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# Research Article
# Amphiphilic Esterified Xylo-Oligosaccharide: Surface-Active Properties and Anti-Microbial Activities

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## Abstract

**Background and Objective:** The lipase catalyzed-esterification of native xylo-oligosaccharide (Xylo) and lauric acid (C-12) was used to synthesize amphiphilic esterified xylo-oligosaccharide laurate (Xylo_L). This study was designed to investigate the surface-active properties and antimicrobial activities at different concentrations of Xylo_L [0-15% (w/w)], compared to Xylo. **Materials and Methods:** Emulsifying activity, emulsifying stability, foamability and foaming stability of Xylo_L was determined, compared to Xylo. The antimicrobial activities of Xylo and Xylo_L were evaluated against *Escherichia coli* (a Gram-negative bacterium) and *Staphylococcus aureus* (a Gram-positive bacterium). **Results:** Esterified modification of Xylo improved the emulsifying ability and prolonged the stability of emulsions, when soybean oil was used as the dispersed phase. Xylo exhibited antimicrobial activities for both *E. coli* and *S. aureus* at all concentrations [5-15% (w/w)]. Xylo_L was less effective at *E. coli* and *S. aureus* inhibition than Xylo at all concentrations [5-15% (w/w)]. **Conclusion:** The antimicrobial activities decreased after the esterification of Xylo to Xylo_L. Xylo_L may suitable as an ingredient of emulsion foods, as an emulsifier and stabilizer with a slight antimicrobial function.

Key words: Amphiphilic oligosaccharide, antimicrobial, esterification, fatty acid, surface-active, xylo-oligosaccharide

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

At present and into the future, customers prefer environmental-friendly and healthy ingredients and foods; hence, many food companies have avoided or reduced using food ingredients and additives which are chemically synthesized or contain chemical agents. Food scientists have attempted to modify natural materials and to improve their functionality using enzymatic reactions to replace the chemically synthesized ingredients following the customer needs. Amphiphilic oligosaccharides synthesized using enzymatic esterification are one of ingredients of interest because they exhibit various properties such as emulsifying and stabilizing abilities[1-3] as well as antimicrobial abilities[4]. These esterified oligosaccharides also synergize a commercial small molecule surfactant (Tween 80) to generate mono-dispersed emulsions[5] with double stabilization film[6] and enhance emulsion stability during storage[7,8], resulting in a reduction of Tween 80 use.

The antibacterial activity of sugar fatty acid esters is dependent on the nature of the carbohydrate core, number and type of esterified fatty acids and the degree of esterification[9]. Watanabe *et al.*[10] reported that among the synthesized carbohydrate esters, galactose and fructose laurates showed the highest growth-inhibitory effect against *Streptococcus mutans*, while the other analogs of hexose laurates showed no antibacterial activity. Smith *et al.*[11] found that lauric ester derivatives of methyl α-D-mannopyranoside and methyl β-D-glucopyranoside showed the best inhibitory effects against *Staphylococcus aureus*. Karlová *et al.*[12] reported that the antimicrobial effects of fatty acid fructose esters decreased rapidly as the length of the fatty acid chain increased. Moreover, sugar fatty acid esters exhibited good antifungal activities against *Penicillium oxalicum* and *Aspergillus tubingensis*[13]. Our previous research reported that esterified maltodextrin (DE of 16) with lauric acid (C-12) inhibited *Escherichia coli* growth with a minimum inhibitory concentration of 5% (w/w). Moreover, *E. coli* inhibition was not found for esterified maltodextrin decanoate (C-10) and palmitate (C-16) in the studied concentration range of 5-20% (w/w). Furthermore, the growth of *Staphylococcus aureus* was not clearly retarded by all these esterified maltodextrins[4]. These reports indicated that esterification with lauric acid could enhance the anti-*E. coli* ability of maltodextrin. Hence, lauric acid was chosen to esterify xylo-oligosaccharide for antimicrobial ability in the current study because lauric acid may have the typical chain length of the hydrophobic part of esterified oligosaccharide to enhance antimicrobial ability.

