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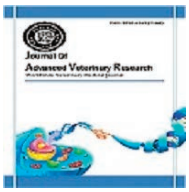

Inhibitory Effect of some Nanomaterials on *Streptococcus* species Producing Biofilm Isolated from some Dairy Farms

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ABSTRACT

Multidrug resistant, *Streptococcus* (*Strep.*) producing biofilm (SPB) considered the most important environmental pathogens associated with concern problems in dairy farms and confers public health hazard. This cohort study aimed to evaluate *Strep.* spp. in dairy farms, antibiotic resistance pattern against 15 antibiotics, and detected the ability of isolates to biofilm formation phenotypically by quantitative (polystyrene), qualitative (Congo Red Agar (CRA)) and Geno-typically by *lmb* and *brpA* genes. The minimum biofilm eradication concentration (MBEC) of chitosan nanoparticles (CNPs) and carvacrol nanoemulsions (CNE) were investigated in vitro by polystyrene method (which is considered the first record, according to the authors knowledge) in field by washing milking utensils. A total of 150 milk samples (cows and buffaloes) were collected, in addition to 48 swabs from milking utensils of different farms in Assiut Governorate, Egypt. The prevalence of *Strep.* spp. was 21.72% of all examined samples and the most frequently isolated pathogen was *Strep. agalactia* (12.6 %). Overall, there were 14% of isolates resistant to vancomycin. *Strep. uberis* revealed the highest resistance to most antibiotics used and 80% of it considered biofilm producers. SPB were classified to strong (23.3%), moderate (32.5%), weak (27.9%) and non-producers (16.3%) by polystyrene method, while, 55.8% considered biofilm producers by CRA. All SPB were positive to *lmb* and only three were negative to *brpA* genes. The prepared nanomaterials were characterized by Transmission Electron Microscope (TEM) and the MBECs were 30% and 20 % for CNPs and CNE, respectively. In addition, complete inhibition to SPB in milking utensils occurred at 10 and 20 minutes from washing by CNE 20% and CNPs 30%, respectively. The present study concluded that most of the isolated *Strep.* spp. are biofilm producers, which are resistant to studied chemotherapeutic agents. CNE is considered more efficient than CNPs for elimination of SPB.

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Introduction

A biofilm is a microbial-derived sessile formed by self-produced polysaccharide matrix extracellular polymeric substances attached to biotic or non-biotic surfaces (sessile cells) (Nilsson *et al*., 2019; Subhaswaraj and Siddhardha, 2020). The microorganisms can be transmitted from the planktonic cells to this sessile mode of life leading to cause severe infection in both of humans and animals and cannot be eradicated by antimicrobial agents (Borges *et al*., 2020). The biofilms formation are considered the most dangerous not only because they form a defensive barrier against other pathogens (in case of the host microbiota), but because they also generate a break line between minor and debilitating infectious diseases, thus promote horizontal spread of resistance determinants and performs human health risk (Rosini and Margarit, 2015; Galié *et al*., 2018).

Strep. spp. can form biofilms on natural or abiotic surfaces such as glass (hydrophilic) or polystyrene (hydrophobic), and also can be detected in closed dairy farm system (Marks *et al*., 2014).

Strep. spp. is considered one of the most important environmental threat (Song *et al*., 2020), which cause several diseases in human, contagious mastitis in dairy animals and recently isolated from different animal's body sites and environmental surroundings of dairy farms and plants (Cobo-Angel *et al*., 2018; Dufour *et al*., 2019). *Strep. agalactia*e was recognized as a pathogen in cattle causing acute or chronic mastitis, which lead to diminishing milk production. Outbreaks in herds are common by this pathogen with a major significance for the dairy industry (Ariffin *et al*., 2019; Cobo-Angel *et al*., 2019). *Strep.* spp. pathogenicity increased with their antimicrobial resistance threating to both human and animal health (Oppegaard *et al*., 2020) rather than their ability to form biofilm (Alves-Barroco *et al*., 2019).

Recently, carvacrol (2-methyl-5-(1-methylethyl) phenol) (CVC), which is a phenolic monoterpene found in the essential oils of herbs like thyme (*Thymus vulgaris*), marjoram (*Origanum majorana*), oregano (Origanum vulgari L.), pepperwort *(Lepidium* sp.) (Campos *et al*., 2018). Its nano-emulsions have

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been studied as anti-biofilm gent, where it acts as antimicrobial agent, consequently, it is widely used as a food additive (Iannitelli *et al*., 2011). Chitosan and carvacrol has good biocompatibility so has been approved for use by the United States Food and Drug Administration as a drug carrier and bioengineering material (Divya and Jisha, 2018). Chitosan nanoparticles could inhibit the growth of various bacteria by disruption of the cell membranes and leakage of the cytoplasm (Divya and Jisha, 2018).

There are several genes responsible for biofilm formation, most important genes are *brpA* and *lmb*. Nevertheless, the environmental condition is responsible for genetic expression and regulation (Santos *et al*., 2020).

