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Gram-Positive and Gram-Negative Antibacterial Activity in Textiles Impregnated with OA-g-CSO Copolymer

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ABSTRACT

Antibacterial textiles impregnated with chitosan copolymer (OA-g-CSO) have shown good antimicrobial performance and are resistant to different strains by inhibiting the growth of bacteria. However, the partial performance of these antibacterial textiles against Gram-positive and Gram-negative strains has not been well evaluated. In this research, two types of OA-g-CSO copolymers with different precursor ratios were prepared, in which the minimum amount of amino substitution was considered to show antibacterial properties on textiles. The performance of these two types of copolymers on textiles was evaluated by examining the time effect of textiles impregnation with OA-g-CSO, the maximum retention time of the OA-g-CSO emulsion, and the detergent effect on the antibacterial textiles.

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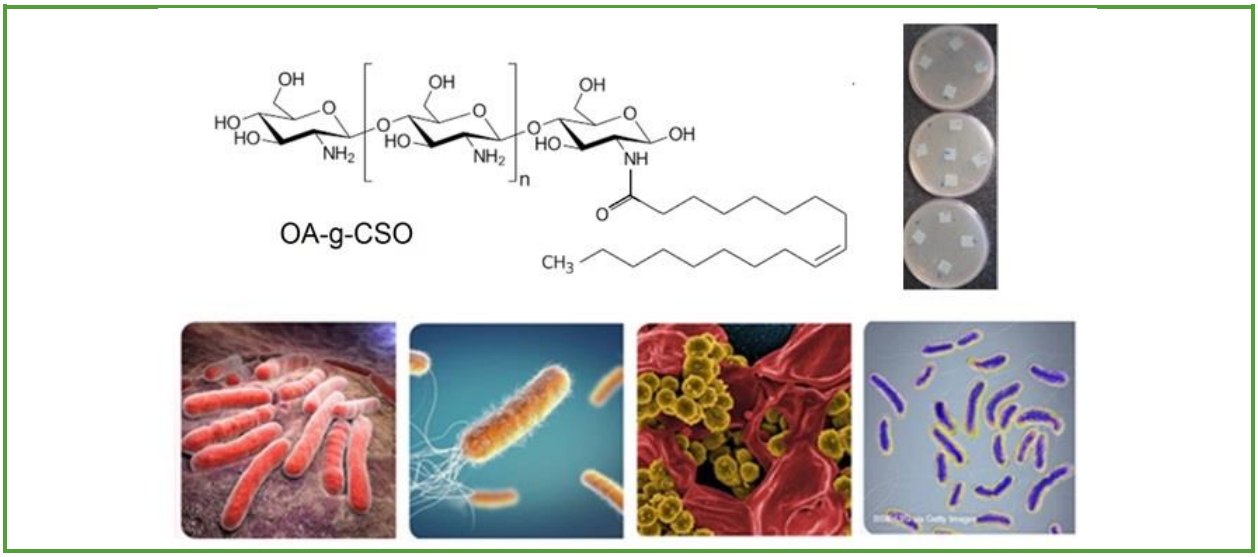
Graphical Abstract



Introduction

Textiles have long been known as media to support the growth of microorganisms such as bacteria and fungi [1, 2]. These microorganisms multiply rapidly when their basic needs are met such as moisture, nutrients, and temperature. Most synthetic textiles are more resistant to microorganism attacks than natural textiles due to their high hydrophobicity [2, 3].

Therefore, in the last few decades, efforts have begun and continue to impregnate natural textile with antibacterial substances. These materials include silver nanoparticles, titanium dioxide, zinc oxide, and other organic or inorganic materials [4].

One of the organic substances with animal origin known as an antibacterial agent is the oligosaccharide chitosan (CSO), which is an acetylated derivative of chitin. This substance is the main component of crustacean shells such as shrimp, lobster, and lobster [5-8].

Chitosan and its derivatives are hydrophilic compounds with low solubility in physiological environments with pH=7.2-7.4 [9]. Chitosan is widely used in medicine [10], antibacterial activity [11-18], water purification [6], and as drug and gene carrier [13].

