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Original Research Article

EXPERIMENTAL TRIALS FOR REDUCING BIOFILM-PRODUCING *Escherichia coli* USING *Nigella sativa* AND OLIVE OILS' NANOEMULSIONS

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Abstract: Dairy products can be contaminated from their surrounding surfaces and equipment with biofilm forming foodborne pathogens such as *Escherichia coli* (*E. coli)*. Biofilm production is regarded as an adaptive mechanism that enhance microbial growth and proliferation. In sight of these facts, this study aimed to investigate of the prevalence of *E. coli* in dairy products (kariesh cheese, ice cream, and pasteurized milk), and dairy plant floors, and their contact equipment. Besides, the abilities of the identified *E. coli* serotypes to produce biofilm were further examined. In a reduction trial, the use of *Nigella sativa* and olive oils' nanoemulsions to reduce *E. coli*-biofilm formation was examined. The obtained results indicated that the prevalence rates of *E. coli* in the examined kariesh cheese, ice cream, pasteurized milk, dairy plant floors, and equipment were 19.05%, 4.76%, 0%, 45.24%, and 30.96%, respectively. Five *E. coli* serotypes were identified in the current investigation namely, *E. coli O26:H11*, *E. coli O55:H7, E. coli O78:H-, E. coli O111:H4, and E. coli O127:H6* at variable rates. Molecular identification of the recovered *E. coli* serotypes revealed that all serotypes harbored *E. coli*-specific *16S rRNA*, and *fim H*, biofilm-coding genes. All identified *E. coli* serotypes had the ability to produce biofilm in the following order: *E. coli O26:H11*, *E. coli O55:H7, E. coli O78:H-, E. coli O111:H4, and E. coli O127:H6*, respectively. *Nigella sativa,* and olive oils' nanoemulsions reduced biofilm production in a concentration dependent manner.

Key words: *E. coli*; biofilm; dairy products; *Nigella sativa*; olive oil's nanoemulsions

Introduction

Dairy products, despite their high nutritional values (1), are implicated in the transmission of several foodborne pathogens such as *Clostridium botulinum* (2), *Staphylococcus aureus* (3), Salmonella *spp.* (4), *Bacillus cereus* (5), and *Escherichia coli* (*E. coli*) (6).

Microbial contamination of dairy products might occur during any step of their processing due to the unhygienic personal practices, use of contaminated raw materials, or via cross-contamination from contaminated equipment or utensils (7, 8).

E. coli is one of the coliforms groups that is considered as a common inhabitant of the intestinal tract of the dairy producing animals. Detection of *E. coli* in dairy products indicates fecal contamination (6). At the same time, *E. coli* is implicated in several cases of human illness outbreaks such as that reported in Colombia (9), Iran (10), India (6), and many African countries (11). *E. coli* has more than 100 pathotypes that play critical roles in *E. coli*-related foodborne infections such as *E. coli* O157, O26, O45, O103, O111, O121, and O145 (12). There are urgent needs for continuous monitoring

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of the occurrence of *E. coli* in foods of animal origin, particularly, dairy products in Egypt.

Biofilm is considered as a protective and invasive mechanism for microorganisms to promote microbial growth and proliferation. Biofilm production increases the microbial efficiency, and their resistance to antimicrobials, and therefore regarded as a major concern for food safety, and public health sectors (13). The biofilm production abilities of different *E. coli* serotypes, particularly those associated with dairy products, have received less attention.

The use of essential oils as natural antimicrobials has increased recently as alternatives to chemical preservatives (14). Of these, antimicrobial activities of *Nigella sativa* oil have been reported against several foodborne pathogens including *Staphylococcus aureus,* and *Salmonella* spp. (14, 15). In addition, olive oil (*Olea europaea L.*) retarded the growth of *Pseudomonas fluorescens* and *Enterobacteriacae* in Fior di latte cheese in a study conducted in Italy (16). However, the protective effects of *Nigella sativa*, and olive oils against *E. coli*-produced biofilm are less investigated.

The objectives of the present study were firstly to investigate the prevalence of *E. coli* in three dairy products among the most commonly consumed dairies in Egypt, including kariesh cheese, ice cream, and pasteurized milk retailed in Sharkia Governorate, Egypt. Secondly, the prevalence of *E. coli* in swabs sampled from the surfaces, and equipment of dairy processing plants were additionally examined. Thirdly, the ability of the identified *E. coli* serotypes to produce biofilm was *in vitro* examined. Finally, a reduction trial for *E. coli*-produced biofilm was employed using *Nigella sativa*, and olive oils nanoemulsions.