This problem may be not only limiting the option for effective treatment but also spreading of the resistance genes from contaminated milk to human normal flora (Oppegaard *et al*., 2020). In addition to that, the formation of mature biofilm protects bacterial cells from antibiotic therapy and impede the host immune system from recognizing the presence of infection (Subhaswaraj and Siddhardha, 2020). Therefore, the treatment of biofilm-related infections requires high doses and long-term use of antibiot¬ics and the resultant inactivation is not always satisfactory. Animal consumption of low concentrations of antibiotics have even been reported to enhance the growth of milk bacterial biofilms (Alves-Barroco *et al*., 2019).

Therefore, novel and innovative approaches are needed for the prevention of biofilm formation and the treatment of formed biofilm related infectious diseases. Recent progress in nano¬technology provides a new approach for eradication of biofilm formation. This study aimed to investigate SPB and its antimicrobial resistance in milk and milking utensils in some dairy farms, as well as studied the effect of chitosan nanoparticles (CNPs) and carvacrol nanoemulsion (CNE) on biofilm formed by *Strep.* spp.

Materials and methods

Sample collection

The study was conducted on different dairy farms located in Assiut province, Egypt. A total of 198 samples (75 for each buffalo's and cow's milk samples (150 for total raw milk samples), and 48 swabs from milking utensils were collected during the period from July to October of 2019. Milk in sterile separate tubes, and swab samples from the inner surface of milking utensils were labeled and carried on ice tank to be transferred with a minimum delay to the Animal Health Research, Assiut laboratory for bacteriological examination.

Streptococcus spp. *isolation and identification*

The samples were incubated into Dextrose azide broth (Himedia Co.) at 37ºC for 24-48 h. Then streaked onto *Streptococcus* selective agar (Sigma-Aldrich) with 5-10% sheep blood. The suspected colonies were subjected to Gram's staining and conventional biochemical tests according to Quinn *et al*. (2004).

Antimicrobial resistance pattern

It was carried out according to Quinn *et al*. (2004) using agar disc diffusion method on Mueller Hinton agar. The isolated *Streptococci* strains were tested against 15 antibiotic discs, which related to different categories of chemotherapeutic agents. The antibiotic discs were penicillin (10 IU), ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), cephradin (30 µg), cefotaxime (3 µg) Ceftiofur (10 µg), oxytetracycline (30 µg), doxycycline (25 µg), streptomycin (10 µg), gentamycin (10 µg), lincomycin (2 µg), vancomycin (30 µg), sulfamethoxazole/trimethoprim (25 µg), erythromycin (15 µg), florofinicole (30 µg) oxoid Hamp-shire, UK. The interpretation of inhibition zones of the tested isolates was carried out according to CLSI (2015)

Biofilm formation assay

Polystyrene method

Biofilm production on polystyrene was carried according to Ferreira *et al*. (2012). Briefly, 100 µl of *Strep.* spp. culture (OD570 =0.6) was added to 100 µl of Trypticase Soy Broth (TSB) supplemented with 0.5% (w/v) glucose, 1% (w/v) sucrose and 1% (w/v) lactose in a 96 well plate and mixed by pipetting. A 200 µl from previously prepared TSB was used as control. The plate was sealed and incubated for 20 h. at 37ºC. The OD570 was measured in a spectrophotometer. The supernatant was carefully removed, and each well was washed twice with sterile saline solution (0.85% w/v) to remove non-adherent bacteria and dried at 65ºC for 1 h. The biofilm was stained with crystal violet (Sigma-Aldrich, St.) 1% (w/v) for 1 min. The wells were washed again gently with sterile distilled water four times (until the control-wells dye was completely removed). The optical density (OD) at 570 nm (OD570=0.6) of the stained biofilm was directly measured in the plate reader. Interpretation of biofilm formation was performed according to the criteria previously described (Stepanović *et al*., 2007) and the isolates were therefore categorized as follows: non-producer (OD≤0.060), weak producer (OD between 0.060-0.120), moderate producer (OD between 0.120-0.240), strong producer (OD > 0.240) as reported by Ferreira *et al*. (2012).

Congo Red Agar (CRA) binding assay

The isolates were cultivated on BHIA (Difco) with 0.08% (w/v) congo red (Sigma-Aldrich, Germany) supplemented with 5% (w/v) sucrose (Elgomhorea Co., Egypt). The strains were inoculated in streaks and incubated at 35°C under aerobic conditions for 48 h. The *Streptococcus* biofilm producer strains formed black colonies, while the non-biofilm producer strains formed red colonies (Kaur *et al*., 2009).

Biofilm genes identification

SPB strains were sent to the Reference Laboratory for Veterinary Quality Control, Biotechnology Unit in Animal Health Research Institute, Dokki, Giza, Egypt, for detection of *brpA* and *lmb* genes as follow:

DNA extraction

DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in Table 1.