Foodborne pathogens continue to be a serious threat to public health worldwide. *E. coli* (a Gram-negative bacterium) and *S. aureus* (a Gram-positive bacterium) are two major foodborne bacterial pathogens frequently involved in foodborne outbreaks[14]. The survival of these microorganisms in food can lead to spoilage or cause infection and illness when consumed. Hence, these two microorganisms were chosen to estimate the antimicrobial activities, to investigate the cause of food spoilage and to decrease the occurrence of these pathogens.

The objectives of current study were to investigate the surface-active properties and to evaluate the antimicrobial activities of esterified xylo-oligosaccharide with lauric acid (C-12) (Xylo_L) against *E. coli* (a Gram-negative food pathogenic bacterium) and *S. aureus* (a Gram-positive food pathogenic bacterium), compared to native xylo-oligosaccharide (Xylo).

## MATERIALS AND METHODS

**Materials:** Xylo-oligosaccharide, extracted from corn, was supplied by San-Ei Gen F.F.I. (Osaka, Japan). The range of the degree polymerization (DP) of the xylo-oligosaccharide was from 2-7. Lipase from *Thermomyces lanuginosus* solution, containing 2% (w/v) lipase, was purchased from Sigma-Aldrich (Buchs, Switzerland). The enzyme activity was about 100,000 U $g^{-1}$; 1g of enzyme hydrolyzes tributyrin and releases 100,000 μM of titratable butyric acid per minute under assay conditions. Lauric acid (C-12), dimethyl sulfoxide (DMSO), ethanol and soybean oil were purchased from Sigma-Aldrich. Criterion™ nutrient broth was used and nutrient agar was purchased from Titan Biotech Co., Ltd. (Bhiwadi, India). All other chemicals used were of analytical grade.

**Microorganisms:** Gram-negative bacteria *Escherichia coli* DMST4212 and Gram-positive bacteria *Staphylococcus aureus* DMST8840 were used as reference stains.

### Methods

**Esterified xylo-oligosaccharide preparation:** Xylo_L was prepared following the method of Udomrati and Gohtani[7]. Xylo and lauric acid were used in the ratio of 1:0.5 (mole of xylose unit/mole of lauric acid). Maltodextrin (1 g) was dissolved in an open flask with 2 mL dimethyl sulfoxide (DMSO), as solvent for both the hydrophilic and lipophilic substrates. The lauric acid was added and then the mixture was stirred using a magnetic stirrer for 10 min. The purchased lipase enzyme solution (350 μL) was added to the mixture.

Then the samples were incubated in a water bath at 60°C for 4 h with stirring using a magnetic stirrer throughout incubation. The ester formed was precipitated by adding ethanol. The ethanol supernatant was poured off after centrifugation at 3,000 rpm for 5 min. Three additional ethanol extractions were performed prior to drying the precipitate in a hot-air oven at 50°C.

**Proton nuclear magnetic resonance ($^1$H NMR) spectra:** The $^1$H NMR spectra of the samples were recorded using a nuclear magnetic resonance spectrometer (NMR; ALPHA 600, JEOL, Japan). Xylo_L was dissolved in DMSO-$d_6$ and the dispersion concentration was 15% (w/w). Measurement occurred at 70°C. All chemical shifts were reported in parts per million (ppm) using tetramethylsilane (TMS) as the reference because it is usually used as an internal standard for NMR measurements at elevated temperature. The maximum possible DS is 2.0, corresponding to the number of OH molecules available on the backbone of the xylo-oligosaccharide. The DS was calculated using Eq. 1:

$$DS = \frac{I_{methyl}}{3I_{anomericXyl.}} \quad (1)$$

where, $I_{methyl}$ is the area of methyl protons of the ester chains at 1.9-2.0 ppm and $I_{anomericXyl.}$ is the area of the anomeric protons of xylo-oligosaccharide at 4.5 ppm[15].

**Interfacial tension measurement:** The interfacial tension between soybean oil and pure water containing Xylo [15% (w/w)] or Xylo_L [5, 10 and 15% (w/w)] was measured using a fully automatic interfacial tensiometer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan) at 25±1°C. The apparatus can automatically determine the oil-water interfacial tension from the maximum volume of a pendant drop detached from a stainless steel needle containing Xylo or Xylo_L suspension immersed in soybean oil excluding the droplet holding time during measurement.

