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Antimicrobial Activity of Nanoemulsion on Cariogenic Planktonic and Biofilm Organisms

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Abstract

Introduction—Nanoemulsions (NE) are a unique class of disinfectants produced by mixing a water immiscible liquid phase into an aqueous phase under high shear forces. NE have antimicrobial properties and are also effective anti-biofilm agents.

Materials and Methods—The effectiveness of nanoemulsion and its components was determined against Streptococcus mutans and Lactobacillus casei by live/dead staining. In vitro antimicrobial effectiveness of nanoemulsion against planktonic Streptococcus mutans, Lactobacillus casei, Actinomyces viscosus, Candida albicans and mixed culture was determined by a serial dilution technique to obtain minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC). In addition, efficacy was investigated by kinetics of killing, adherence and biofilm assays.

Results—Compared to its components, nanoemulsion showed notable antimicrobial activity against biofilm organisms, up to 83.0% kill within 1 min. NE dilutions ranging from 243 to 19683 were effective against planktonic S. mutans, L. casei, A. viscosus, C. albicans and mixed culture of these four strains as shown through MIC/MBC assays. NE showed antimicrobial activity against planktonic cells at high dilutions, confirmed by time kill studies. The level of adhesion on glass surface was reduced by 94.2 to 99.5 % in nanoemulsion treated groups ($p < 0.001$). 4-day-

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old S. mutans, L. casei, A. viscosus, C. albicans and mixed cultures biofilms treated with NE showed reductions of bacterial counts with decreasing dilutions ($p < 0.001$).

Conclusion—These results suggest that nanoemulsion has effective anti-cariogenic activity against cariogenic microorganisms and may be a useful medication in the prevention of caries.

Keywords

Nanoemulsion; cariogenic microorganisms; anti-caries; adherence; biofilm organisms

Introduction

Cariogenesis is the production of dental caries and can be defined as a multi-step process involving demineralization and remineralization rather than unidirectional demineralization of the tooth surface. Dental caries occurs by the action of acidogenic and aciduric bacteria, which interact with microorganisms in biofilms on dental surfaces. The dental biofilm is formed via adhesion of planktonic bacteria to a cellular pellicle coating the oral surface. Several types of bacteria participate in the formation of the dental biofilm [1]. More than five *Streptococcus* species and *Actinomyces viscosus* are regarded as early colonizers of tooth surfaces, while mutans streptococci such as *Streptococcus sobrinus* and *Streptococcus mutans* are considered to be late colonizers of the dental biofilm. Other species of microorganisms that are present in the oral cavity, such as *Candida albicans* and *Lactobacillus casei*, may also cause pathologies, particularly under specific conditions. Biofilm formation initiates plaque formation and the synthesis of glucan from sucrose by *S. mutans*, which is catalyzed by glucosyltransferases (GTFases). Dental plaque metabolizes the carbohydrates contained in foods, releasing organic acid metabolites that demineralize tooth surfaces, resulting in dental caries [2].

The prevention and control of dental caries is still considered a public health problem of the world's population. Nanoemulsions are a unique class of disinfectants produced by mixing a water immiscible liquid phase into an aqueous phase under high pressure. This procedure yields a uniform population of high energy droplets which are only kinetically stable, and which have the appearance and consistency of whole milk [3]. Nanoemulsions have broad biocidal efficacy against bacteria, enveloped viruses, and fungi by disruption of their outer membranes. The mixed immiscible preparations of soybean oil and water represent a new generation of disinfectants that selectively disrupt membranes of bacteria, fungi in dilutions up to 1:1000 and enveloped virus [4, 5, 6, 7, 8].

The nanoemulsion employed in this work contains cetylpyridinium chloride (CPC), a quaternary ammonium salt. CPC is an effective antiplaque agent regulated by the Food and Drug Administration. It has been proposed for inclusion in a range of products for dental use, such as mouth rinses, toothpastes, varnishes, orthodontic adhesives and liners for glass ionomer cements [9-14]. The safety and efficacy of CPC have been evaluated extensively and proven based on cytotoxicity data collected from many animal studies [15-17]. Therefore, the use of a CPC-containing nanoemulsion to control the adhesion and biofilm formation of cariogenic bacteria on the tooth surface is a logical approach to prevent this common oral disease.