Table I. Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
brpA	TGAAGCTAAGTTGAATGCTGC GAACCACCATCAGACAAGGT	534	94˚C 5 min.	94˚C 30 sec.	42˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Alves-Barroco *et al*. (2019)
lmb	AGTCAGCAAACCCCAAACAG GCTTCCTCACCAGCTAAAACG	397	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Kaczorek *et al*. (2017)

PCR amplification

The volume of the PCR reaction mixture was 25 µl contained 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied Biosystem 2720 thermal cycler (Biometra, Gottingen, Germany).

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp and 100 bp plus DNA ladders (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Effect of chitosan nanoparticles (CNPs) and carvacrol nanoemulsion (CNE) on biofilm formation

Preparation of CNPs and CNE

Nano chitosan was prepared following the procedure described by Calvo *et al*. (1997). A 2.5 mg/mL chitosan solution was prepared by dissolving chitosan in a 0.05% (v/v) acetic acid solution and leaving it under stirring for 24 h. The pH was adjusted to 5.5 with a 0.5 M sodium hydroxide solution and diluted in deionized water to the final desired concentrations. Sodium Tripolyphosphate (TPP) was dissolved in deionized water to a final concentration of 0.25 mg/mL. Then, the TPP solution was added to the chitosan solution drop wise (0.3 mL/min) at different TPP: chitosan ratios under vigorous magnetic stirring at room temperature. The resulting suspension was then left to gelify for 30 min. 2 v/v% Tween 80 was dissolved in double-distillated water at room temperature. The mixture was shaken with a magnetic type stirrer for 10 minutes to get a homogeneous solution. The essential oil was added slowly and mixed with a direct driven stirrer for 15 minutes. The resulting crude emulsion was sonicated using a 25 kHz ultrasonic Homogenizer (USH650, max power: 650 watt) (Ghosh *et al*., 2013). Then they were characterized by Transmission Electron Microscopy (TEM) Model JEOL-JEM-100CX II in the Electron Microscopy Unit, Assiut University, Egypt.

The minimum biofilm eradication concentration (MBEC) of CNPs and CNE

To measure the ability of CNPs and CNE to disrupt preformed biofilms, the strong biofilm producer bacteria (OD>0.240nm) were grown for 24 h in 96-well plates as mentioned previously (section: on polystyrene). The medium was then removed and biofilms on the wall of the wells were exposed to 200 µL of tenfold serial dilutions (0, 10, 20, 30, 40, 50, 60 ml.) of tested compounds were added. The plates were then incubated for another 24 h at 35°C. The treated biofilm was washed three times with phosphate-buffer saline (PBS), air dried, and stained with 200 µL of 0.1% crystal violet for 30 min. All wells were washed again twice with 200 µL of sterile distilled water prior to addition of 200 µL of 95% ethanol, and the OD was measured at 540 nm. Measurements were done in triplicate, and each experiment was repeated three times (Nilsson *et al*., 2019). Percent of biofilm inhibition formation was calculated using the following equation (Jadhav *et al*., 2013):

Biofilm inhibition (%) = OD (biofilm) - {OD (biofilm + extract)-OD (extract)}/OD (biofilm) X 100

CNPs and CNE application in dairy farms

The most effective concentrations (20% CNE and 30% CNPs on MBEC) were used to wash the milking utensils in the farms that previously selected for this study. Utensils swab samples were taken at zero time, 5, 10, 15, 20, 25 and 30 min. The samples were delivered to the laboratory in ice bag at a temperature of 4–6°C within 1–3 h. The samples were retested for detection of *Strep.* spp. contamination as previously mentioned.

Statistical analysis

The statistical analysis was performed using program GraphPad Prism 5.04 (GraphPad, Inc., San Diego, USA). The data represented by using the Microsoft Excel Spreadsheet.

Results

Assay the incidence of Strep. spp. in dairy farms samples

It was found that *Strep.* spp. could be detected in 21.72% of total 198 samples collected. The highest incidence was for *Strep. agalactia* followed by *Strep. uberis*, and the lowest was for *Strep. pyogenes* in percentages of 12.6, 5.1 and 4.1, respectively. Most contaminated samples were milking utensils (45.8%), which were considered the most important source for infection transmission (Table 2).

Detection of multidrug resistance pattern to the isolated Strep. spp. and its relationship with biofilm formation

Most *Strep.* spp. (97.7%) were resistant to streptomycin, 72.1 % to oxytetracycline, penicillin (69.8%) and sulfamethoxazole/trimethoprim (55.8%) (Table 3). The highest multidrug was resistance exhibited by *Strep. uberis*, all its isolates resisted streptomycin (100%), 80% of it resist oxytetracycline, penicillin, 70% was resistant to lincomycin and 60% to gentamycin, sulfamethoxazole/trimethoprim (Table 3). Followed by *Strep. agalactia* then nearly the lowest one in multidrug resistance was *Strep. pyogenes*. On the other hand, the strongest biofilm formation by polystyrene method were observed by *Strep. uberis* (40%), *Strep. agalactia* (20%) then *Strep. pyogenes* (12.5%). Also, by CRA, 80% of *Strep. uberis*, 56% of *Strep. agalactia* and 25% of *Strep. pyogenes* were biofilm producers (Table 3 and Fig. 1).