Despite extensive research on other antibacterial substances [19-24], but chitosan is still one of the important options to choose. Many studies have shown that chitosan inhibits the growth of microbes. A minimum inhibitory concentration (MIC) of 0.05-0.1% (w/v) of chitosan is sufficient against many common bacterial species. [5, 7, 8].

Chitosan is still considered and studied as a biocompatible antimicrobial material because it has a high capacity to increase the antimicrobial property by making changes in its structure [9].

As an example, oleic acid-grafted chitosan oligosaccharide (OA-g-CSO) with different degrees of amino substitution (DS) have shown antibacterial properties. The OA-g-CSO with different DS has been synthesized by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling reaction. The DS includes the number of OA groups per 100 amino groups in the CSO structure [14].

The antibacterial activity of this copolymer has been investigated against *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S. aureus*).

Antibacterial test has shown that this copolymer has good antibacterial activity against *E. coli* and *S. aureus* bacteria [13].

The antibacterial mechanism of chitosan and its derivatives is not clear, but it is generally accepted that primary amine groups create positive charges that interact with negatively charged residues on the cell membrane of bacteria [8, 11].

Currently, one of the best ways against bacteria is to destroy their cell membrane and make it ineffective [15].Most of the bacteria are divided into two groups, Gram-positive (G+) and Gram-negative (G-), based on their cell membrane composition. This classification is based on different interaction of the cell membrane compounds with the chemical compounds of the dye, in Gram-staining which is related to different composition of the cell membrane, as displayed in Figure 1.

The external membrane of Gram-positive bacteria is composed of peptidoglycan molecule with the chemical formula $C_{40}H_{67}N_9O_{21}$ which exists only in prokaryotes, but in Gram-negative bacteria, the peptidoglycan layer is more internal with lower thickness [16-18].

Despite extensive research on the antibacterial properties of OA-g-CSO copolymer, not sufficient research has been done on the antimicrobial performance of chitosan-impregnated fabric. Chitosan has often been investigated as an antimicrobial agent when it is in the form of a thin film [11], which is different from when chitosan is placed on a textile or fabric. In addition, the chitosan antimicrobial research has often been conducted on a special type of bacteria such as Lactobacillus [8].

On the other hand, there is no comprehensive report on the performance of this copolymer against Gram-positive and Gram-negative bacteria. Accordingly, in this article, an attempt has been made to compare the antibacterial effects on Gram-positive and Gram-negative bacteria by examining the antibacterial fabric impregnated with this copolymer.

In this research, two types of bacteria (Gram-positive and Gram-negative) were selected to investigate textiles impregnated with OA-G-CSO; *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*) which are Gram-positive and *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) which are Gram-negative (Figure 2). Therefore, this study is somehow new and innovative. The investigations include OA-g-CSO concentration, saturation time (IT), retention time (RT), and the detergent effect on the antibacterial performance of textile impregnated with this copolymer.

Experimental

Materials and Methods

Chitosan with the empirical formula $(C_8H_{13}NO)_n$, with white color and molecular weight 5000 kDa (Da=1 g/mol) was used with deacetylation degree of 75%. Oleic acid with empirical formula $C_{18}H_{34}O_2$, molecular weight 282.46 g/mol was used. EDC (EDC-HCl salt form) was used as a catalyst with empirical formula $C_8H_{17}N_3$. The molecular mass of $C_8H_{17}N_3$.HCl is 191.70 g/mol. It is frequently used at pH=6-4 as a active ingredient of carboxyl group. DI water is used to prepare solutions.