**Viscosity and density measurement:** The viscosity of the Xylo [15% (w/w)] solution and the Xylo_L [5, 10 and 15% (w/w)] suspensions was measured using a vibrational viscometer (SV-10, A and D Company, Ltd., Tokyo, Japan) at 25±1°C. The densities of these suspensions or solutions were measured using a density meter (DA-130 N, Kyoto Electronics Manufacturing, Co., Kyoto, Japan).

**Emulsifying activity and emulsifying stability:** The assay for emulsifying activity was modified from the method of Zhang *et al.*[16] using soybean oil as the test substance. The aqueous phase consisted of various concentrations of Xylo and Xylo_L [0-15% (w/w)]. Aqueous phase and soybean oil were used in the ratio of 1:1. The initial height ($H_0$, cm) was measured of the mixture of the aqueous phase and soybean oil. Emulsification was performed using a high speed homogenizer (Ultra turrax T25, IKA Janke and Kunke, Germany) at 8,000 rpm for 2 min. After storing the emulsions for 10 min, the height of the emulsion layer ($H_1$, cm) was measured. Then, storage emulsions were allowed to stand for 24 h at room temperature; some emulsions clearly separated into a serum layer at the bottom and an emulsion layer at the top. Since the density of the liquid oil was lower than that of the aqueous phase, the oil droplets tended to move upward. The height ($H_2$, cm) of the emulsion layer after storage for 24 h was measured. The emulsification activity (%) and emulsifying stability were calculated using Eq. 2 and 3, respectively:

$$\text{Emulsifying ability (\%)} = \frac{H_1}{H_0} \times 100 \quad (2)$$

$$\text{Emulsion stability (\%)} = \frac{H_2}{H_1} \times 100 \quad (3)$$

**Foamability and foaming stability:** The aqueous phase was prepared of varied concentrations of Xylo and Xylo_L [0-15% (w/w)] and then poured solution or suspension (10 mL) into 50 mL tubes, the height of each aqueous phase was measured ($H_o$, cm). Aqueous phase was mixed by a homogenizer at 13,500 rpm for 2 min and the foam height ($H_2$, cm) and the total height ($H_1$, cm) were determined immediately. After storage of 50 min at room temperature, the foam height ($H_3$, cm) was remeasured. The foamability and foaming stability were calculated using the following Eq. 4 and 5, respectively:

$$\text{Foamability (\%)} = \frac{H_1 - H_0}{H_0} \times 100 \quad (4)$$

$$\text{Foam stability (\%)} = \frac{H_3}{H_2} \times 100 \quad (5)$$

**Microscopic analysis:** A scanning electron microscope (SEM; SU3500, Hitachi, Japan) was used to determine the microstructure of the Xylo and Xylo_L powders at 3,000× and 4,500× magnification.

**Antimicrobial activity:** The antimicrobial activity of Xylo_L was compared with Xylo using the broth dilution method. Three different sample concentrations [5, 10 and 15% (w/w)] were prepared by dissolving Xylo or Xylo_L in 0.9% nutrient broth (NB) before autoclaving. The tested cultures (*E. coli* and *S. aureus*) were suspended in sterile water and adjusted to an optical density (OD) of 0.20±0.05 at a wavelength of 660 nm in order to control the number of cells. The 0.5 µL of the culture suspension was mixed with 5 mL of the sample in NB or in a control tube (0%, without sample) to obtain starter culture of about $10^6$ CFU $mL^{-1}$. A sample (10 µL) of the mixture was dropped and spread in one partition of a sterile plate containing nutrient agar. The plates were incubated at 37°C for 24 h before colony counting commenced as the start of culturing (0 h). The test tubes containing inoculated test samples were incubated at 35°C with shaking at 120 rpm. After 24 h of incubation, each 10 µL sample was dropped and spread on one partition of a sterile plate containing nutrient agar. The plates were incubated at 37°C for 24 h before colony counting of the start culture (24 h). The experiment was done in duplicate and data were reported as colony forming units per milliliter (CFU $mL^{-1}$) compared with NB as the control and with Xylo as a positive control.