Material and Methods

Preparation of nanoemulsion and bacterial strains

The oil-in-water nanoemulsion has an oil phase of soybean oil (25% v/v of the total emulsion), cetylpyridinium chloride (CPC) (1% w/v), and Triton X-100 (10% v/v) in 65% v/

v deionized water. The mixture was emulsified with a microfluidizer (M-110L, Microfluidics, Newton, MA) by 2 passes at 20,000 psi and at room temperature. Particle size was measured using laser light scattering (Dynamic Light Scattering, Brookhaven Instruments, Holtsville, NY) [4]. In the present study, brain heart infusion (BHI) from Difco Laboratories (Detroit, MI) was used to culture *Streptococcus mutans, Lactobacillus casei, Actinomyces viscosus, Candida albicans* and mixed cultures of the four strains. For biofilm and adherence assays, cells were grown in BHI with 2% sucrose supplementation.

Activity of nanoemulsion and its components

S. mutans and *Lactobacillus casei* were grown in 4-well Lab-Tek chamber slides with cover slide (Nalge Nunc International, Naperville, IL) for 24 hrs at 37°C with BHI broth and 2% sucrose supplementation. The grown biofilm was treated with nanoemulsion, or with 1% CPC, 10% Triton X-100, or a combination of 1% CPC and 10% Triton X-100 for 1 min. Biofilms were stained with L 7012 LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit from Molecular Probes Inc. (Eugene, OR) as described by Neu and Lawrence [18]. The live/dead stain, stored at −20 °C, was warmed to room temperature and centrifuged prior to use. The staining solution, containing two components SYTO9 and propidium iodide, was mixed in equal quantities and applied to the wells for 15 min. The samples were immediately examined via the Olympus FV1000 confocal system on an IX81 microscope (Olympus Life Science, Center Valley, PA). Excitation wavelengths were 488 and 543. Image analysis was conducted as described elsewhere (Al-Ahmad et al., 2007). In order to quantify the ratio of green and red areas of the analyzed biofilm points, a maximal projection of each image stack was built using the program LSM Image Browser (Zeiss, Oberkochen, Germany). Using the image analysis program Image J1.42q (Wayne Rasband National Institute of health, USA), red and green projections were converted into merged black and white (B/W) images. In order to determine the area covered by all cells, live or dead, B/W intensity thresholds were manually set for each of the measured biofilm areas. The resulting red and green ratios were analyzed for their statistical significance.

Minimum inhibitory (MIC) and bactericidal (MBC) concentrations

Nanoemulsion was serially diluted with standardized cell suspension (10^7 CFU/ml) of *S. mutans, L. casei, A. viscosus, C. albicans* and mixed cultures of the four strains and incubated at 37°C overnight. The highest dilution where there was no growth was recorded as the MIC. For MBC testing, aliquots were plated onto Brain Heart Infusion (BHI) agar and incubated overnight at 37°C. The highest dilution where there were no survivors was recorded as the MBC.

Time kinetics

Overnight bacterial cultures of *S. mutans, L. casei, A. viscosus, C. albicans* and mixed cultures of the four strains were added to 1, 10, 100 and 250 dilutions of the emulsion in microtiter plates. After the addition of each culture, 1 ml samples were taken at 1, 5, 15, 30 and 60 min from inoculated emulsions and immediately diluted in 9 ml of BHI. For viable counts, BHI broth samples were incubated at 37°C for 24 h. The optical density of the resulting broth was read at 490 nm [4].

Adherence assay

Overnight bacterial cultures containing 10^7 CFU/ml of *S. mutans, L. casei, A. viscosus, C. albicans* and mixed cultures of the four strains were added to nanoemulsion in different dilutions (1, 10, 100 and 250) in 5ml glass test tubes. The tubes were incubated aerobically (to simulate conditions in the mouth) at 37°C for 24h. Attached bacteria were fixed with 5ml of methanol per tube for 15 min. The tubes were then emptied and air dried. Each tube was

then stained for 5 min with 5 ml of 1% (v/v) crystal violet. Excess stain was rinsed off by placing the tube under running tap water. The tubes were air dried and the dye bound to the adherent cells was removed with 5 ml of 33% (v/v) glacial acetic acid per tube. The optical density of the resulting solutions was read at 595 nm.

Chlorhexidine digluconate 0.12% (v/v) (SigmaAldrich, St. Louis, MO) was used as positive control. At least three independent experiments were carried out for each experimental condition. In each case, the mean optical density of the negative control was subtracted from the readings of all other samples.

