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Trails to suppress *Bacillus cereus* growth producing biofilm and Cereulide toxin in kariesh cheese using oat and rosemary

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Abstract

Nowadays, there is an interest in the consumption of food without synthetic additives and using of natural preservatives. The prevalence of *Bacillus cereus* (*B. cereus*) in a total of 150 samples in some dairy products (raw milk, zabadi balady and Kariesh cheese (50 for each) marketed in Assiut city were investigated. The highest percentage of *B. cereus* existence was noticed in raw milk followed by Kariesh cheese (42, 30%, respectively). While the lowest percentage of *B. cereus* in zabadi balady was 18 %. Moreover; the overall isolation rate of *B. cereus* in all of the examined samples was 30 %. Detection of Biofilm production by Bacillus cereus showed that 28 isolate (62.2%) were positive using Congo Red Agar (CRA). Detection of emetic (cereulide toxin) gene and other virulence genes (*ces, hbl* and *cytK*) were detected by PCR. All tested isolates have *cytK* gene and one isolate has the three genes. The antibacterial effect (MIC) of oat and rosemary at different concentration in kariesh cheese were investigated. The results revealed that rosemary could inhibit *B. cereus* at 1st day while, oat at 3rd day .Also, the acceptability of these plants in kariesh cheese was evaluated in terms of flavor, appearance and palatability. The study recommended that rosemary should be used as a natural food additive in kariesh cheese in Egyptian markets due to its highest antimicrobial effect and good acceptability.

Keywords: *Rosemary, Oat, Milk, Kariesh cheese, Cereulide Toxin, Biofilm and B.cereus.*

Introduction

B. cereus is an aerobic spore-forming bacterium widely distributed in the environment. This bacterium produces one emetic toxin and at least three different enterotoxins that are responsible for the separate emetic and diarrheal syndromes, respectively **(Yang *et al.,* 2017).** Vegetables and dairy products, including pasteurized dairy products, are the main sources of *B. cereus*, and different studies indicated that it appears impossible to completely avoid its presence in milk samples (**Stenfors *et al.,* 2008 and Forero *et al.,* 2018).** In fact, thermal milk treatment processes select this bacterium because they eliminate competitive microbiota but are not sufficient to kill *B. cereus* spores **(Andersson *et al.,* 1995).**

B. cereus produces food poisoning with diarrhea and/or emesis and causes outbreaks that are often underestimated **(Papan *et al.,* 2019).** The emetic syndrome is generated by the emetic toxin, cereulide (**Logan, 2012**), encoded by the *ces* gene cluster (*cesHPTABCD*), located on a plasmid in strains belonging to a particular lineage of *B. cereus* **(Økstad and Kolstø, 2011)**. The diarrheal syndrome is associated to different enterotoxins coded in the bacterial chromosome. The hemolysin BL (HBL) is encoded by the *hbl* operon (*hblCDA* or *hblCDAB* that includes the *hblB* pseudogene), and the nonhemolytic enterotoxin (NHE) is encoded by the nhe operon (*nheABC*) **(Logan, 2012).** The cytotoxin K encoded by the *cytK* gene, has a high cytotoxic activity, and is the most common variant **(Stenfors *et al.,* 2008).** A biofilm can be defined as aggregation of microbes cells stick to each other and to a surface. These adherent cells are frequently embedded within a

self-produced matrix of extracellular polymeric substance (EPS). Biofilm is a survival strategy of microbes in which they are protected against antibiotics, chemical disinfectants and environmental challenges **(Arampatzi *et al*., 2011 and Zuberi and Nadeem, 2017).**

Aromatic and medicinal plants are already used in pharmaceutical industries for their active ingredients and in food industries as flavorings. More recently, because of the potential antimicrobial effect they have attracted the increased interest of food scientists and technologists as natural preservatives **(Costa *et al.,* 2015 and Gouvea *et al.,* 2017).** Moreover, medicinal plants such as fennel, oregano, oats, rosemary, dill, cumin, pepper, sage, thyme, and parsley demonstrated satisfactory *in vitro* antimicrobial activity against pathogens and spoilage microorganisms associated with cheese contamination (**Hassanien *et al.,* 2014; Moro *et al.,* 2014 and Caleja *et al.,* 2015).** The natural extracts considered safe, and having specific properties as antioxidant, antidiabetic, antimutagenic, antitoxigenic and antibacterial effects thus indicating great potential in their use as preservatives **(Nieto *et al.,* 2018).**