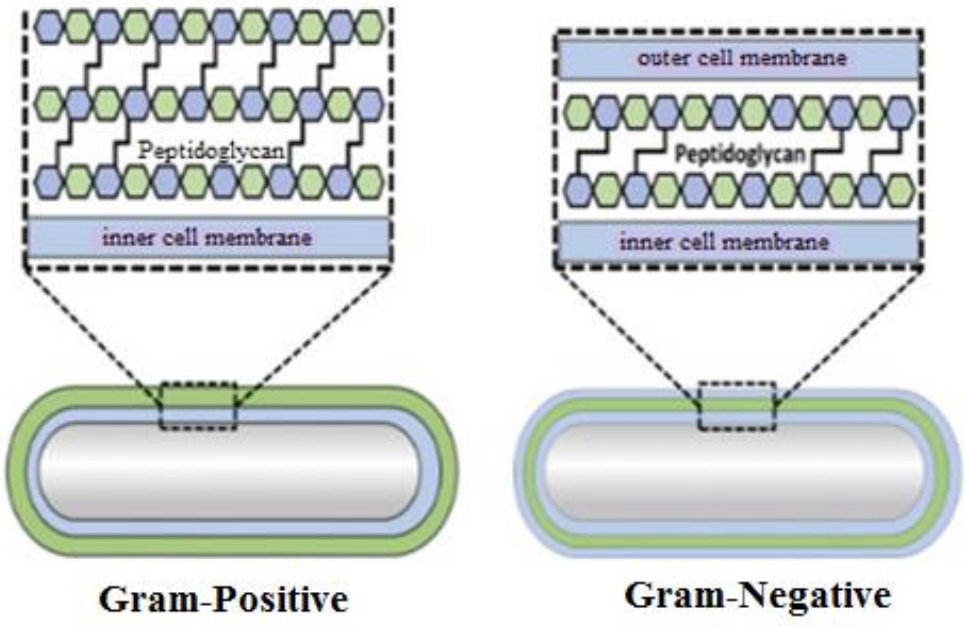


Figure 1. The difference between Gram-positive and Gram-negative bacteria in different cell membrane compositions

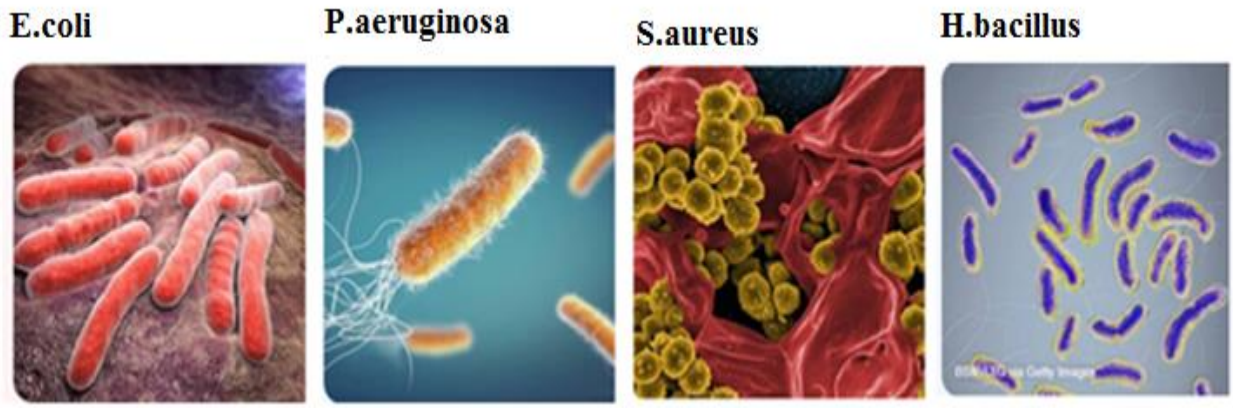


Figure 2. A view of Gram-positive and negative bacteria

Preparation of OA-g-CSO

The CSO was coupled with OA using EDC catalysts and forms copolymer OA-g-CSO. To synthesize copolymer OA-g-CSO, often the same molar ratios of all three materials CSO, OA, and EDC is used [14]. In this situation, every single CSO macromolecule is coupled with a single OA, Figure 3.

However in this article, instead of a molar ratio of 1:1:1, different molar ratios, i.e. 1:0.024:0.0026 and 1:0.240:0.0260 have been used to check the antibacterial behavior of the copolymer with the least substitution of oleic acid and the least amount of DS.