**Statistical analysis:** Data were subjected to one way analysis of variance (ANOVA) followed by Duncan's multiple range test at the 95% confidence level ($p<0.05$) using the SPSS statistical software (IBM, USA).

## RESULTS AND DISCUSSION

**Surface activities of Xylo and Xylo_L:** The degree of substitution (DS) of Xylo_L was 0.05, as shown in Table 1. The DS value was rather low because of the restriction of the catalyzed-enzymatic reaction and contamination of precipitated-Xylo with ethanol. The interfacial tension of the Xylo solution-soybean oil interface at a concentration of 15% (w/w) was 26.4 mN $m^{-1}$ and the interfacial tension of Xylo_L [15% (w/w)] decreased to 23.5 mN $m^{-1}$. The Xylo_L had greater oil interfacial activity in water-soybean oil due to its amphiphilic molecules. These results were in agreement with Udomrati and Gohtani[2] who investigated Xylo and Xylo_L dispersions at the *n*-hexadecane interface. The interfacial tension of Xylo_L tended to decrease as the concentration increased (Table 1) because of increase of adsorbed Xylo_L on the oil droplet surface. There was minimal difference between the viscosity of Xylo and Xylo_L (Table 1) at the same concentration of 15% (w/w). The viscosity of Xylo_L increased as the concentration increased due to an increase in the number of polysaccharide molecules per unit volume of the aqueous phase[17].

The capability of an emulsifier is indicated by emulsifying activity parameters[18]. Emulsion stability is used to estimate efficient emulsion stabilization of an emulsifier during storage. The emulsifying activity and emulsion stability of Xylo and Xylo_L at different concentrations using soybean oil as dispersed phase are presented in Fig. 1. The emulsifying activity of Xylo_L was much higher than that of Xylo at the same concentration because of the latter having more effective surface-active properties, which was confirmed by the lower interfacial tension value (Table 1). The emulsion stabilization mechanism of esterified oligosaccharides may be due to their hydrophobic part being absorbed at the oil/water interface and forming a dense stabilization layer of hydrophilic loops that provide steric repulsion between the surfaces of the oil droplets[3]. The emulsifying activity of Xylo_L tended to increase with increasing concentration because more absorbable Xylo_L covered the surface of the soybean oil droplets.

There was no emulsion-stabilizing capacity for Xylo after storage for 24 h. However, the emulsion stability values of all emulsions containing Xylo_L were higher than 75%. The emulsion stability values of emulsions containing Xylo_L tended to decrease with increasing Xylo_L concentration. This may have been caused by the emulsion stabilization film of Xylo_L surrounding oil droplet surface not being strong enough to inhibit the coalescence of oil droplets at high concentration because the attractive force between the oil droplets increased progressively as the concentration of oligosaccharide increased[17].

Table 1: DS values of Xylo_L, interfacial tensions of aqueous phase-soybean oil interface, densities and viscosities of aqueous phase containing Xylo at concentration of 15% (w/w) and Xylo_L with varying concentrations (5-15% (w/w))

| Aqueous phase | DS | Density (kg/$m^3$) | Interfacial tension (mN $m^{-1}$) | Viscosity (mPa.s) |
|---|---|---|---|---|
| Xylo | - | | | |
| 15% (w/w) | | 1011 | 26.4±0.2$^a$ | 2.72±0.02$^b$ |
| Xylo_L | 0.05 | | | |
| 5% (w/w) | | 1015 | 24.2±0.2$^b$ | 1.41±0.01$^d$ |
| 10% (w/w) | | 1031 | 23.8±0.1$^{bc}$ | 2.05±0.00$^c$ |
| 15% (w/w) | | 1060 | 23.5±0.2$^c$ | 2.98±0.00$^a$ |

*Different lowercase letters (a,b,c) within a same column indicate significantly different at the 95% confidence level. Values are mean±standard deviation