Rosemary (*Rosmarinus officinalis*) is a small ever-green plant, belonging to the Labiatae family. It grows basically in the basin of the Mediterranean Sea. Active substances present in Rosmarinus have a potent antioxidant and antibacterial activity and are widely used in the food industry **(Djeddi *et al.,* 2007 and Martínez *et al.,* 2019).** Oat (*Avena sativa*) is a cereal for human consumption with high contents of soluble dietary fiber, well-balanced protein, energy in the form of carbohydrate and oil, as well as several vitamins and minerals. In a addition, oats contained abundant antioxidant compounds such as tocols, and phenolic compounds which has antioxidant, antimicrobial, anti-inflammatory, antiallergic and anticarcinogenic activities **(Hao *et al.,* 2014).** So, this study aimed for isolation of *B. cereus* from some dairy products for detection of the most virulent diarrheal and emetic genes as well as biofilm production and to study the inhibitory effect of oat and rosemary on isolated *B. cereus in vitro* (MIC) and *in vivo* (in Kariesh cheese).

Materials and Methods

Sample collection

A total of 150 samples including random samples of raw milk, zabadi balady and Kariesh cheese (50 for each), were collected from different markets and dairy shops in Assiut City, Egypt. The samples were collected in package as marketed to the consumer and sent to the laboratory in an insulated box with a minimum of delay to be examined.

Enumeration ,Isolation and identification of *B. cereus:*

samples were incubated onto BHI at 37°C for 24 hr. then a loopful from the samples were cultured on Mannitol egg yolk phenol red polymyxin (MYP) agar medium for isolation of *Bacillus cereus*. Suspected colonies stained with gram stain and examined under oil emersion lens of microscope **(Tallent *et al.,* 2012)**. Pure cultures of the isolate were Biochemical identified according to **Quinn *et al* . *(2002).***

Detection of biofilm production by *B. cereus:*

The isolates were cultivated onto Congo red agar (CRA) and incubated aerobically at 37° Cfor 24 - 48 hours (**Kaur *et al.,* 2009).** A positive result was indicated by black colonies with a dry crystalline consistency while negative usually remained pink colonies.

Detection of *B. cereus* toxic and virulence genes

Some selected *B. cereus* positive strains (n=10) were sent to the biotechnology unit of Reference laboratory for Veterinary Quality Control on Poultry Production in Animal Health Research Institute, Dokki, Giza, Egypt, for detection of the following virulence *ces, cytk* and *hbl* genes.

DNA extraction

DNA extraction from samples was performed using the QIA amp 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 (I).

PCR amplification

PCR primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

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 were 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.

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

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ces	GGTGACACATTATCA TATAAGGTG GTAAGCGAAC-CTGTCTGTAACAACA	1271	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	Ehling-Schulz *et al.*, 2006
cytK	ACA GAT ATC GGI CAA AAT GC CAA GTI ACT TGA CCI GTT GC	421	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
hbl	GTA AAT TAI GAT GAI CAA TTTC AGA ATA GGC ATT CAT AGA TT	1091	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	

Determination of Minimum Inhibitory Concentration (MIC)

The rosemary and oats used in this research was obtained from Plant Department, Faculty of Agriculture, Al Azhar University, Assiut branch, Egypt. Each plant was ground in a laboratory mortar to be ready use. The (MIC) of Rosemary and Oat were established using the broth dilution method, as described by **Quinn *et al.*(2002)** .This test was carried out by preparing two-fold serial dilutions of Rosemary and Oat (10%, 5%, 2.5%, 1.25%, 0.625% and 0.312% w/v) was prepared separately using sterile Muller Hinton broth. Each tube was inoculated with a suspension of the 100 µL from *B. cereus* that contains approximately 10^5 CFU/ml. The tubes together with the control tube (tubes contained broth only) were incubated aerobically at 37 °C for 24h. The lowest concentration of the antibacterial that inhibits growth of the organism as detected by lack of visible turbidity was designated the MIC.

Effect of Oat and Rosemary plants on *B. cereus:*

Manufacture and treatment of Kariesh cheese.