Materials and methods

Collection of samples:

A total of 125 samples (25 each of kariesh cheese (50 g), ice cream (50 g) of local produce, and pasteurized milk (200 ml/package)) were collected from dairy processing plants at Zagazig city, Sharkia Governorate, Egypt. In addition, 50 swabs in sterile quarter strength Ringer's solution were collected from surfaces (floors, walls, operators' hands, mates, and processing tables) (25 swabs), and equipment (25 swabs) of these dairy processing plants. All collected samples were transferred cooled without delay to Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Egypt for microbiological examination.

Sample preparation:

Ten grams/ml from each sample was homogenized aseptically in buffered peptone water 0.1%, or sodium citrate 2% in case of cheese (90 ml) for 2 min at 2500 rpm to obtain a sample homogenate (17).

Isolation of E. coli:

A loopful from each homogenate was directly streaked onto Eosin Methylene blue (EMB) agar (Oxoid Limited, Hampshire, UK), then incubated at 37°C for 24 h (17). Typical *E. coli* colonies (appear greenish, metallic with dark purple center), and suspected colonies were purified and sub-cultured onto nutrient slope agar (Oxoid Limited, Hampshire, UK), kept frozen in Eppendorf tubes containing glycerol at -80°C for further investigations. *E. coli* identification was done based on staining and biochemical tests including Eijkman test, catalase, oxidase, indole, methyl red, VP test, H_2S production, and Gelatin liquefaction tests (17).

Serodiagnosis of E. coli:

E. coli confirmed isolates were serologically identified using diagnostic *E. coli* antisera sets (Hardy Diagnostics, Ohio, USA) (18).

Bacterial DNA preparation & amplification reaction of E. coli 16S rRNA & fim H genes:

DNA from sero-diagnosed *E. coli* was extracted (19). Molecular confirmation of the identified *E. coli* serotypes was done via detection of the 16S rRNA gene using PCR. Similarly, biofilm-coding gene (fimbria adhesin) (*fim H*) among the identified *E. coli* isolates was detected using PCR. The used primers in the present study were prepared using https://primer3plus.com/cgi-bin/dev/primer3plus.cgi, and presented in Table 1. The amplification

Table 1: The used primers in the present study

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	Accession number
16S rRNA (F)	**5'** CTTTCAGCGGGGAGGAAGG **'3**	390	MK918474
16S rRNA (R)	**5'** TCAACCTCCAAGTCGACATCGT **'3**		
Fim H (F)	**5'** GAGAAGAGGTTTGATTTAACTTATTG **'3**	498	KJ866866
Fim H (R)	**5'** AGAGCCGCTGTAGAACTGAGG **'3**		

reaction for PCR was performed on a Thermal Cycler (Master cycler, Eppendorf, Germany). The PCR cycling conditions started with an initial denaturation at 95°C for 5 min, followed by 40 cycles consist of 15 sec denaturation at 95°C, annealing for 30 sec at 60°C, and extension for 1 min at 72°C. A final extension step for 7 min at 72°C was employed, followed by a holding at 4°C. DNA fragments were run on 1.5% agarose gel electrophoresis (AppliChem, GmbH, Germany) in 1x TBE buffer stained with ethidium bromide and visualized on a UV transilluminator. DNA Ladder (100 bp, Qiagen, GmbH) was used to determine the fragment sizes.

Detection of biofilm production among E. coli isolates:

A 96 well-flat bottom tissue culture plate was used for biofilm detection among the identified *E. coli* serotypes using the method of Karigoudar *et al.* (20). Briefly, *E. coli* isolates from each serotype were refreshed in Tryptone Soy Broth (TSB) (Oxoid Limited, Hampshire, UK) overnight at 37°C. The obtained *E. coli* aliquots were diluted at 1:100 in TSB. Then five wells/each *E. coli* serotype were filled with 0.2 ml of the diluted cultures. The culture plate was then incubated for 24 h at 37°C. After incubation, the content of the cultured wells was gently removed. Then wells were washed three times with buffered PBS (ThermoFisher Scientific, UK). Wells were then stained with 0.1% crystal violet and left to dry at room temperature. Optical density (OD) of the stained wells was determined by an ELISA plate reader at a wavelength of 570 nm. For quality assurance, wells filled with only TSB served as negative control to check nonspecific binding of the media. The mean OD value of the negative control was deducted from all the test OD values. The experiment was repeated twice to confirm reproducibility. *E. coli* O157:H7 was used as a positive control, while TSB was used as a negative control in this experiment.

