

RESEARCH ARTICLE

Evaluation of Antibiofilm Activity of *Nigella sativa* and Olive Oils' Nanoemulsions against *Bacillus cereus* from Dairy Processing Plants

Salah F.A. Abd-El Aal, Alaa M. A. Mohamed*, Asmaa S. M. Mohamed

Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt

*Corresponding author: dr.alaa.mohamed.tawfik@gmail.com

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Abstract

Kariesh cheese, ice cream, and pasteurized milk are among the popular dairy products with high nutritive values. These products are commonly consumed in Egypt at all ages. However, the surrounding surfaces and equipment might lead to contamination of such dairies with foodborne pathogens such as *Bacillus cereus*. Microbial biofilm production is a developed mechanism that enhances survival and resistance to harsh environment along with spores. This study aimed to investigate the prevalence of *B. cereus* in dairy products including kariesh cheese, ice cream as well as pasteurized milk, dairy plant surfaces, and equipment. Besides, the ability of the identified *B. cereus* isolates to produce biofilm were further examined. In a prevention trial, *Nigella sativa* and olive oils' nanoemulsions were used at a concentration of 1%, and 2% to reduce *B. cereus* biofilm formation. The obtained results revealed isolation of *B. cereus* from the examined kariesh cheese, ice cream, pasteurized milk, dairy plant surfaces, and equipment at 44%, 16%, 8%, 72%, and 68%, respectively, with average counts of 3.69 ± 0.23 , 2.58 ± 0.19 , 2.15 ± 0.21 , 5.36 ± 0.39 , and 4.46 ± 0.59 log 10 cfu/g, respectively. Molecular confirmation of the recovered *B. cereus* isolates revealed that all isolates harbored *B. cereus*-specific *16S rRNA*, and *Cal Y*, biofilm-matrix protein. All *B. cereus* isolates had the ability to produce biofilm. *Nigella sativa*, and olive oils' nanoemulsions reduced *B. cereus*-produced biofilm in a concentration-dependent manner.

Keywords: *B. cereus*; biofilm; dairy products; *Nigella sativa*; olive oil's nanoemulsions.

Introduction

Dairy products are rich sources of essential amino acids, vitamins such as vitamin D, and minerals such as calcium, and magnesium. Therefore, dairy products are regarded as important sources to provide humans with major part of their needs from proteins, fatty acids, vitamins, and minerals. Dairy products such as kariesh cheese, ice cream, and pasteurized milk are very popular foods among all ages, particularly children in Egypt [1]. At the same time, dairy products are involved in the transmission of several foodborne pathogens such as *Bacillus cereus* (*B. cereus*) [2].

Dairy products might be contaminated with a vast array of microorganisms during any stage of the manufacture processes,

storage, or distribution. In general, the level of personal hygiene, the use of contaminated raw milk or other ingredients, and the sanitary conditions of the used equipment and utensils, and other contact surfaces affect the microbial load of the retailed dairy products [3, 4].

Bacillus cereus is one of the spore-forming bacteria that is associated with many cases of foodborne toxi-infections. The elimination of *B. cereus* from different food matrices, particularly dairy products is considered as a challenging issue in the food industry. In this context, *B. cereus* was frequently isolated from dairy products such as soft cheese and yoghurt in Ghana [5], artisan cheeses made in Mexico [6], powdered infant formula in China [7], raw

and pasteurized milk in China [8]. However, the prevalence of *B. cereus* in dairy products such as kariesh cheese, ice cream, and milk retailed in Egypt, as well as in their contact surfaces in the dairy plants had received less attention.

Biofilm is produced by various array of bacterial species, particularly among those with high abilities to grow on different food matrices. Biofilm production is regarded as a protective mechanism that enhance bacterial growth and proliferation on food subjects and their contact surfaces [9]. Biofilm production by foodborne pathogens with public health importance has become an issue of concern for food safety, hygiene, and public health sectors, as it increases microbial efficiency, and antimicrobial resistance [10].