Kareish cheese was prepared from skim milk that was pasteurized at 74ºC for 15 seconds. Then, the previously prepared rosemary /oat were mixed with skimmed milk at concentration 1.25% (w/v). Also, previously prepared inoculum of *B. cereus* was added then a sample was taken to determine the initial count. The inoculated skim milk was salted to a concentration of 1 %. Rennet starter was added at ratio of 2% **(Alnemr *et al.*, 2013)**. First, milk was divided into equal portions of four groups (group one: negative control: no plants or bacterial strains are present; group two: positive control: inoculated only with *B. cereus* at 10^5 CFU/ml; group 3, 4: inoculated with *B. cereus* and rosemary /oat in a ratio of 1.25% each

group were repeated three trials. The treated milk was incubated at 30ºC for overnight until coagulation and cheese was obtained. Treated cheeses as well as control samples were stored at refrigeration temperature (4±2ºC) for 11 days. Counts were taken from the finished cheese after second day and every 2 days for *B. cereus* count and pH measurement **(Tiwari and Awasthi, 2014 and Han *et al.*, 2015)**.

Sensory evaluation of kariesh cheese manufactured.

The Karish cheese was manufactured as previously mentioned and according to **Alnemr *et al.*,(2013)** briefly as follow: Skim milk was heated at 74°C for 15sec. and then cooled to 32°C. Rennet starter for cheese was added in ratio 2 % in milk. First, milk was divided into equal portions of three groups (group one: negative control: no plants; group 2,3: inoculated with rosemary /oat in a ratio of 1.25 % for sensory evaluation. The treated milk was incubated at 30ºC for overnight until coagulation and cheese was obtained. Treated cheeses as well as control samples were stored at refrigeration temperature (4±2ºC). Twenty-three consumers were selected in teams of different ages, sex and education to taste the samples. The perception of consumers toward cheese with various treatments was studied with respect to three different attributes (flavor, appearance and palatability). The level of agreement was scored as strongly agree (SA), agree (A), disagree (D), and strongly disagree (SD) (**Nelson and Torut, 1981**).

Statistical Analysis

The statistical analysis was performed using programs Graph Pad Prism 5.04 (GraphPad, Inc., San Diego, USA). Least significant differences were used at $p < 0.05$. The data represented by using the Microsoft Excel Spreadsheet.

Results

Table (1). The prevalence of *B.cereus* in the commercial dairy products tested

Tested products	No. of samples	No. (%) of positive samples	*Bacillus cereus* Count in Positive Samples (log CFU g^{-1} or mL^{-1})			Contamination Level (log CFU g^{-1} or mL^{-1})					
			Minimum	Maximum	Average ± SD	<1	≥1-2	≥2-3	≥3-4	≥4-5	≥5
Raw milk	50	21 (42)	1.0	4.8	2.4±0.43	29	9	5	2	3	2
Zabadi balady	50	9 (18)	1.0	2.0	1.1±0.31	41	6	1	2	0	0
Kariesh cheese	50	15 (30)	1.0	3.0	1.3±0.54	35	7	3	3	1	1

CFU, colony forming unit, SD, standard deviation

Table (2). Biofilm production of *Bacillus* spp. by CRA method

Tested products	No. of isolates	Biofilm production			
		Positive		Negative	
		No.	%	No.	%
Raw milk	21	15	71.4	6	28.6
Zabadi balady	9	3	33.3	6	66.7
Kariesh cheese	15	10	66.7	5	33.3
Total	45	28	62.2	17	37.8

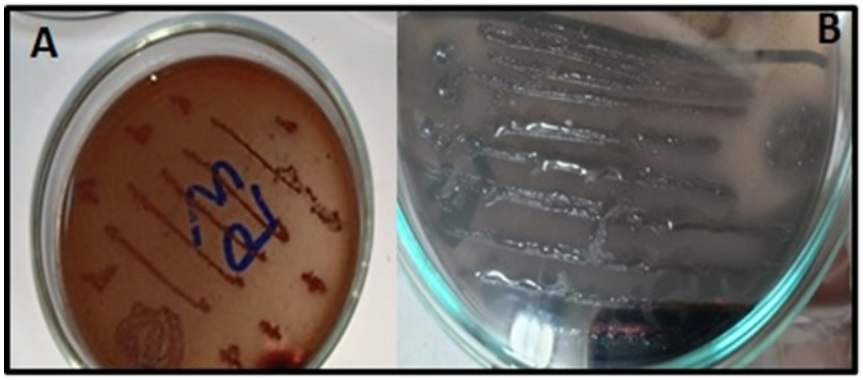


Fig. (1): CRA showed negative biofilm formation (A) and black pigmentation indicate positive biofilm formation (B)