Biofilm assay

Biofilm formation of *S. mutans, L. casei, A. viscosus, C. albicans* and mixed cultures of the four strains in plastic microplates was performed as previously described by Stepanovic et al. [19]. A 20 μl portion of an overnight broth culture (10^7 CFU/ml) was added to each well of a 96-well tissue culture plate and was incubated aerobically, with mild agitation at 70 rpm, for 72 h at 37 °C. Every 12 h, the medium containing suspended bacterial cells was removed and an equal volume of fresh medium was added. After removing the supernatant media, the biofilms were treated with 1, 10, 50, 100, 200 and 250 μl emulsion for 30 min at room temperature and without agitation. Following this, the emulsion was removed and the wells were gently washed twice with sterilized distilled water. Quantification of viable cells in biofilms in plastic microplates was performed as previously described by Stepanovic et al. [19]. Attached bacteria were fixed with 250 μl of methanol per well for 15 min. The microplates were then emptied and air dried. Each well was then stained for 5 min with 250 μl of 1% (v/v) crystal violet. Excess stain was rinsed off by placing the microplate under running tap water. The microplates were air dried and the dye bound to the adherent cells was removed with 250 μl of 33% (v/v) glacial acetic acid per well. The optical density of the resulting solutions was read at 595 nm on a microplate reader.

Chlorhexidine digluconate 0.12% (v/v) (SigmaAldrich, St. Louis, MO) was used as positive control. At least three independent experiments were carried out for each experimental condition. In each case, the mean optical density of the negative control was subtracted from the readings of all other samples.

Statistical procedure

Experiments were performed in triplicate. Each experiment was done with five replications of the same concentration with individual microorganisms. The means of five values were used for calculation of means and standard deviations. The assumptions of equality of variances and normal distribution of errors were checked for the response variables. Significant differences among the groups were established with ANOVA to analyze the factors X and Y followed by Tukey's test. *P*-values of $p \leq 0.001$ were considered significant. For ANOVA analysis, concentrations of nanoemulsion and exposure time were considered factors in the kinetic study. Nanoemulsion concentration and microorganism group were considered factors for the adherence and biofilm study.

Results

Microfluidizer emulsification resulted in a narrow distribution of droplets with a mean diameter of 308 nm. This nanoemulsion (NE) was used for all experiments.

Effectiveness of nanoemulsion was greater than that of its components at 1 min, with cetylpyridinium chloride (CPC), Triton X-100 (TRI) and CPC/TRI showing lower activity, Fig 2. Compared to the control, nanoemulsion showed 83% inhibition of *S. mutans* and *Lactobacillus casei*.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of nanoemulsion (NE) for *Streptococcus mutans* (MIC 19683 and MBC 2187 dilutions), *Lactobacillus casei* (MIC 2187 and MBC 729 dilutions), *Actinomyces viscosus* (MIC 19683 and MBC 2187 dilutions), *Candida albicans* (MIC 2187 and MBC 729 dilutions) and mixed culture (MIC 2187 and MBC 243 dilutions) was tested. Higher MIC was observed for *A. viscosus* and *S. mutans* and no visible colonies were observed at 2187 dilution for *A. viscosus* and *S. mutans*. Inhibitory concentrations of NE used was 9-to 27- fold smaller than that of chlorhexidine (Fig. 3).

Killing curves of nanoemulsion and chlorhexidine digluconate against *S. mutans, L. casei, A. viscosus, C. albicans* and mixed culture strains are shown in Fig. 4. In the present experiments we observed that nanoemulsion had faster and more powerful bactericidal activity than chlorhexidine. During incubation with cell suspensions, NE considerably reduced the number of survivors; lower dilutions of NE resulted in the fewest survivors, with a dilution of 1 showing the highest activity. Percentage reduction compared to chlorhexidine was 92.4% (*A. viscosus*), 88.6 (*C. albicans*), 85.2 (*L. casei*), 74.9 (*S. mutans*) and 88.2% for mixed organisms.

The inhibitory effect of NE on adherence of cells to the glass surfaces of test tubes was examined using growing cells of *S. mutans, L. casei, A. viscosus, C. albicans* and mixed culture. NE inhibited the adherence of the growing cells (Fig 5). When the data were subjected to ANOVA, it was found that adhesion of all strains was significantly affected by nanoemulsion ($p < 0.001$). The level of adhesion on glass surface was reduced 94.2 to 99.5 % in nanoemulsion-treated groups when compared to controls. Highest adherence was observed with *A. viscosus* and *C. albicans*. Adherence of *S. mutans* was reduced 96% with nanoemulsion and but only 74 % with chlorhexidine digluconate treated with 1 dilution.