Chemical food preservatives have been used in food industry for decades. However, much concern has been raised for their residual levels in the final food products, and their potential health risks [11]. Therefore, food industry sector is seeking for friendly alternatives to antimicrobials to be used during the manufacture of different dairy products for the purpose of extension of the shelf life, reduction of the microbial loads, and production of new products with unique aroma and flavor. Essential oils are regarded as excellent candidates to be used as natural additives to achieve such purposes [12]. *Nigella sativa* oil (black seed oil) has been used since ancient times in Egypt for the treatment of arthritis, diabetes, asthma, hypertension, and obesity [13]. Olive oil (*Olea europaea L.*) is one of the major oils used in Mediterranean diet. It is rich in monounsaturated fatty acids and believed to play essential role as an antioxidant [14]. Both *Nigella sativa*, and olive oils are among the essential oils with broad spectrum antimicrobial activities [15]. However, the protective effects of *Nigella sativa*, and olive oils against *B. cereus*-produced biofilm are less investigated.

In sight of the previous facts, this study was undertaken to investigate the prevalence of *B. cereus* in three of the most commonly consumed dairies in Egypt, including kariesh cheese, ice cream, and pasteurized milk. In addition, the prevalence of *B. cereus* in swabs

samples from the surfaces, and equipment of dairy processing plants were examined. The ability of the identified *B. cereus* to produce biofilm was *in vitro* examined. Besides, *Nigella sativa* and olive essential oils' nanoemulsions antibiofilm activities were investigated against biofilm producing-*B. cereus*.

Material and Methods

Collection of samples

A total of 125 samples including kariesh cheese (100 g), ice cream (100 g) of local produce, and pasteurized milk (200 mL/package) (25 samples each) 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 (walls, floors, and 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

Ice cream samples were left to melt at room temperature, then 10 mL from each ice cream or pasteurized milk samples were pipetted into 90 mL of 0.1% buffered peptone water followed by preparation of serial decimal dilutions. Regarding cheese samples, ten grams from each sample were homogenized aseptically in 90 mL sodium citrate for 2 min at 2500 rpm to obtain a sample homogenate, followed by preparation of serial decimal dilutions [16].

Count, isolation, and identification of *B. cereus*

For *B. cereus* counts, 0.1 mL from each prepared serial dilution was streaked onto agar plates containing *B. cereus* agar base (Oxoid, UK) supplemented with Egg Yolk Emulsion (Oxoid) and Polymyxin B Selective Supplement (Oxoid). Cultured plates were incubated at 37°C for 24 h and observed for growth. *B. cereus* colonies with blue appearance (typically mannitol-negative) and lecithinase positive (zone of precipitation around colonies) were counted and selected

from each plate and sub-cultured on nutrient agar (Oxoid). *B. cereus* colonies were further identified by phenotypic and biochemical tests including cell shape and motility, hemolysis, production of catalase, oxidase, urease and lecithinase, nitrate reduction, fermentation of D-glucose, maltose, D-xylose, lactose and D-mannitol, and growth at a temperature of 10°C [17].

Bacterial DNA preparation & amplification reaction of *B. cereus* 16S rRNA & *Cal Y* genes

DNA was extracted from ten selected refreshed *B. cereus* colonies [18] using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, USA). Molecular confirmation of the identified *B. cereus* was done via detection of the 16S rRNA gene using PCR. Similarly, biofilm-matrix protein (*Cal Y*) among the identified *B. cereus*

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 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 (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.

Table 1. The designed primers' sequences of *B. cereus* 16S rRNA & *Cal Y* genes

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	Accession number
16S rRNA (F)	5' CGGCTTCGGCTGTCACCTTAT '3		NR_074540.1
16S rRNA (R)	5' ACGCTTGCCACCTACGTATT '3	347	
<i>Cal Y</i> (F)	5' AGCAGCATTGGGGTTAGCTT '3		NC_011725.1
<i>Cal Y</i> (R)	5' AGGCTCACTTTGTTTATCCCAGT '3	322	

Detection of biofilm production among *B. cereus* isolates

Biofilm detection among the recovered *B. cereus* isolates was conducted using a 96 well-flat bottom tissue culture plate according to Kwon *et al.* [19]. In short, initially refreshed *B. cereus* isolates in Trypticase Soy Broth (TSB) were diluted at 1:100 in TSB. Then five wells/each *B. cereus* isolate 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. Washing of the wells three times with buffered PBS was proceeded. Staining of the wells with 0.1% crystal violet was followed, and then 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, only TSB-filled wells served as negative control. The mean OD value of the negative control was deducted from all the test OD values. The experiment was repeated twice to confirm reproducibility. *B. cereus* ATCC 11778 (ATCC, Manassas, USA) was used as a positive control.