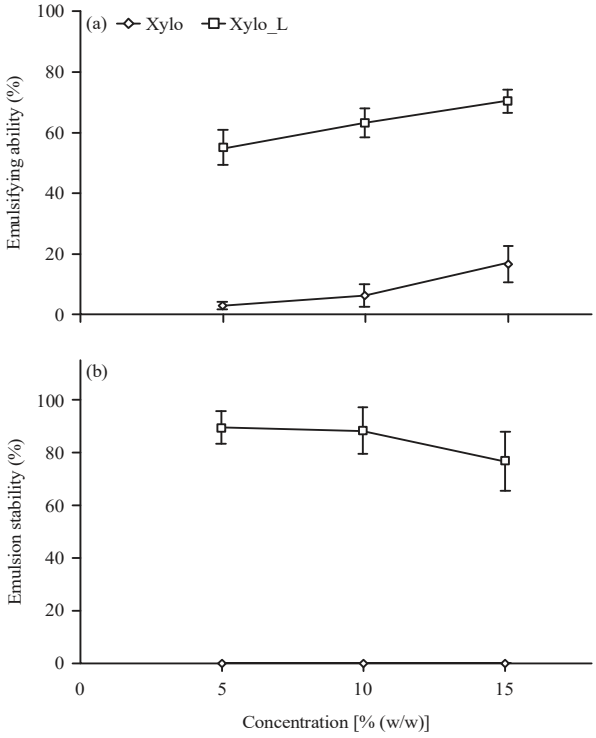


Fig. 1(a-b): (a) Emulsifying ability and (b) Emulsion stability as function of Xylo and Xylo_L concentration, using soybean oil as the dispersed phase
Values are mean±standard deviation

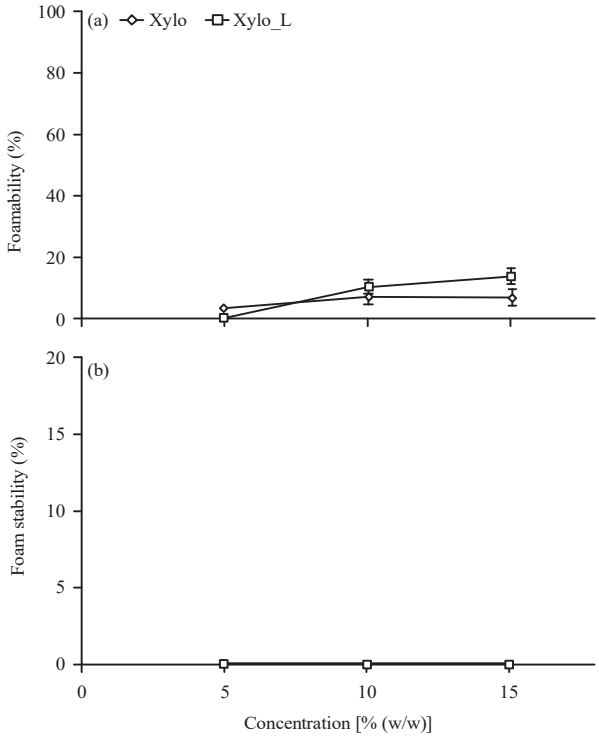


Fig. 2(a-b): (a) Foamability and (b) Foaming stability as a function of Xylo and Xylo_L concentration
Values are mean±standard deviation

Xylo and Xylo_L had small foamability as shown in Fig. 2. The foamability of Xylo_L was slightly higher than for Xylo at concentrations of 10 and 15% (w/w). Neither Xylo nor Xylo_L expressed foaming stability. Consequently, we concluded that Xylo_L was surface-active and had emulsifying properties but foaming properties were not clearly exhibited.

**Microstructure of Xylo and Xylo_L:** Spherical particles of Xylo of various sizes were observed under the SEM (Fig. 3a-b) and the particles were agglomerated as small floc. The Xylo_L sample consisted of small, non-uniform particles with a rough surface and agglomeration was apparent, similar to Xylo because Xylo_L still had high hydrophilicity because of its low DS value (Table 1) that induced water absorption and agglomeration. The difference in the particle shape between Xylo and Xylo_L may have been due to the different drying processes. Commercial Xylo may involve a spray drying process, while the experimental modified Xylo_L was dehydrated and the ethanol was evaporated using a hot-air oven, after which the dried, large particles were broken down into small particles using grinding in a mortar that resulted in various shapes and sizes (Fig. 3c-d).