NE markedly inhibited *S. mutans, L. casei, A. viscosus, C. albicans* and mixed culture biofilm formation at quantities as low as 100, 200 and 250μl added to each microplate well, respectively ($p < 0.001$). Highest inhibition of biofilm formation was observed at the 250 μl level (Fig. 6).

Discussion

The application of nanoemulsion as an antimicrobial agent is a new and promising innovation [3]. The investigation into the use of nanoemulsion as an antimicrobial agent was prompted by the known problem of development of antimicrobial resistant strains experienced with the use of existing agents due to the widespread, and sometimes inappropriate, use of antibiotics, disinfectants and antiseptics [20]. These drawbacks justify further research and development of new antimicrobial agents targeting specific pathogens while being safe for the host. Since the mechanism of action of NE appears to be the non-specific disruption of bacterial cell membranes, NEs would not result in the development of resistant strains. Due to their intrinsic features, NE can be further diluted in aqueous solutions and stored at a broad range of temperatures for up to 2 years. Nanoemulsions have been reported to have extensive bactericidal, sporicidal and virucidal effects [4, 5, 6, 7, 21]. NE at biocidal concentrations is nontoxic in short term application to skin, mucous membranes, and the gastrointestinal tract [7].

The addition of a cationic halogen-containing compound such as cetylpyridinium chloride places a positive surface charge on the nanodroplet by being incorporated as a co-surfactant [22]. Bacteria in dental plaque have a net negative surface charge [23, 24]. In addition, exopolysaccharide chains, which vary in size from 103 to 108 kDa, are usually negatively

charged, sometimes neutral or rarely positively charged [25]. Positively charged nanodroplets should have increased potential to interact with the biofilm cells and matrix.

In addition to the above proposed action, quaternary ammonium salts such as CPC have antimicrobial activity of their own, apparently through multiple mechanisms. One mechanism is thought to be due to disruption of intermolecular interactions, causing a dissociation of cellular membrane lipid bilayers, compromising cellular permeability controls, and inducing leakage of cellular contents [26]. Longer exposure times may result in additional breakdowns of intracellular material which are indicative of autolysis [26, 27]. In addition, CPC has an inhibitory action against fructosyltransferases, extracellular enzymes which synthesize fructans from sucrose, which then play an important role in the progression of dental caries by serving as an extracellular nutrition reservoir for bacteria [28].

The application of nanoemulsions for the control of cariogenic biofilm has not been widely investigated, hence the objective of this present study. In this study, we showed that one NE has antimicrobial activity against cariogenic planktonic and biofilm *S. mutans, L. casei, A. viscosus, C. albicans* and mixed culture. MIC and MBC of NE on four strains and mixed culture show significant effectiveness, even at high dilutions. Myc et al., [29] found that the MIC of a different nanoemulsion never exceeded 0.1% of NE. When compared with earlier reports our NE showed very high activity [8, 20].

The nanoemulsion activity in the present study was 9- to 27-fold higher than that of chlorhexidine, which is currently the most potent antimicrobial agent against oral biofilm. However, chlorhexidine is limited in its applications in oral care as it may produce detrimental side effects such as tooth discoloration [30].

Time kinetics assays showed NE to reduce survivors up to 92.4% (*A. viscosus*), 88.6% (*C. albicans*), 85.2% (*L. casei*), 74.9% (*S. mutans*) and 88.2% (mixed culture). Our results are in agreement with previous findings that emulsion had bactericidal properties against Gram-positive and enteric pathogen species [7, 8, 20].

Since, in the dental caries process, adherence of *S. mutans, L. casei, A. viscosus* and *C. albicans* to the tooth surface occurs in the initial stage, inhibition of adherence is essential for the prevention of dental caries. The anti-cariogenic potential of NE may be attributed to their ability to inhibit the attachment of bacteria. NE is significantly effective in inhibiting the growth of four strains by inhibition of adherence to glass surfaces. Initial adherence and colonization thus form the key events in biofilm formation [31]. In addition, the *in vitro* anti-adherence of *Candida albicans* to oral buccal mucosal cells by low concentrations of CPC has been described [32, 33].