Protective effects of *Nigella sativa* and olive oils nanoemulsions against biofilm producing- *B. cereus*

The inhibitory effects of *Nigella sativa* and olive oils nanoemulsions against biofilm production by *B. cereus* were tested by mixing of the oil's nanoemulsions (prepared in Nakaa Nanotechnology Network, NNN) at two concentrations (32 µg/mL; 1%), and (64 µg/mL; 2%), based on their minimum inhibitory concentrations [20], with refreshed

B. cereus isolates in TSB and incubation for 24 h at 37°C. Then the content of each well was hygienically disposed and followed by staining with crystal violet, 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 \pm SD, where $p < 0.05$ is considered significant [19].

Results

The obtained results in Figure 1 revealed the isolation of *B. cereus* from the examined kariesh cheese, ice cream, pasteurized milk, dairy plant surfaces, and equipment at concentrations of; 44% (11 out of 25 samples), 16% (4 out of 25 samples), 8% (2 out of 25 samples), 72% (18 out of 25 samples), and 68% (17 out of 25 samples), respectively.

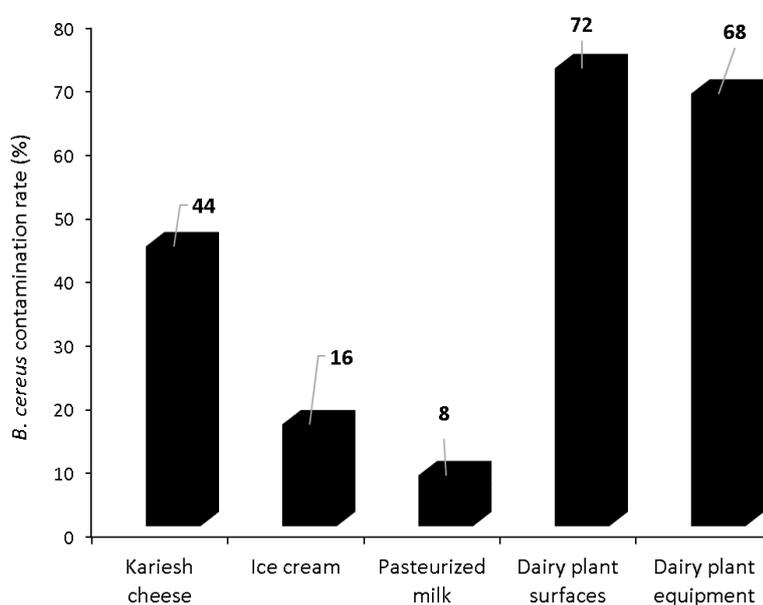


Figure 1. Prevalence rates (%) of *B. cereus* in the examined kariesh cheese, ice cream, pasteurized milk samples, and in swabs from dairy plant surfaces, and equipment.

The obtained results in Figure 2 indicated that the mean \pm SD values of *B. cereus* counts at the examined kariesh cheese, ice cream, pasteurized milk, dairy plant surfaces, and equipment were 3.69 ± 0.23 , 2.58 ± 0.19 , 2.15 ± 0.21 , 5.36 ± 0.39 , and 4.46 ± 0.59 log 10 cfu/g, respectively. *B. cereus*-specific *16S*

rRNA gene and *Cal Y* biofilm matrix protein, which is responsible for the adhesion of *B. cereus* to the host cell surfaces, were detected at the isolated *B. cereus*, and this reflects the potential abilities of such isolates to produce biofilm (data are not shown).