Table 2. Incidence of the different isolated *Strep.* spp. recovered from examined samples.

Isolated *Strep.* spp.	Types of examined samples						Total	
	buffalo's milk samples		Cow's milk samples		Milking Utensils swabs			
	No./75	%	No./75	%	No./48	%	No./198	%
Strep. agalactia	5	6.67	7	9.33	13	27.1	25	12.6
Strep. uberis	2	2.67	3	4	5	10.4	10	5.1
Strep. pyogenes	2	2.67	2	2.67	4	8.3	8	4.1
Total	9	12	12	16	22	45.8	43	21.72

Table 3. Antimicrobial resistance pattern of the isolated *Streptococci*.

	Strep. agalactia		*Strep. uberis*		*Strep. pyogenes*		Total	
Antibiotic discs	No./25	%	No./10	%	No./8	%	No./43	%
Penicillin (10 IU)	17	68	8	80	5	62.5	30	69.8
Ampicillin (10µg)	7	28	4	40	1	12.5	12	27.9
Amoxicillin/clavulanic acid (30µ g)	1	4	1	10	1	12.5	3	7
Cephradin (30µg)	11	44	5	50	3	37.5	19	44.2
Cefotaxime (3µg)	8	32	4	40	2	25	14	32.6
Ceftiofur (10µg)	0	0	0	0	1	12.5	1	2.3
Oxytetracycline (30µg)	18	72	8	80	5	62.5	31	72.1
Doxycycline (25µg)	9	36	5	50	2	25	16	37.2
Streptomycin (10 µ g)	24	96	10	100	8	100	42	97.7
Gentamycin (10µg)	11	44	6	60	3	37.5	20	46.5
lincomycin (2µg)	12	48	7	70	4	50	23	53.5
Vancomycin (30µ g)	3	12	1	10	2	25	6	14
Sulfamethoxazole/Trimethoprim (25µg)	13	52	6	60	5	62.5	24	55.8
Erythromycin (15µ g)	6	24	5	50	1	12.5	12	27.9
Florofinicole (30µg)	1	4	1	10	0	0	2	4.7

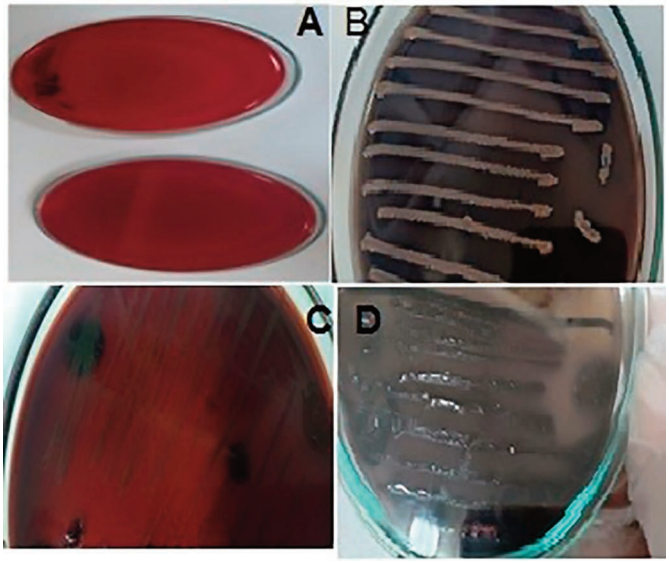

Fig. 1. Detection of biofilm formation *Strep.* spp. by CRA. A: negative control, B: dark red colonies indicated non-biofilm producers, C, D: black colonies, biofilm producers

The ability of *Strep.* spp. to form biofilm not only assayed phenotypically, by quantitative (polystyrene), qualitative (CRA) methods but also, genetically by detecting the presence of prbA and *lmb* genes as clarified in Figs. 2 and 3.

Structural properties of CNPs and CNE

The shape and particle sizes were obtained by TEM (Fig. 4).

The effect of CNPs and CNE on SPB

By enhancement biofilm formation firstly polystyrene method was used at different concentrations from CNPs and CNE to detect the MBEC and as shown in Fig. 5. CNE at 20% concentration and 30% of CNPs have biofilm eradication effect so, these concentrations were used by washing of dairy utensils.

According to the available literatures, this study is considered the first recorded in application of CNPs and CNE in dairy farm utensils in Assiut city, Egypt. The results revealed that CNPs could eradicate SPB at 30% for 20 min., while CNE could eradicate SPB at 20% for just 10 min., as shown in Fig. 6.