In the first experiment, 1 g of CSO was dissolved in 100 ml solution 1% (v/v) acetic acid in DI water under stirring at room temperature for 15 min. The resulting solution,

which was a viscous colloidal, was poured into a 500 mL round bottom flask equipped with a thermometer. According to Table 1, 0.0014 g of oleic acid was dissolved in 15 ml of methanol and immediately added to the flask, and then 0.0010 g of EDC was dissolved in 15 mL of methanol and added to the flask.

The polymerized solution was stirred in the flask for 24 h at a temperature of 60 °C on the stirrer-heater to complete the formation process of OA-g-CSO and result AE1 emulsion. The same experiment was repeated with the same amount of CSO (1 g), but different amounts of oleic acid (0.0140 g) and EDC (0.0100 g) to prepare another polymerized solution, emulsion AE2, AE1, and AE2 were two OA-g-CSO antibacterial emulsions were employed to impregnate textiles. The selection of these two molar ratios, i.e. 1:0.024:0.0026 and 1:0.240:0.0260 to obtain the minimum DS process required to produce antibacterial properties in the fabric is not available in the literature. Obviously, a wider range of concentrations can be investigated, which was not targeted in this research. The control sample in this research is chitosan itself without the additional DS process.

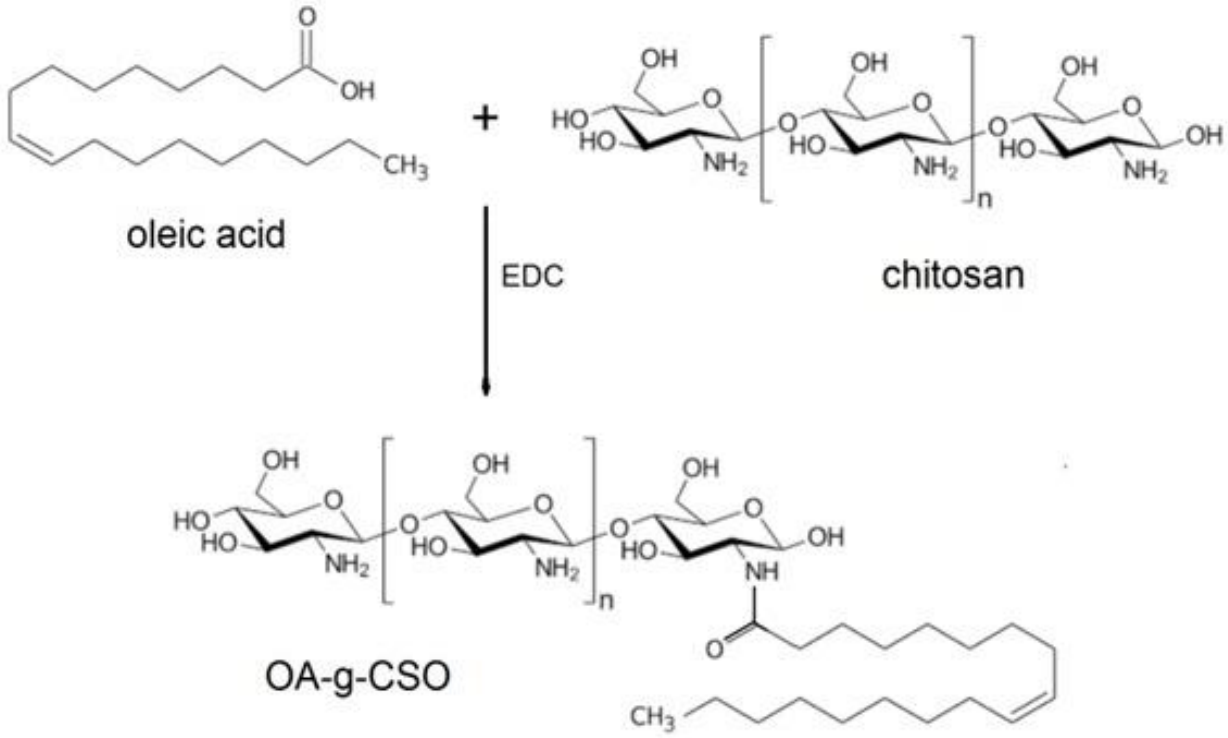


Figure 3. Oleic acid-grafted chitosan oligosaccharide (OA-g-CSO)