The capacity of NE to reduce biofilms of *S. mutans, L. casei, A. viscosus and C. albicans* was evaluated. Generally, exposure to emulsion for 30 min resulted in maximum reductions of biofilms and effectively reduced the numbers of the planktonic form. In addition, Al-Adham et al [34] found that a microemulsion was highly effective against biofilms of *P. aeruginosa* and *S. aureus*. Also, previous reports found that biofilm microorganisms are very resistant to typical cleaning agents, yet are susceptible to nanoemulsions [35]. However, in the present study, complete elimination of biofilm did not occur, possibly due to short exposure times. Further work needs to be carried out to investigate the mechanisms of the interactions between emulsions, cells in suspension and biofilms [20] and to improve the efficacy of NE against biofilms composed of organisms involved in the caries process.

The separate mechanisms of action of both nanoemulsions and CPC may be operating to reduce biofilm formation. It is possible that the positively charged emulsion remains

attached to the biofilm for a longer time than does chlorhexidine and is therefore able to prevent the formation of further biofilm.

In conclusion, the nanoemulsion employed in this work has improved efficacy against microorganisms involved in the caries process in excess of the efficacy of the unemulsified components. CPC-containing nanoemulsion with droplets of mean diameter 308 nm showed remarkable inhibitory effects on the growth of cariogenic *S. mutans*, *L. casei*, *A. viscosus* and *C. albicans* at very high dilution. NE effectively inhibited adherence of these strains to glass surfaces and subsequent biofilm formation. The anti-adherence and anti-biofilm behavior suggest that NE could be useful for the development of promising anti-cariogenic agents.

Acknowledgments

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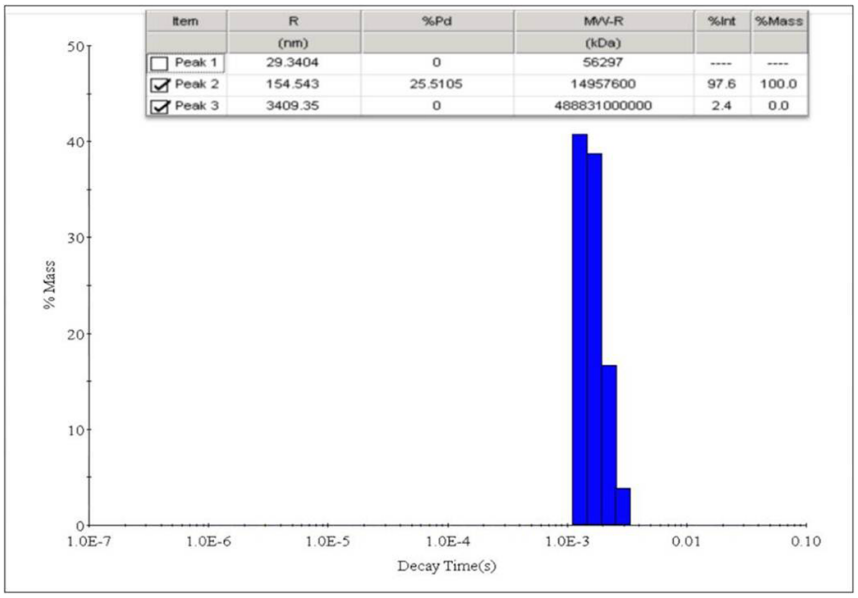