Discussion

The incidence of the pathogenic biofilm producing bacteria in dairy farms of developing countries is increased. *Strep.* spp. is the most pathogenic, wide spread organisms that are associated with dairy farm infections and generate important

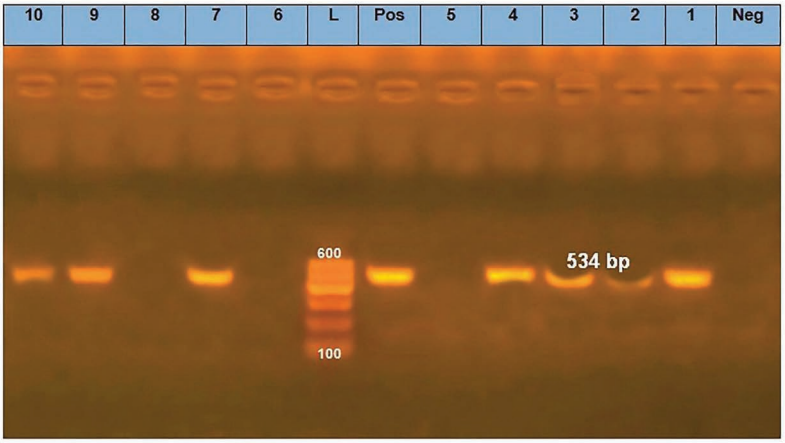

Fig. 2. The amplified prbA gene of biofilm producers *Strep.* spp. recovered from dairy farms samples. L: Molecular marker, Lane pos.: Positive control, Lane Neg.: Negative control, Lanes 1-4, 7, 9-10: positive isolates, Lane 5, 6, 8: negative isolates.

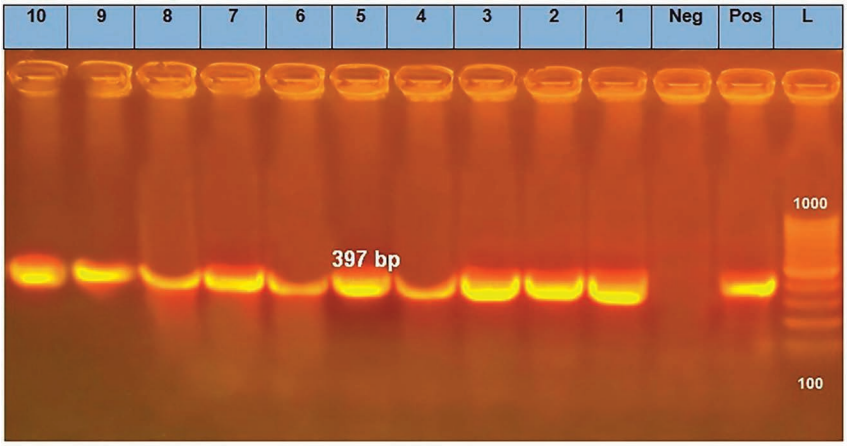

Fig. 3. The amplified *lmb* gene of biofilm producers *Strep.* spp. recovered from dairy farms samples. L: Molecular marker, Lane pos.: Positive control, Lane Neg.: Negative control, Lanes 1-10: positive isolates.

losses in the dairy industry in Egypt. They considered as a risk factor for food poisoning in humans (Ariffin *et al*., 2019; Ahmed *et al*., 2020). Results from the present study revealed that *Strep.* isolates were detected in 12, 16 and 45.8% of buffalo's milk, cow's milk and milking utensils, respectively, which are similar to Hawari and Al-Dabbas (2008), Wyder *et al*., (2011) and Tian *et al*., (2019). While, higher results were detected by Sabry (2015) and Ahmed *et al*., 2020). The overall isolation rate of *Strep.* spp. was 21.72% in the examined samples (Table 2). These findings were coordinated with that cited by Ahmed el al. (2020), but the recorded percentages were lower than that reported by El-Jakee el al. (2013), Sabry (2015) and AbdEL-Tawab *et al*. (2017), who observed that Streptococcal isolation rate were 55%, 40% and 65.3%, from buffalo's milk, cow's milk and milking utensils, respectively.

The current study found that the percentage of *Strep. agalactiae*, *Strep. uberis* and *Strep.* pyogenes were 27.1, 10.4 and 8.3% respectively, in milking utensils, lower percentages in cow's milk (9.33, 4.0, 2.67%) and the lowest percentages were detected in buffalo's milk (6.67, 2.67 and 2.67%) (Table 2), which indicated the highest probability of utensils contamination. Nearly higher results were recorded in milk by Minst *et al*. (2012), Cobo-Angel *et al*. (2019) and Tian *et al*. (2019), nearly similar results were reported by El-Jakee *et al*. (2013) and Sabry (2015), and lower than that was detected by Abd EL-Tawab *et al*. (2017).

The presence of *Strep.* spp. in raw milk could be attributed to the bad hygienic measures during the production and handling of milk in the studied dairy farms, since it is liable to contaminate milk through different sources as dust, air, water, equipment, milkers and handlers (Alekish *et al*., 2013). Especially it could be isolated in the highest incidence (45.8%) in milk utensils. The risk of infection with zoonotic *Strep.* spp. appears greatest in those who drink unpasteurized milk. In human, *Strep.* spp. may in fact have caused historical milk-borne outbreaks of septic sore throat and scarlet fever, which was attributed to hemolytic *Strep.* pyogenes (Cunningham, 2008; Beres *et al*., 2010). Also, Cobo-Angel *et al*. (2019) recorded the transmission of group B *Streptococcus*, between people and dairy cattle, which resulted in neonatal death, and reported as an emerging pathogen in adults and also, caused bovine intra-mammary infections.