Table 1. Amounts of precursors to prepare antibacterial emulsion (AE) of OA-g-CSO

Material	CSO/gr	OA/gr	EDC/gr	AE
Molecular weight	5000	282.46g/M	191.7g/M	-
Molar ratio (1:1:1) in ref [14]	1.0000	0.0564	0.3834	-
Molar ratio (1:0:0) as a control	1.0000	-	-	AE0
Molar ratio (1:0.024:0.0026)	1.0000	0.0014	0.0010	AE1
Molar ratio (1:0.240:0.0260)	1.0000	0.0140	0.0100	AE2

Impregnation of textile sample with OA-g-CSO

A number of textile samples with dimension of 2 × 2 cm^2 were washed with detergent, rinsed with distilled water, and immersed in AE1 or AE2 emulsions. The temperature of all emulsions was kept constant at 60 °C, then the samples were removed from the emusions after different times, washed with distilled water, and dried on a heater at 100 °C. Some samples were washed with detergents to check the stability of the OA-g-CSO copolymer on textiles. Some samples were immersed in the solutions after one to three weeks to check the effect of the retention time on the quality of the emulsions. Finally, the impregnated samples with OA-g-CSO were infected with bacteria to evaluate their bacterial resistance. The differences in the impregnated samples include differences in the use of different antibacterial emulsions (AE), retention time (RT), saturation time (IT), and detergent effect (DET).

Antibacterial test

Antibacterial tests had been done using bacterial strains including, *E. coli* and *P. aeruginosa* as Gram negative bacteria (G-), and also *S. aureus* and *B. subtilis* as Gram positive bacteria (G+). In all measures, the standard specific antibiotics were used to ensure uniform conditions for all tests. In addition, comparative study based on standards recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) was used [17]. Proprietary standard antibiotics of Streptomycin, penicillin, and chlorophyll was used for Gram-positive and Gram-negative, the case of fungal, respectively. All strains before use were stored in environments containing 11% (v / v) dimethyl sulfoxide at -70 °C. Before use, bacterial and fungal strains were grown on Mueller Hinton agar plates [16, 17] and sabouraud dextrose agar (Merck, Germany at 37 °C), respectively.

One colony of bacterial samples was cultured on nutrient agar (N-agar) with Nutrient Broth (NB) 8 g and 16 g and incubated for 24 h at 37 ºC. After two consecutive subcultures, samples rejuvenation was ready to do the next steps [16].

Seminal fluid was contained microorganisms suspensions in the liquid cultivation medium with about 106 single colony (CFU) [17]. For preparation of cellular colonies, the pure bacterial cell suspension was added to 10 ml cultivation media of the NB and after 30 s Vertex was placed on the shaker-incubator for 4 h. The concentration of samples in this interval equals to half McFarland turbidity visually or equivalent of approximately 1.5×106 CFU/ml. The pure colonies were used to produce seminal fluid [18].

To assess the antibacterial disc diffusion method, 100 µl of under examination seminal fluid bacterial and fungal strains were grass cultivated with sterile swabs on Mueller Hinton agar plate's sterile environments. Antibiotic disc (6 mm) was saturated with 30 µl of each of the tested extracts, and then solvent on disc was evaporated in laboratory temperature. Resulted disc was mounted onto under investigation of grass cultured plates. Plates were incubated at 37 ºC in an incubator, Heraeus-Cytoperm 8088 [17].