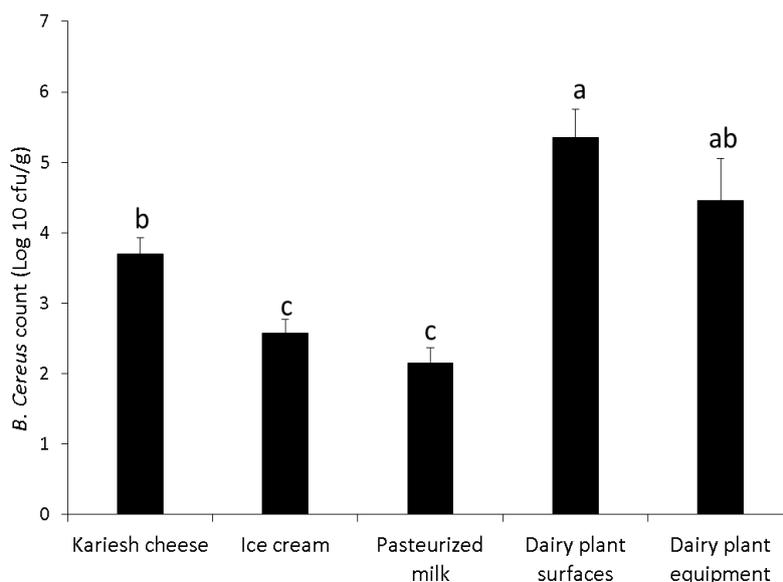


Figure 2. *B. cereus* counts (Log 10 CFU/gm) in the examined kariesh cheese, ice cream, pasteurized milk samples, and in swabs from dairy plant surfaces, and equipment. Columns carrying different letter (a, b, c) are significantly different at $p < 0.05$

Biofilm production abilities among the recovered *B. cereus* were further investigated. The obtained results revealed that all tested *B. cereus* isolates had the ability to produce biofilm in the range of (OD value) 0.554 to 1.985 (Figure 3). In a trial to reduce *B. cereus*-produced biofilm, *Nigella sativa*, and olive oils' nanoemulsions were used at two

concentrations (1%, and 2%). Interestingly the two used nanoemulsions reduced the biofilm production in a concentration dependent phenomenon, particularly *Nigella sativa*, and olive oils' nanoemulsions at 2% achieved the highest reduction rates (66.75%, and 77.33%, respectively) (Figure 4).

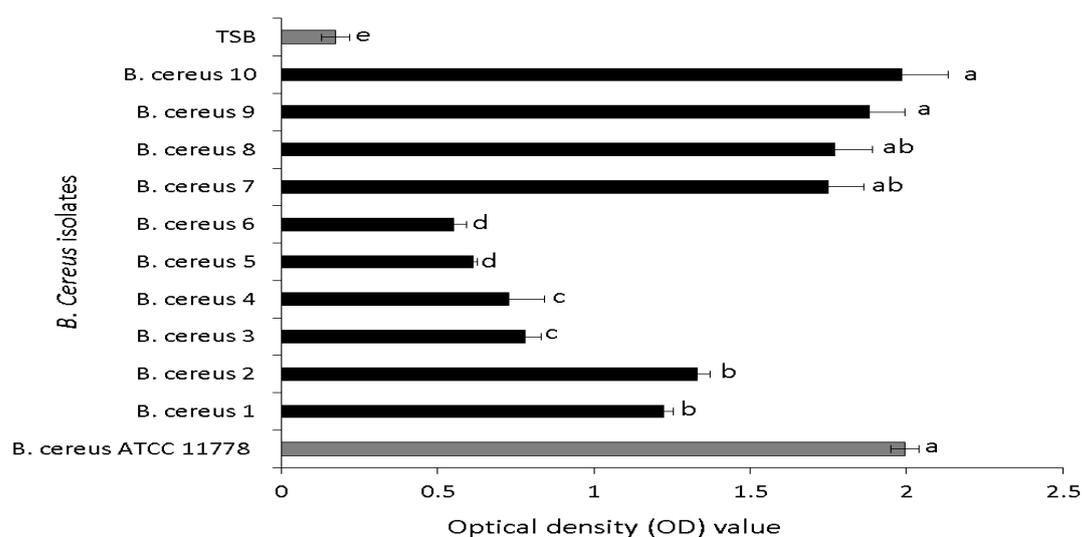


Figure 3. Biofilm producing ability of *B. cereus* isolates recovered from the examined kariesh cheese, ice cream, dairy plant surfaces and equipment. Columns carrying different letter (a, b, c, d) are significantly different at $p < 0.05$.