The results of the antimicrobial resistance pattern (Table 3), revealed that *Strep. uberis* isolates were highly resistant to streptomycin (100%), 80% for oxytetracycline and penicillin, 70% for gentamycin and 60% for lincomycin and sulfamethoxazole/trimethoprim. *Strep. agalactia* also resist the same mentioned antibiotics but at lower percentages. These results were coincide with results reported by many authors (Boutet *et al*., 2005; Nunes *et al*., 2007; Pitkala *et al*., 2008, Rüegsegger *et al*., 2014; Kia *et al*., 2014). On the other hand, these results disagree with those obtained by Moges *et al*. (2011) and El-Jakee *et al*. (2013), who reported that *Strep. agalactia* was sensitive to sulfisoxzale and kanamycin by 100 % and 80%, respectively. the obtained findings revealed that *Strep.* pyogenes was resistant for most antibiotics as streptomycin (100%), 62.5% for penicillin, oxytetracycline and sulfamethoxazole/trimethoprim, 50% for Lincomycin and 25% for vancomycin. Results from this study were concordant with other studies (Camara *et al*., 2013; Khan *et al*., 2013). The multidrug resistance of *Strep.* spp. represents a public health hazard problem, which may be transmitted from animals to human through dairy products (Minst

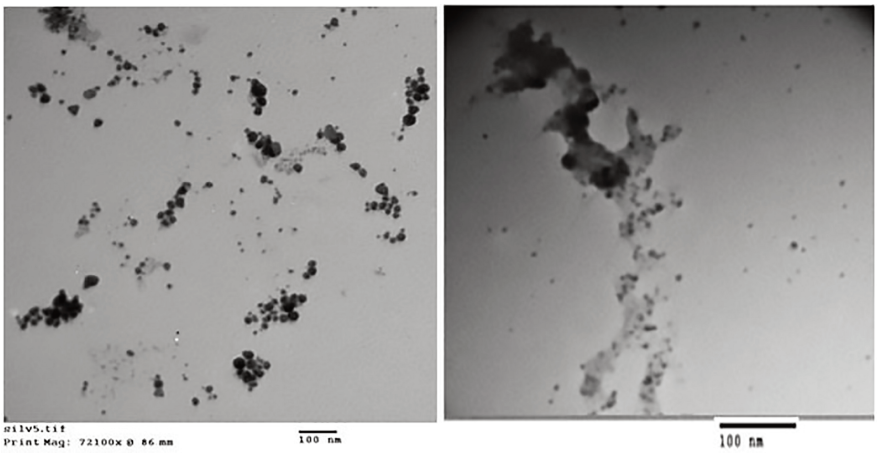

Fig. 4. TEM of CNPs with average size 35.7 nm (left), and TEM of CNE with average size 78.8 nm (right)

Table 4. Biofilm formation detection in *Strep.* spp. by using different methods.

Degree of Biofilm forming	Tested Strains											
	Strep. agalactia			*Strep. uberis*			*Strep. pyogenes*			*Strep.* spp. (total)		
	No. (25)	%	Mean ± SE	No. (10)	%	Mean ± SE	No. (8)	%	Mean ± SE	No. (43)	%	Mean ± SE
Polystyrene method												
Non producers	3	12	0.026±0.006	0	0	0	4	50	0.032±0.004	7	16.3	0.03±0.003
Weak producers	8	32	0.085±0.003	2	20	0.094±0.007	2	25	0.088±0.003	12	27.9	0.09±0.003
Moderate producers	9	36	0.176±0.005	4	40	0.18±0.009	1	12.5	0.184±0.023	14	32.5	0.18±0.005
Strong producers	5	20	0.282±0.006	4	40	0.294±0.013	1	12.5	0.271±0.014	10	23.3	0.29±0.006
CRA inoculation												
Non producer	11	44	----------	2	20	----------	6	75	----------	19	44.2	----------
Biofilm producer	14	56	----------	8	80	----------	2	25	----------	24	55.8	----------

et al., 2012). The ability to biofilm formation may maximize the antibiotic resistance pattern of isolated strains, since biofilm formation confers many advantages to the microbial cells in a food industry environment, such as chemical protection against disinfectants, antimicrobials, physical resistance against desiccation, or mechanical resistance against liquid streams in pipelines (Galié *et al*., 2018).