The diameter of inhibition zone (IZ) around the disc was determined after 18 h by caliber. Textile samples (TS) with dimensions of 1×1 cm^2 were cut and used to determine the antibacterial effect. It should be noted that in this study the cultivation standard conditions of non-photosynthetic microorganisms were used. To quantify and assess the effects of antibacterial numerical radius zone of inhibition is compared to the sample edge. In

the following studies, the antibacterial effects have been reported based on the diameter of zone and millimeter scale.

Results and Discussion

Figure 4 demonstrates antibacterial effects in some Textile samples (TS) impregnated with AE1 and AE2 after contaminating with germs; *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. These effects were shown by inhibition zone (IZ) from the edge of textile sample per mm. To avoid crowding, only the results of TS4, 5, and 6 have been shown, respectively, and for the rest of the samples, only the numerical results have been reported. According to Table 2, antibacterial test results were reported for all textile samples (TS).

The TS1 is control sample without impregnating with AE1 or AE2, but contaminated with germs. No inhibitory effect seen in TS1. The samples of TS2-10 and TS11-20 were impregnated with AE1 AE2, respectively, within different conditions. In general, checking the results of the report in Table 2 indicate that the effectiveness of AE1 solution is only on Gram-positive bacteria and the AE2 effectiveness is on both Gram-positive and negative bacteria. Applying a retention time of one week for both AE1 and AE2 solutions does not have a special effect on the antibacterial performance of emulsions, and this shows the stability of the solution containing chitosan.

Decreasing the impregnating time in both series of textile samples causes a decrease in performance. The use of detergents in both series of impregnated samples with AE1 and AE2 weakens the antibacterial effect of the textiles.

In particular, TS1 which is not impregnated with any antibacterial emulsions did not show

The antibacterial emulsion of OA-g-CSO with lower concentrations has an inhibitory

any antibacterial activity and inhibition zone. Samples dipped in AE1 solution have responded only to Gram-positive bacteria. The examination of samples 2 and 3 showed that the minimum time of impregnation with AE1 solution should be 6 h. The 2 h impregnation is not enough to have antibacterial activity in textile. The TS 4, 5, and 6 showed that the retention time of AE1 from 1 to 3 weeks has a partially negative effect on the antibacterial performance of antibacterial textiles and reduces the inhibition zone. Examining the effect of detergent in the TS 7, 8, and 9 illustrated that the impregnated samples with AE1 during 2 h to 6 h, after employing detergent, have not any antibacterial properties.

Comparison of TS 10 with TS3 showed that increasing the impregnation time from 6 h to 8 h weakens the performance of the antibacterial activity of textile. The TS 12, 13, and 14 showed that the minimum time of impregnation with AE2 is 6 h, and with the increase of impregnation time, the IZ increases. The 2 h of impregnation time was not enough to achieve antibacterial activity. The TS 15, 16, and 17 exhibited that the retention time of AE2 from 1 to 3 weeks has a partially negative effect on the antibacterial performance of impregnated textile in AE2 solution and reduces the inhibition zone.

The effect of detergent in TS18, 19, and 20 showed that the impregnated samples with AE2 during 2-6 h, after washing with detergent, have shown a weaker antibacterial effect and the inhibition zone, IZ, is greatly reduced with compared to TS12, 13, and 14. Comparison of sample TS 11 with 14 showed that increasing impregnation time from 6 h to 8 h weakens the performance of the antibacterial activity on the textile.