Protective effects of Nigella sativa and olive oils' nanoemulsions against biofilm production by E. coli:

The inhibitory effects of *Nigella sativa* and olive oils nanoemulsions against biofilm production by *E. coli* O26:H11 (The highest biofilm-producing *E. coli* serotype in the present study) were tested by mixing of the oil's nanoemulsions (prepared in Nakaa Nanotechnology Network NNN) at two concentrations (1%, and 2%), based on their minimum inhibitory concentrations (21), with refreshed *E. coli* O26:H11 isolates in TSB and incubation for 24 h at 37°C. Then the content of each well was hygienically disposed and followed by crystal violet staining and OD reading as previously mentioned.

Statistical analysis:

Data were analyzed using the one-way ANOVA procedure of SPSS v.23 (SPSS Inc., Chicago, Illinois, The USA). Tukey's multiple comparison tests were used to test significant variations. Data were expressed as means ±SD, with a P-value of 0.05 is considered significant.

Results

The obtained results in Figure 1 revealed that the prevalence rates of *E. coli* in the examined kariesh cheese, ice cream, pasteurized milk, dairy plant surfaces, and equipment were 19.05%, 4.76%, 0%, 45.24%, and 30.96%, respectively. Five *E. coli* serotypes were identified in the current investigation namely, *E. coli* O26:H11, *E. coli* O55:H7, *E. coli* O78:H-, *E. coli* O111:H4, and *E. coli* O127:H6 at variable rates.

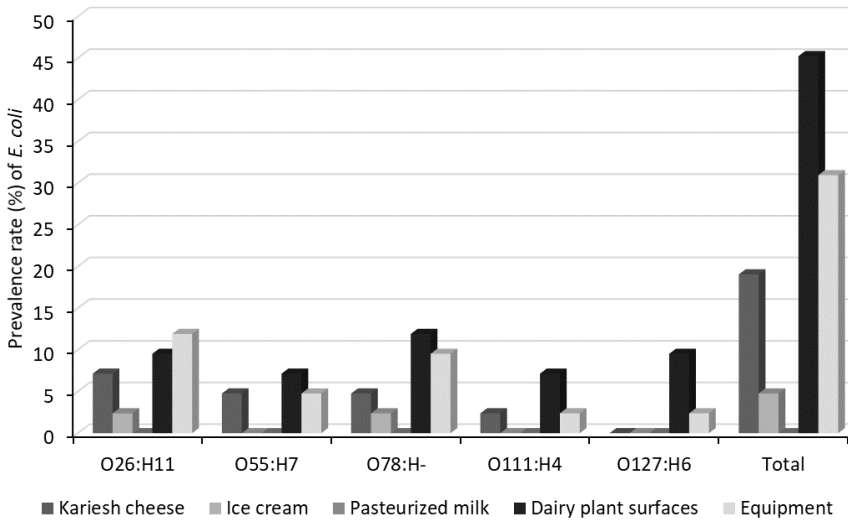

Figure 1: Prevalence rates (%) of different E. coli serotypes in the examined kariesh cheese, ice cream, pasteurized milk samples, and in swabs from dairy plant surfaces and equipment

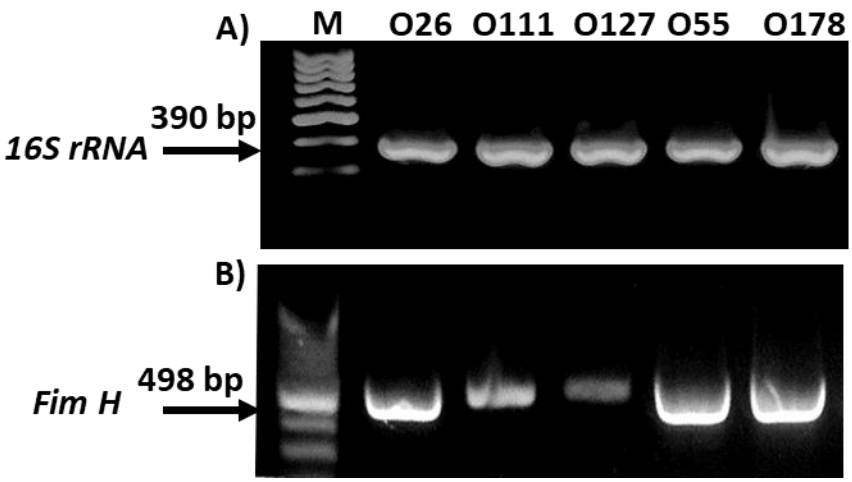

Figure 2: PCR amplification of A) E. coli-specific 16S rRNA, B) fim H-biofilm producing genes among different E. coli identified serotypes recovered from the examined kariesh cheese, ice cream, dairy plant surfaces and equipment