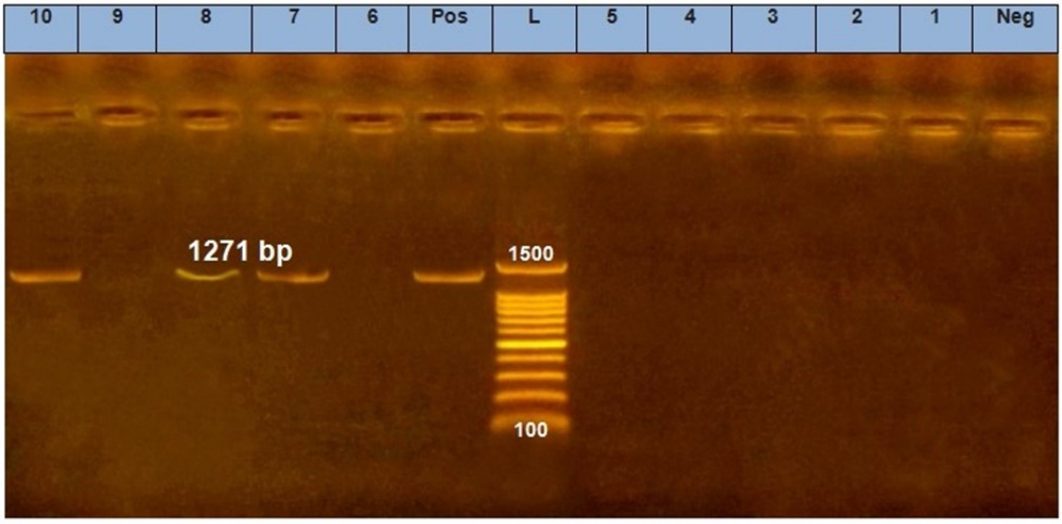


Fig. (2): The amplified *ces* gene of *B. cereus* recovered from different types of dairy samples
L: Molecular marker; **Lane pos.:** Positive control; **Lane Neg.**: Negative control; **Lanes 1-6, 9**: negative isolates; **Lane 7, 8, 10:** positive

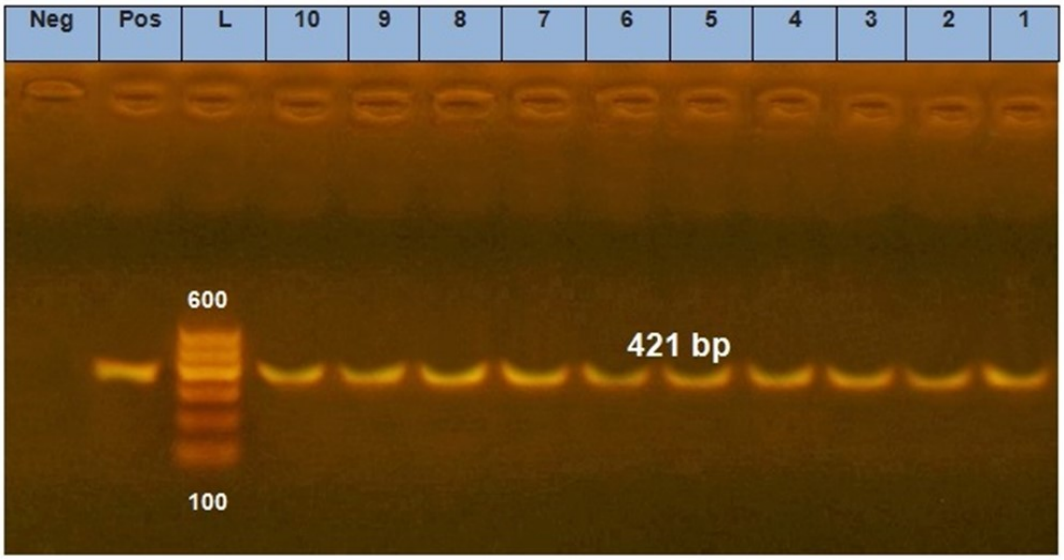


Fig. (3): The amplified *Cytk* gene of *B. cereus*
L: Molecular marker; **Lane pos.:** Positive control; **Lane Neg.**: Negative control; **Lanes 1- 10:** positive isolates**.**