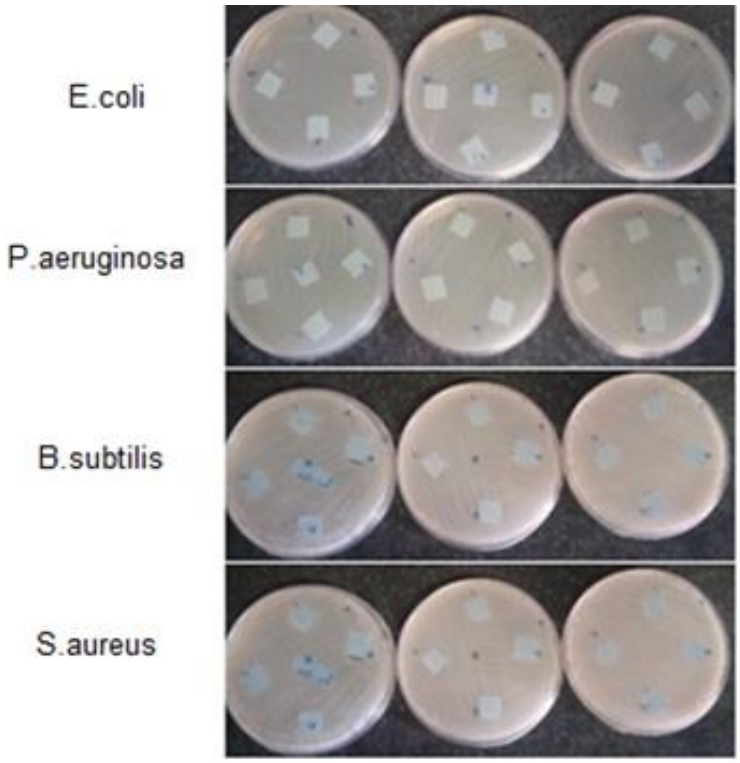


Figure 4. TS4, TS5, and TS10 with antibacterial effects against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*

Table 2. Antibacterial result on textile impregnated with OA-g-CSO

TS	AE	RT (week)	IT (h)	DET	*B. subtilis* *G+* IZ	*S. aureus* *G+* IZ	*E.coli* *G-* IZ	*P. aeruginosa* *G-* IZ
1	-	-	-		-	-	-	-
2	AE1	-	2		-	-	-	-
3	AE1	-	6		1.5-2	2-3	-	-
4	AE1	1	6		2-3.5	2-3.5	-	-
5	AE1	2	6		2-3.5	1	-	-
6	AE1	3	6		2-3	-	-	-
7	AE1	-	2	yes	-	-	-	-
8	AE1	-	4	yes	-	-	-	-
9	AE1	-	6	yes	-	-	-	-
10	AE1	-	8		≥1	≥1	-	-
11	AE2	-	8		1	-	-	-
12	AE2	-	2		≥1	1	1	1
13	AE2	-	4		1	1	1	1
14	AE2	-	6		1-2	1-2	1-2	1-2
15	AE2	1	6		1	2	-	-
16	AE2	2	6		1	-	-	-
17	AE2	3	6		-	-	-	-
18	AE2	-	2	yes	1	1	-	-
19	AE2	-	4	yes	1	1	1	1
20	AE2	-	5	yes	-	-	-	-

Effect only on Gram-positive bacteria *B. subtilis* and S. *aureus*, and also with higher concentrations on both types of Gram-positive bacteria *B. subtilis* and *S. aureus* as well as bacteria Gram-negative *E. coli* and *P. aeruginosa*.

Persistence of OA-g-CSO emulsions with both concentrations has reduced and negative Effects on the antibacterial property of impregnated textile.

The use of detergent in impregnated textile with has destructive effects on antibacterial performance. The minimum and maximum time required to impregnate textile with OA-g-CSO and make it antibacterial is 4 h and 6 h, respectively. The impregnation time of 2 h was insufficient and more than 6 h was a waste of time because it did not show better results than the impregnation time of 6 h.

Conclusion

Antibacterial tests were performed using bacterial strains including Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, as well as Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*. Two antibacterial emulsions (AE) containing OA-g-CSO with different ratios of precursors were used. Textile samples were used to determine the antibacterial effect of OA-g-CSO copolymer on the textiles. It is clear that the AE1 efficacy with at least degrees of amino substitution (DS) was only on Gram-positive bacteria and the AE2 efficacy with higher DS was on both Gram-positive and Gram-negative bacteria. Applying a retention time of one week for both AE1 and AE2 solutions has a negative effect on antibacterial performance. Decreasing the saturation time in both series of textile samples decreases the antibacterial performance of the fabric. The use of detergents in both series of textile samples weakens the antibacterial effect of the fabric.

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No potential conflict of interest was reported by the authors.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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