Figure 2A demonstrated the detection of *E. coli*-specific 16S rRNA gene which confirm serological identification of the obtained *E. coli* isolates. Furthermore, detection of *fim H* gene which is responsible for the adhesion of *E. coli* to the host cell surfaces, and this indicates potential abilities of such isolates to produce biofilm (Figure 2B).

We further extended our study to investigate biofilm production abilities among the identified *E. coli* serotypes using 96-well approach. The obtained results revealed that all identified serotypes had the ability to produce biofilm in the following order: *E. coli* O26:H11 (OD value = 1.22 ± 0.003), *E. coli* O55:H7 (0.83 ± 0.04), *E. coli* O78:H- (0.78 ± 0.05), *E. coli* O111:H4 (0.63 ± 0.01), *and E. coli* O127:H6 (0.61 ± 0.01), respectively (Figure 3). In a trial to reduce *E. coli* O26:H11-produced biofilm, *Nigella sativa,* and olive oils' nanoemulsions were used at two concentrations (1.0, and 2%). Interestingly the two used oils reduced biofilm production in a concentration dependent manner (Figure 4).

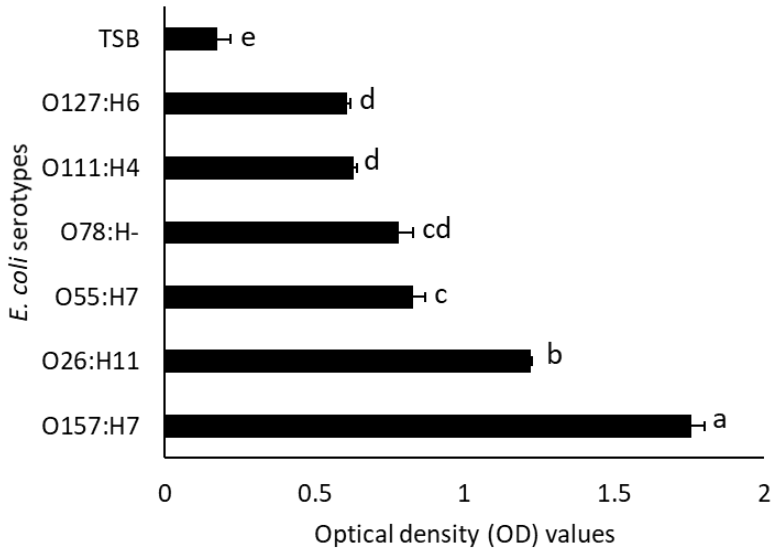

Figure 3: Biofilm producing ability among different E. coli identified serotypes. recovered from the examined kariesh cheese, ice cream, dairy plant surfaces and equipment. Columns carrying different letter (a, b, c, d, e) are significantly different at $p < 0.05$

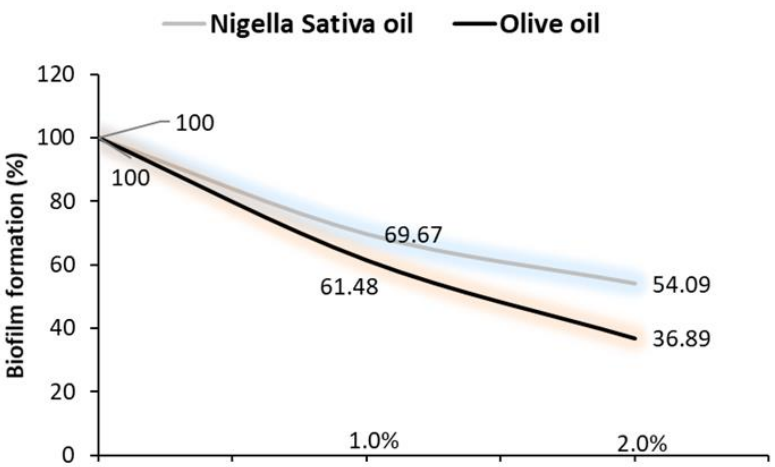

Figure 4: Protective effects of Nigella sativa, and olive oils' nanoemulsions against E. coli O26:H11produced-biofilm at 0, 1.0, and 2.0% concentrations