Fig.1.
Light scattering image showing droplet size distribution of nanoemulsion

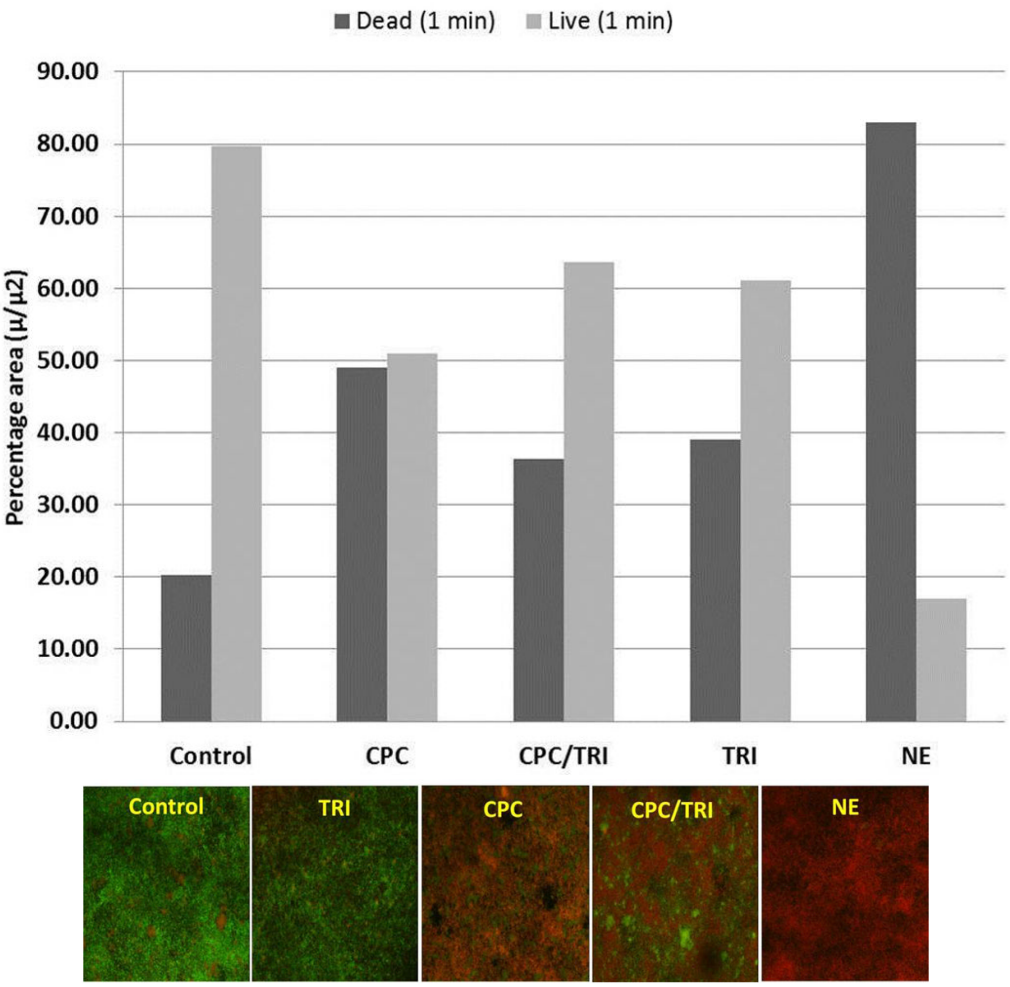

Fig. 2.

Effect of nanoemulsion and its components against *S. mutans* and *Lactobacillus casei* at 1 min. (CPC-Cetylpyridinium chloride; CPC/TRI - Cetylpyridinium chloride/ Triton X-100; TRI - Triton X-100; NE- Nanoemulsion)

Fig.2b Confocal micrographs of live/dead staining of *S. mutans* and *Lactobacillus casei* at 1 min. (CPC-Cetylpyridinium chloride; CPC/TRI - Cetylpyridinium chloride/ Triton X-100; TRI - Triton X-100; NE- Nanoemulsion)

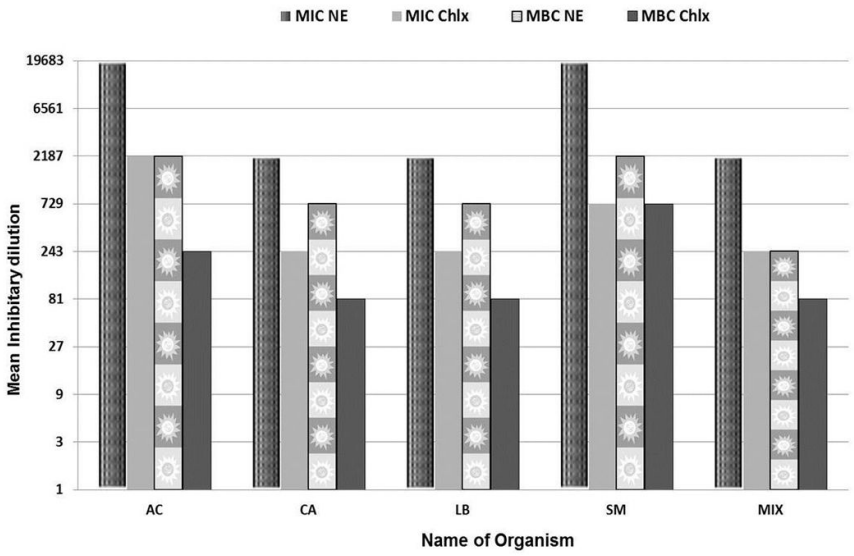

Fig. 3.
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration of nanoemulsion