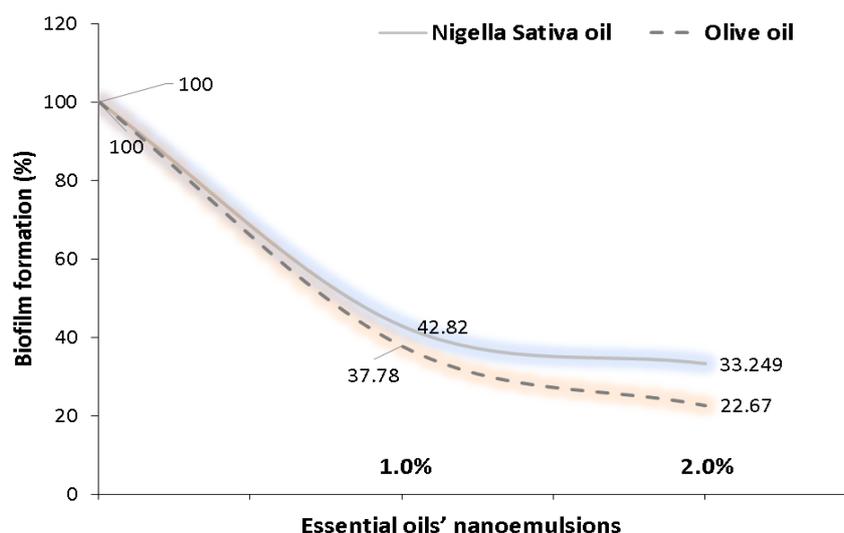


Figure 4. Protective effects of *Nigella sativa*, and olive oils' nanoemulsions against *B. cereus*-produced biofilm at 0, 1%, and 2% concentrations.

Discussion

Despite the recent advances in food industry field, *B. cereus* remains a challenging problem because of its effects on both consumer safety and shelf-life of dairy products [21, 22]. *B. cereus* has the ability to produce diarrheal and emetic food poisoning if contaminated dairies were consumed [23]. In the current investigation, *B. cereus* was isolated from dairy products including kariesh cheese, ice cream, and pasteurized milk, which indicates either contamination of the raw milk with heat resistant *B. cereus* or post-pasteurization contamination. *B. cereus* was also isolated from swabs collected at the dairy processing plants. This might explain contamination of the examined dairy products with *B. cereus*. Cross-contamination of the dairy products from the surrounding surfaces, and equipment are regarded as possible mean of transmission of *B. cereus* to such dairy products [5]. This agrees with Mugadza *et al.* [24] who isolated *B. cereus* from raw milk, pasteurized milk, and filler nozzles. In addition, Chang *et al.* [8] isolated *B. cereus* at 33.3% from buffalo raw milk and 15.3% from pasteurized buffalo milk from three provinces in China. Kariesh cheese had the highest *B. cereus* isolation rates and counts compared with ice cream,

and pasteurized milk. This reflects the poor hygiene adopted during the manufacture process of kariesh cheese. In general, kariesh cheese is commonly produced by local farmers in villages at a minimum level of hygiene. Therefore, the chance of kariesh cheese to be contaminated with a wide array of microorganisms is relatively high when compared with other dairy products. Likely, Owusu-Kwarteng *et al.* [5] isolated *B. cereus* from West African soft cheese at 35%, dairy plant soils at 72%, and raw milk at 47%, respectively. Furthermore, Adame-Gómez *et al.* [6] isolated *B. cereus* from artisan cheeses sold in Mexico at 29.48%. Additionally, Martínez *et al.* [25] isolated *B. cereus* from milk and cheese retailed in Cuba at 23.2 and 24.2%, respectively.

Biofilm is a complex microbial ecosystem formed by the coalescence of bacterial colonies on the surfaces and regarded as an adaptive mechanism to enhance microbial growth, and proliferation [26]. The achieved results in the current study revealed detection of *Cal Y*, a biofilm-matrix protein, in all tested *B. cereus* isolates using PCR. This genotypic characteristic of the identified *B. cereus* agreed to the phenotypic behavior as all identified *B. cereus* isolates had the ability to produce biofilm using a 96-well approach.

In agreement with recorded results, Radmehr *et al.* [27] found that 53.7% of the isolated *B. cereus* from pasteurized milk had the ability to form biofilm. Furthermore, Chang *et al.* [8] isolated biofilm-producing *B. cereus* with multidrug resistance from raw and pasteurized buffalo milk retailed in China. Besides, Fei *et al.* [7] recorded biofilm formation at both the phenotypic and genotypic levels among *B. cereus* isolated from powdered milk sold in China.

