KDNα2,6lactulose showed a slight damage to on the cell morphology, rough outer surface and small leak holes (Fig. **7** b,d,e). Unsmoothed cell envelopes were observed in the sample of 8 μg/mL kanamycin treatment (Fig. **7** c). The changes in cell morphology indicated that the KDN-containing sialylated lactuloses had some effects on *S. aureus* cells.

The morphological alterations on the cells confirmed that KDN-containing sialylated lactuloses possessed bacteriostatic activity. Changes in cell morphology indicated the effect of KDN-containing sialylated lactuloses on membrane permeability and integrity. This caused damage to bacterial cell walls and cell membranes, and leakage of intracellular material spilled around [25; 48].

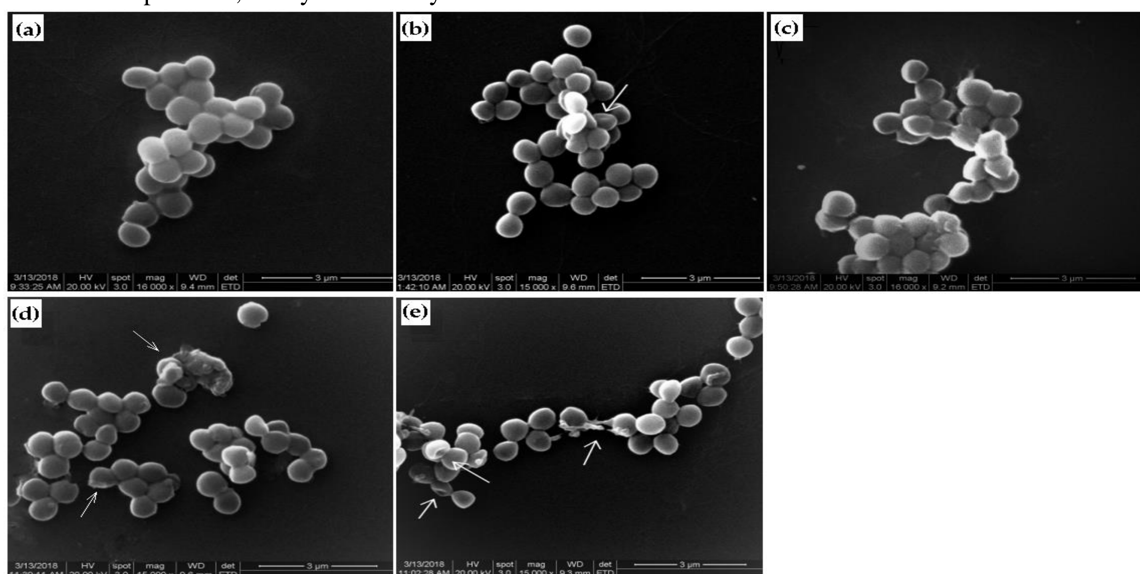

Fig. 7: (a), control; (b), lactulose; (c) , Kanamycin; (d) KDNα2,3lactulose; (e), KDNα2,6lactulose.

Conclusions

KDNα2,3lactulose and KDNα2,6lactulose were efficiently synthesized by an OPME sialylation method from commercial available mannose. These two sialyllactuloses showed certain antibacterial activity against S. aureus. KDNα2,3 lactulose has the higher inhibitory effects on S. aureus than that of KDNα2,6lactulose. SEM, Fluorescence analysis, and protein leakage results indicated that KDNα2,3lactulose and KDNα2,6lactulose could slightly disturb nucleic acid synthesis or cause membrane leakage in S. aureus cells. Although the antibacterial activities of these two KDN-containing sialylated lactuloses were not remarkable, our work could advance the use of sialylated oligosaccharides and their derivatives as potential antibacterial agents or healthy food material. In order to explore other biological functions of these two compounds, we further plan to carry out the immunocompetence determination of these two sialylated lactuloses, and investigate their effects on the intestinal microflora in mice by high-throughput sequencing of 16S rRNA. This will lay the foundation for future research and application.

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