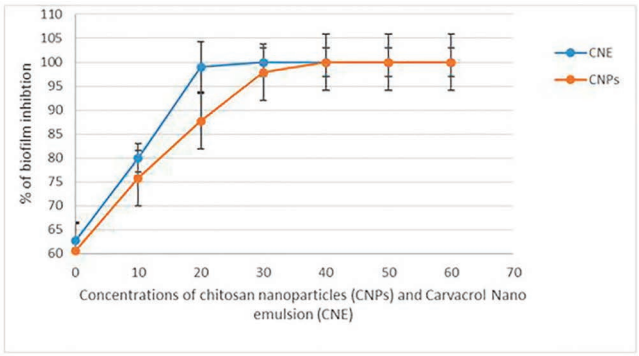

Fig. 5. The Minimum Biofilm Eradication concentration (MBEC) of CNPs and CNE on biofilm.

Biofilm formation can be considered as a developmental process, which is characterized by multiple-step structural changes in biofilm architecture and composition, coordinated by a complex net of regulatory genes and environmental signaling via signal transduction (Donlan and Costerton, 2002). In the current study, both qualitative and quantitative methods used to confirm the ability of isolated *Strep.* spp. to produce biofilm. Table 4, clarified that all *Strep. uberis* isolates could form biofilm on polystyrene surface indicating that they were the most hazardous strains among studied species, where 20%, 40% and 40% of them showed week, moderate and strong biofilm formation, respectively. While, 12% from *Strep. agalactia* isolates were not biofilm producer, 32% weak, 36% moderate and 20% strong producer. Moreover, 36/43 (83.7%) of *Strep.* spp. had biofilm production capabilities. *Strep.* pyogenes has the ability to produce biofilm on a polystyrene and classified as 12.5% for moderate and strong and 25% for weak producer, but the remaining not able to develop biofilm in polystyrene. The current results are similar to that recorded by Courtney *et al.* (2009); Ebrahimi *et al.* (2013) and Alves-Barroco *et al.* (2019).

The CRA method is a fast, reproducible, and presents an advantage: the colonies remain viable in the medium for further analysis (Courtney *et al*., 2009). Therefore, this method was chosen to improve the ability of isolated *Strep.* spp. to produce biofilm matrix. All moderate and strong biofilm producers' isolates from polystyrene can produce extracellular polysaccharides and give black colony with or without black pigment on CRA as clarified in Table 4 and Fig. 1. This result was in agreement with Genteluci *et al.* (2015); Rosini and Margarit (2015); Young *et al.* (2016) and Moliva *et al.* (2017). Also by CRA method, *Strep. uberis* was the highest biofilm producers (80%), which was higher than *Strep. agalactia* (56%) and *Strep. pyogenes* (25%).

As the correlation between a constant black colony forming phenotype on CRA and presence of *brpA* and *lmb* genes, act as complementary approached for accurate and rapid identification of biofilm matrix bacteria (Mariana *et al*., 2009). The isolated strains of *Strep.* spp., which proved to produce biofilm in polystyrene and CRA were subjected to PCR as a confirmatory test. All tested strains gave positive *lmb* gene, but with *brpA*, only 70% of the tested strains were positive as clarified in Figs. 2 and 3. *Strep. uberis* was contained both the tested genes, which confirmed the results obtained by the previously mentioned methods. Genes associated with intercellular communication systems, sensing systems, carbohydrate metabolism, and adhesion have been described as important and required for the adaptation of *Streptococci* in biofilms (Roberts *et al*., 2010; Marks *et al*., 2014). Among them

brpA (lytR) encoding *brpA* and *lmb* regulatory protein has been described as important regulator of biofilm formation (Wen *et al*., 2006, Bitoun *et al*., 2014). Indeed, the *brpA* gene codes for a predicted surface-associated protein with apparent roles in biofilm formation, autolysis, and cell division. Deficiency of BrpA drastically weakens the ability of the deficient *Strep.* to survive to low pH, oxidative challenge, cell envelope stress and its ability to accumulate and develop into mature biofilms decreases drastically (Doern *et al*., 2009; Roberts *et al*., 2010; Bitoun *et al*., 2014). *lmb* gene, its primary function is lamining-binding protein and promote adherence to host laminin (Pires *et al*., 2005).

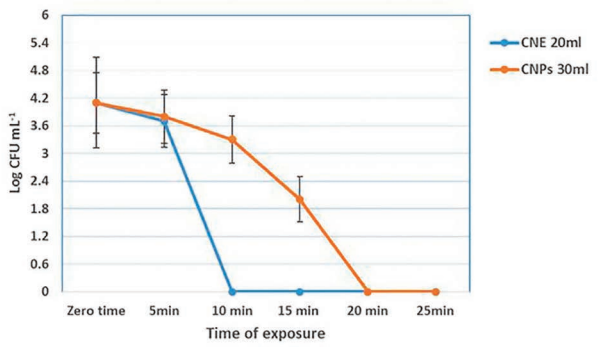

Fig. 6. The velocity of CNPs and CNE to eradicate completely SPB pathogens after application in a dairy farm.