**Antimicrobial activity of Xylo and Xylo_L:** The antibacterial activities of Xylo and Xylo_L against *E. coli* and *S. aureus* were investigated based on the inhibition concentration (Fig. 4). The results showed that both Xylo and Xylo_L produced antibacterial activity against *E. coli* and *S. aureus* in a concentration-dependent manner [5-15% (w/w)]. In addition, concentration-dependent antibacterial activity of chitosan was reported by Shanmugam *et al.*[19] and the antibacterial activity increased with increased concentration of chitosan[20]. Xylo displayed a higher antibacterial activity against *E. coli* than for *S. aureus* at the highest concentration [15% (w/w)]. At the low concentration of Xylo [5% (w/w)], the colony count for *S. aureus* (9.52±0.17 log CFU $mL^{-1}$) was significantly lower than for the control (0% Xylo; 10.06±0.11 log CFU $mL^{-1}$). On the other hand, the colony count for *E. coli* at the same Xylo concentration [5% (w/w)] was 10.80±0.02 log CFU $mL^{-1}$ and was significantly greater than the control (9.83±0.11 log CFU $mL^{-1}$) because of the increased carbohydrate source. However, the same trend was apparent from increasing the Xylo concentration [5-15% (w/w)] that led to a significant decrease in the colony count for *E. coli* and *S. aureus* due to the antimicrobial ability of Xylo[21,22] and the increased osmotic pressure as the concentration increased.

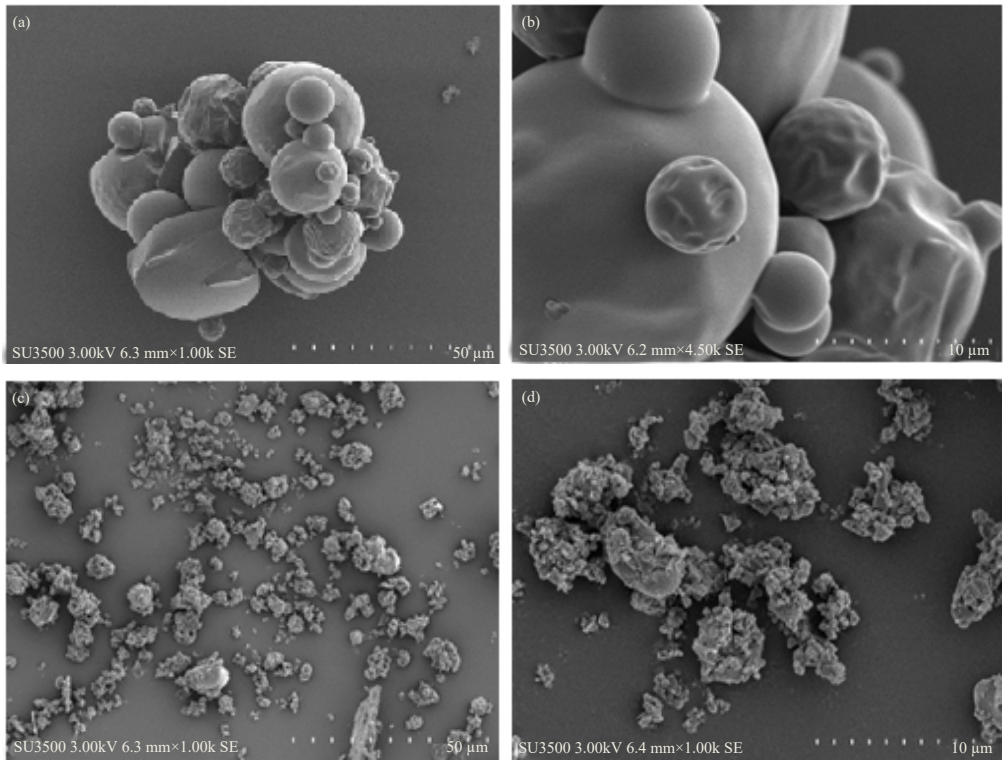


Fig. 3(a-d): Scanning electron microscope micrographs of (a and b) Xylo and (c and d) Xylo_L at (a and c) 3,000× magnification and (b and d) 4,500× magnification