Nigella sativa, and olive oils' nanoemulsions were regarded as friendly candidates with significant antimicrobial properties. The obtained results in the present study indicated that the two used oils' nanoemulsions had significant reduction effects against *B. cereus*-produced biofilm in a concentration-dependent manner. Olive oil is rich in flavonoids and polyphenols which could explain its significant antimicrobial activities [28]. While, *Nigella sativa* is rich in longifolene, thymoquinone, thymohydroquinone, α -thujene, and p-cymene which are known for their strong antibacterial activities [29]. These essential oils act on the mitochondrial membrane in the bacteria, promote the coagulation of the cellular proteins, and affect the proton pump and ion channels [30]. In agreement with the recorded results in the present study, Fei *et al.* [30] reported that olive oil polyphenol extract inhibit the vegetative cells of *B. cereus* isolated from raw milk. Besides, thyme, and thymol essential oils nanoparticles had significant antibiofilm activities [31], and strong antibacterial activities against *E. coli* O157:H7, *B. cereus*, *Salmonella* Typhimurium, *Proteus mirabilis*, and *Pseudomonas fluorescens* [32].

Conclusion

This study demonstrated isolation of biofilm producing-*B. cereus* from dairy products and their contact surfaces indicating inadequate hygienic measures adopted during their manufacture. Therefore, strict hygienic measures should be taken during all steps of the manufacture, distribution, and storage of such products. Besides, *Nigella sativa*, and

olive oils' nanoemulsions are recommended as friendly food additives with antibiofilm activities; while caution should be given for the concentrations used of such nanoemulsions to avoid any undesirable sensory alterations.

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Conflict of interest

None

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الملخص العربي

مخاطر الباسيلس سيريس في مصانع منتجات الألبان مع بعض تجارب الاختزال

صلاح فتحى أحمد عبدالعال , الاء محمد عبدالوهاب توفيق , أسماء صلاح محمد محمد

قسم مراقبة الاغذية – كلية الطب البيطري – جامعة الزقازيق - 44511

يعتبر الجبن القريش والأيس كريم والحليب المبستر من بين منتجات الألبان الشائعة ذات القيمة الغذائية العالية حيث يشيع استهلاك هذه المنتجات في مصر لدي جميع الأعمار. ومع ذلك ، قد تؤدي الأسطح والمعدات المحيطة بعمليات التصنيع إلى تلوث مصنعات الألبان بميكروبات التسمم الغذائي مثل الباسيلس سيريس. يعد إنتاج الأغشية الحيوية الميكروبية آلية متطورة تعزز نمو الميكروبات وانتشارها. هدفت هذه الدراسة إلى تقصي مدى تواجد الباسيلس سيريس في منتجات الألبان مثل الجبن القريش والأيس كريم والحليب المبستر وكذلك بعض الأسطح والادوات المستخدمة في مصانع الألبان. بالإضافة إلى ذلك ، تم فحص مقدرة عزلات بكتيريا الباسيلس سيريس على إنتاج الأغشية الحيوية الميكروبية. وفي محاولة لتقليل إنتاج مثل تلك الاغشية تم استخدام مستحلبات زيت حبة البركة وزيت الزيتون بنسبة 1% ، و 2%. هذا وقد أظهرت النتائج عزل بكتيريا الباسيلس سيريس من الجبن القريش المختبرة والأيس كريم والحليب المبستر وأسطح مصانع الألبان وبعض الادوات المستخدمة فيها بنسبة 44% و 16% و 8% و 72% و 68% على التوالي بمتوسط 0.23 ± 3.69 ، 0.19 ± 2.58 ، 0.21 ± 2.15 ، 0.39 ± 5.36 ، و 0.59 ± 4.46 لوغ 10 لكل مستعمرة بكتيرية على التوالي. كما أظهر الفحص بتفاعل البلمرة المتسلسل احتواء كل المعزولات علي الجينات التأكيدية لوجود بكتيريا الباسيلس سيريس والمسئولة عن إنتاج الأغشية الحيوية الميكروبية. وقد أوضحت النتائج قدرة كل من مستحلبات زيت حبة البركة وزيت الزيتون علي الحد من قدرة معزولات الباسيلس سيريس علي إنتاج الأغشية الحيوية الميكروبية