The difficulty of eliminating the biofilm on the surface by conventional control methods, highlights the urgent need for alternative antibacterial and anti-biofilm agents, with much attention in the medical world (Zodrow *et al*., 2012). Using nano¬particles to inhibit bacterial growth and biofilm formation is an increasingly attractive approach to both prevention and treatment of multidrug resistance and biofilm formation infections (Subhaswaraj and Siddhardha, 2020). CNPs and CNE are natural materials with excellent physicochemical, antimicrobial and biological properties (nontoxicity, biocompatibility, and biodegradability), which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans. Due to these unique properties, they can find a wide array of applications (Divya and Jisha, 2018).

In the current study, the average size of CNPs and CNE were 35.7 and 78.8 nm respectively, as shown in Fig. 4. The size of nanomaterial with high surface areas makes it easier to interact with bacteria and inhibition of biofilm formation (Bharde *et al*., 2008). As a new approach to treat infec¬tion, nanoparticles have many advantages, including high surface-to-volume ratios and nanoscale sizes (Kalishwaralal *et al*., 2010). Milking utensils made from stainless steel for easily cleaning but with mechanical cleaning and longtime using some scratches are formed, so bacteria and raw materials can stick and form biofilm on them. Therefore, many researches recommended CNPs (Jindal *et al*., 2016; Swartjes and Veeregowda, 2016; Aliasghari *et al*., 2016), CNE (Nostro *et al*., 2009; Iannitelli *et al*., 2011) or CNE loaded CNPs (Keawchaoon and Yoksan, 2011; Campos *et al*., 2018). CNPs and CNE find multifaceted applications due to their nontoxicity, biodegradability and antimicrobial properties especially against the multidrug resistant microorganisms, in addition to the nanoparticles properties such as surface and interface effect, small size and quantum size effects. So, they are used in biomedical industries, agriculture, genetic engineering, food industry, environmental pollution control, water treatment, paper manufacture and photography (Campos *et al*., 2018; Divya and Jisha, 2018).

As shown in Fig. 5, nearly complete destruction to biofilm occurred at 30 ml and 20 ml for CNPs and CNE, respectively, with significant difference in OD between the different concentrations of CNPs and CNE used and control. Chitosan causes bacterial death rather than reduction in bacterial adhesion (Busscher *et al*., 2008). In the current investigation, biofilm viability after CNPs treatment was lower than that of the control. CNPs may play a crucial role against the pool of persisted cells that in most cases represent the main obstacle to an effective biofilm eradication and disruption of planktonic bacterial cells (Keawchaoon and Yoksan, 2011; Iannitelli *et al*., 2011). Due to the presence of biofilm water channels (pores) for nutrient transportation, nanoparticles may dif¬fuse through the pores and play an antibacterial function in the biofilm (Kalishwarala *et al*., 2010).

In vitro study as clarified in Fig. 6, could not detect *Strep.* spp. after application of CNPs (30 ml) and CNE (20 ml) at 20 min and 10 min, respectively, which indicated inhibition and bactericidal effect that prevent biofilm formation. This result was in agreement with Aliasghari *et al.* (2016) and Ikono *et al.* (2019), who recorded that nano-chitosan can inhibit the viability of biofilm cells with the effectivity of more than 90%. Previous studies attributed this effect to alteration of bacterial membrane, so inhibit sessile colony formation and deter biofilm architecture completion in dairy utensils (Jindal *et al*., 2016; Swartjes and Veeregowda, 2016).

The antibacterial properties of nanoparticles distinguish them from their bulk chemical counterparts. One such property is their large surface area to volume ratio, which creates a higher number of functional sites, which can enhance the influence of NPs on a given microorganism and can be applied in diverse industrial environments (Beyth *et al*., 2015). Another interesting approach consists in exploiting the effectiveness of different nanocomposite materials toward reducing bacterial adhesiveness. For example, the chemical compounds in CNPs and CNE were able to reduce biofilm formation on polystyrene polymer by inhibiting extracellular matrix biosynthesis genes (Nostro *et al*., 2009; Aliasghari *et al*., 2016).

Bacterial adhesiveness is influenced by multiple chemical and physical properties of the surface, including hydrophobicity, electrical charge and functional groups, all of which can determine the kinetics of bacterial adhesion (Campoccia *et al*., 2013). Bacteria-repelling surfaces are usually composed of inert materials where the repellent property is provided by self-assembled monolayers, polymer brushes, hydrogel coatings or by manipulating the surface morphology or topography. Alternatively, a coating of intrinsically antibacterial and anti-biofilm materials as CNPs and CNE confer this repelling activity (Swartjes and Veeregowda, 2016).

Conclusion

The current study concluded that different species of SPB were identified in cow's milk, buffalo's milk and milking utensils, which perform a serious economic and health issue for animal and human. Most of the isolated *Strep.* spp. is biofilm producer, where all *Strep.* ubris, most of *Strep. agalactia* (80%) and few of *Strep.* pyogenes (25%) produce biofilm. The isolated strains resist different chemotherapeutic agents. All of tested strains harbored *lmb*, while only 70% have *brpA* genes. CNPs and CNE are considered efficient product for elimination of SPB in milking utensils even in low concentrations and within 10- 20 minutes.

Conflict of interest

The authors declare that there are no competing interests.

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