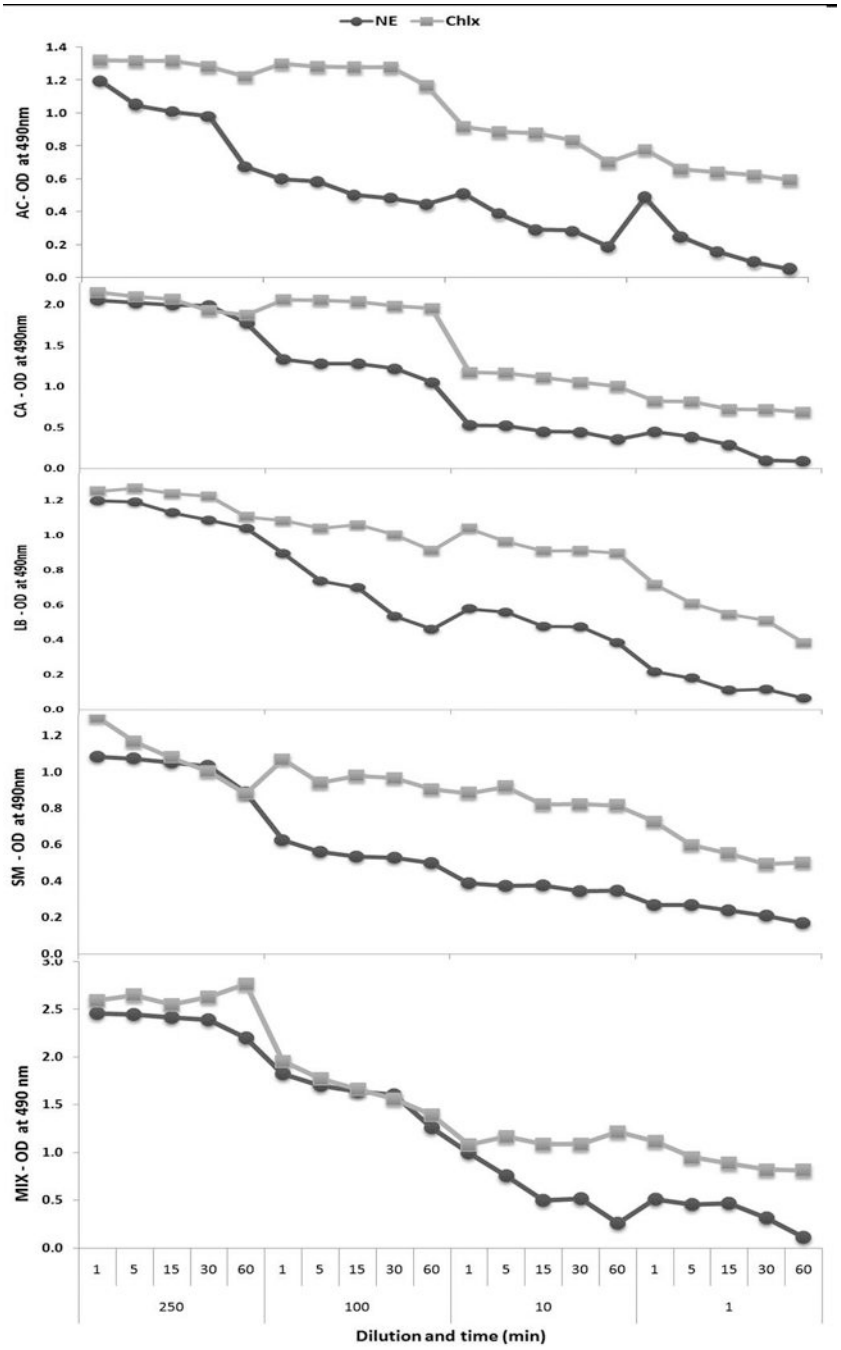

Fig. 4.

Effect of nanoemulsion and chlorhexidine digluconate on time kinetics (1, 5, 15, 30 and 60 mins) of *A. viscosus* (AC), *C. albicans* (CA), *L. casei* (LB), *S. mutans* (SM) and mixed culture of the four strains (MIX) with different dilutions (1, 10, 100 and 250) (NE- Nanoemulsion; Chlx- Chlorhexidine digluconate).