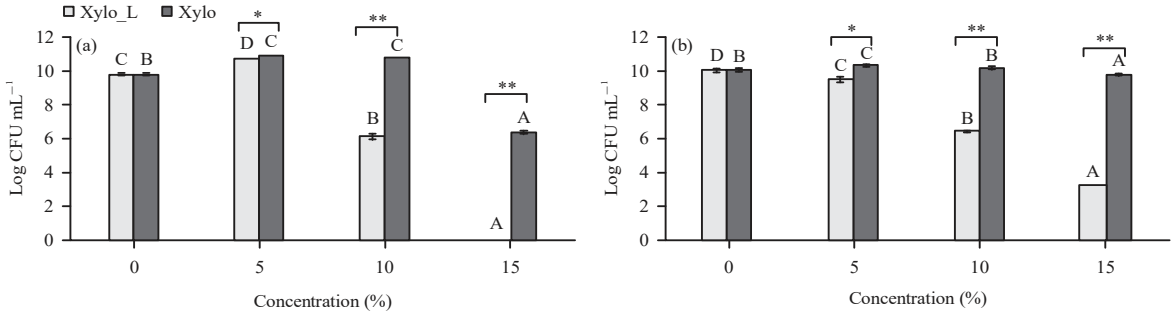


Fig. 4: Number of colony forming units (CFU $mL^{-1}$) of (a) *E. coli* and (b) *S. aureus* after 24 h treatment with Xylo and Xylo_L at different concentrations

Values are Mean±standard deviation; bars with different superscripts (A-D) are significantly ($p<0.05$) different in number of colony forming units among different concentrations for the same sample. * and **indicate significant differences at $p<0.05$ and 0.01 respectively, for Xylo and Xylo_L at the same concentration

Xylo 15% (w/w) was the most effective at inhibiting *E. coli* as no colonies were observed. However, Xylo_L was less effective at *E. coli* inhibition than Xylo at all concentrations [5-15% (w/w)]. This may be attributed to the esterification perhaps reducing the antimicrobial activity of Xylo. Furthermore, Xylo_L might have been limited by the increased osmotic pressure in the system due to its lower solubility in water (84.82%) compared with Xylo. Although, Xylo_L had higher surface activity than Xylo (Fig. 1), it also had low inhibitory ability with *E. coli* and *S. aureus*. This may have been due to the fact that antimicrobial activity not only depended on surface activity but also on the structure and shape of the saccharide fatty acid esters[23]. The colony count for *E. coli* increased with increasing concentration of Xylo_L (0-10%)

but then significantly decreased at the higher Xylo_L concentration [15% (w/w)] because the osmotic pressure in the system increased with the increased concentration producing results similar to those for *S. aureus* inhibition. However, the colony count for *S. aureus* at the highest concentration [15% (w/w)] of Xylo_L was slightly lower than the control [0% Xylo_L (w/w)]. The results suggested that Gram-positive bacteria were more resistant to Xylo_L than Gram-negative bacteria which was in agreement with Pantoa *et al*.[4] who found that maltodextrin ester was more effective at inhibiting the growth of *E. coli* than those of *S. aureus*. On the other hand, Zhao *et al*.[13] concluded that the outer membrane of Gram-negative bacteria restricted diffusion of sugar esters through their lipopolysaccharide covering. Moreover, Nobmann *et al*.[24] found that lauric acid and derivatives had higher activity against Gram-positive bacteria.

## CONCLUSION

Xylo_L clearly exhibited emulsifying properties using soybean oil as dispersed phase but its foaming properties were rather low. The antimicrobial ability of Xylo was reduced following modification with a fatty acid using esterification. The current study suggested that even though Xylo_L antibacterial activity against *E. coli* and *S. aureus* was concentration-dependent as it also was for Xylo, a higher concentration of Xylo_L was required in order to produce antibacterial activity. Hence, using a higher concentration of Xylo_L might increase the antibacterial activity. Xylo_L may suitable as an ingredient of emulsion foods, as an emulsifier and stabilizer with a slight antimicrobial function.

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