Discussion

E. coli is a major foodborne pathogen that is responsible for many cases of human hospitalization. For instances, *E. coli* from unknown food source was implicated in food poisoning outbreaks in seven states of US, with eleven hospitalizations, and one case of death (22). In the present study, *E. coli* was isolated from dairy products including kariesh cheese and ice cream, but not from pasteurized milk. These products are among the most consumed dairies in Egypt. This agrees with Elzhraa *et al.* (23) who isolated *E. coli* from some heat-treated dairy products consumed in Mansoura city, Egypt. Globally, *E. coli* was isolated also from dairy products worldwide (24) such as India (6), Iran (10), and several African countries (11). *E. coli* was also isolated from swabs sampled at the dairy processing plants. This might explain contamination of the examined dairy products with *E. coli.* Cross-contamination of the dairy products from the surrounding environment such as dairy plant floors, and other contact surfaces such as utensils and equipment are regarded as possible mean of transmission of *E. coli* to such dairy products (7, 8). *E. coli* O26:H11, *E.*

coli O55:H7*, E. coli* O78:H-*, E. coli* O111:H4*, and E. coli* O127:H6 were the identified *E. coli* serotypes in the present study using both serology and molecular approaches. These isolates were similarly isolated from dairy products in several reports (6, 10, 11, 23, 24).

The obtained results in the present study demonstrated the detection of *fim H*, a biofilm-coding gene, in all identified serotypes using PCR. Interestingly, such genotypic behavior of the identified *E. coli* serotypes was confirmed at the phenotypic level as all identified *E. coli* isolates had the ability to produce biofilm using a 96-well approach. Besides, *E. coli* O26:H11 had significantly the highest ability to produce biofilm as well as the most intense DNA band for *fim H* gene. In agreement with the recorded results, Tajbakhsh *et al.* (25) reported that biofilm formation was corresponding to the presence of virulence-associated genes including *fim H* among uropathogenic *E. coli* isolated from clinical samples in Iran. Furthermore, Zhao *et al.* (26) reported a positive correlation between biofilm formation and virulence factors in uropathogenic *E. coli* isolated from urinary tract infections.

There is an increasing demand to find friendly alternatives to chemical preservatives for food with clear and significant antimicrobial activities. In this regard, *Nigella sativa*, and olive oils' nanoemulsions were used at two concentrations to experimentally test their anti-biofilm activities. Interestingly, the two used oils' nanoemulsions had significant protective effects against *E. coli*-produced biofilm in a concentration-dependent phenomenon. For instances, *Nigella sativa* oil's nanoemulsions reduced biofilm formation at 30.33% (1.0%), and 45.90% (2.0%), respectively. While olive oil's nanoemulsion could achieve higher protection rates at 38.52% (1.0%), and 63.11% (2.0%), respectively. The antibacterial activities of the olive oil were attributed to its high content of flavonoids and polyphenols (21). *Nigella sativa* essential oil is rich in longifolene, thymoquinone thymohydroquinone, α- thujene, and p-cymene which are known for their strong antibacterial activities (27). Likely, Song *et al.* (28) recorded an antibiofilm activity for grapefruit seed extract against *Staphylococcus aureus* and *E. coli*. Besides, thyme, and thymol essential oils nanoparticles had significant antibiofilm activities (29), and strong antibacterial activities against *E. coli* O157:H7, *Salmonella* Typhimurium *Proteus mirabilis*, *Serratia marcescens, Yersinia enterocolitica*, and *Pseudomonas fluorescens* (30). The proposed mechanisms for the antimicrobial effects of the examined essential oils involved loss of the mitochondrial membrane in the bacteria, coagulation of the cellular proteins, and affecting the proton pump and ion channels, which can be detected and confirmed using electron microscopy (27).

Conclusion

This study demonstrated the isolation of different *E. coli* serotypes with biofilm-producing abilities from dairy products and their contact surfaces indicating inadequate hygienic practices during their manufacture. Therefore, strict hygienic measures should be followed during all steps of the manufacture, distribution, and storage of dairy products. Furthermore, using *Nigella sativa*, and olive oils' nanoemulsions could be considered as friendly food additives with antibiofilm activities and multiple applications.

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The authors declare no conflict of interest.

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