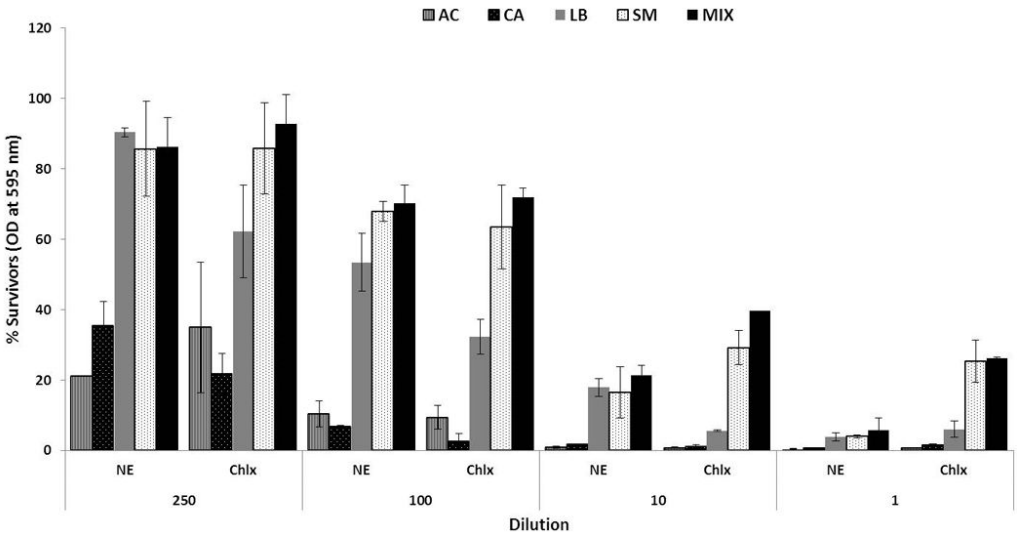

Fig. 5.

Inhibitory effect of nanoemulsion on glass surface adherence of *A. viscosus* (AC), *C. albicans* (CA), *L. casei* (LB), *S. mutans* (SM) and mixed culture of four strains (MIX). (NE- Nanoemulsion; Chlx- Chlorhexidine digluconate

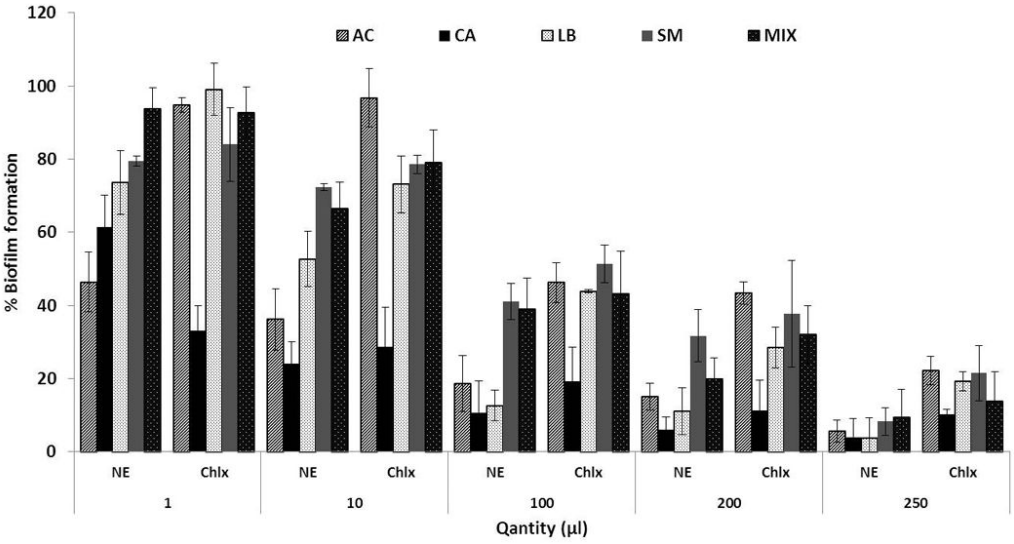

Fig. 6.

Inhibitory effect of nanoemulsion and chlorhexidine digluconate on biofilm formation of *A. viscosus* (AC), *C. albicans* (CA), *L. casei* (LB), *S. mutans* (SM) and mixed culture of the four strains (MIX) (NE- Nanoemulsion; Chlx- Chlorhexidine digluconate)