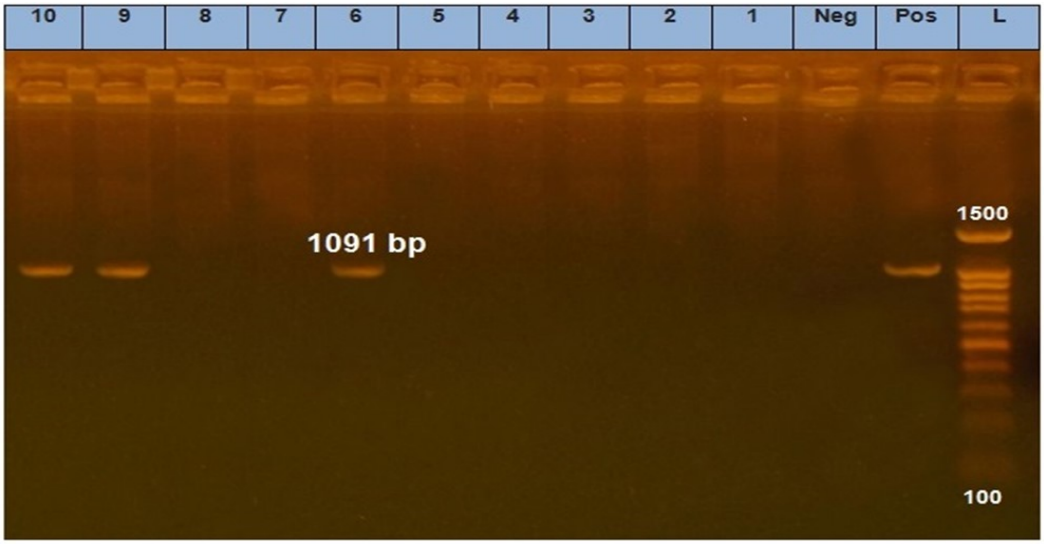


Fig. (4): The amplified *Hbl* gene of *B. cereus*
L: Molecular marker; **Lane pos.:** Positive control; **Lane Neg.**: Negative control; **Lanes 1-5, 7, 8**: negative isolates; **Lane 6, 9, 10:** positive isolates**.**

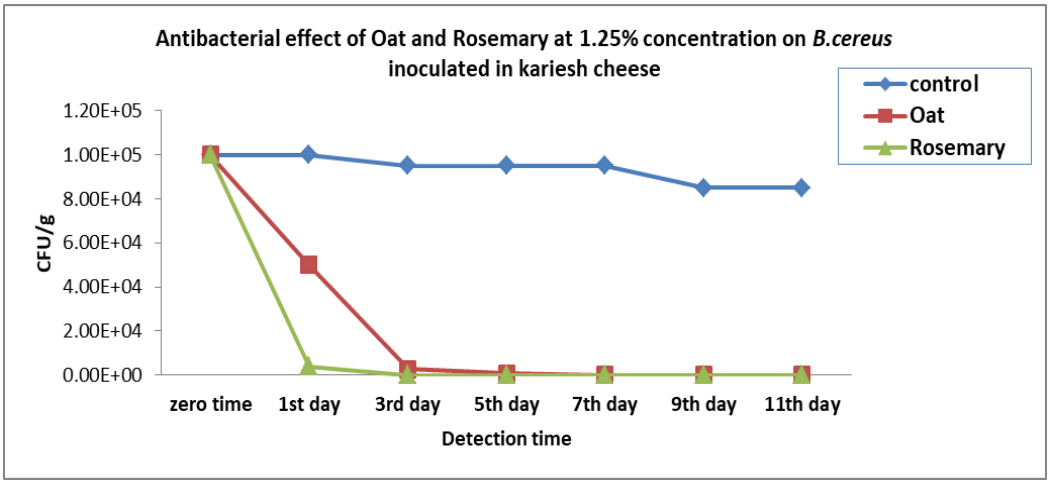
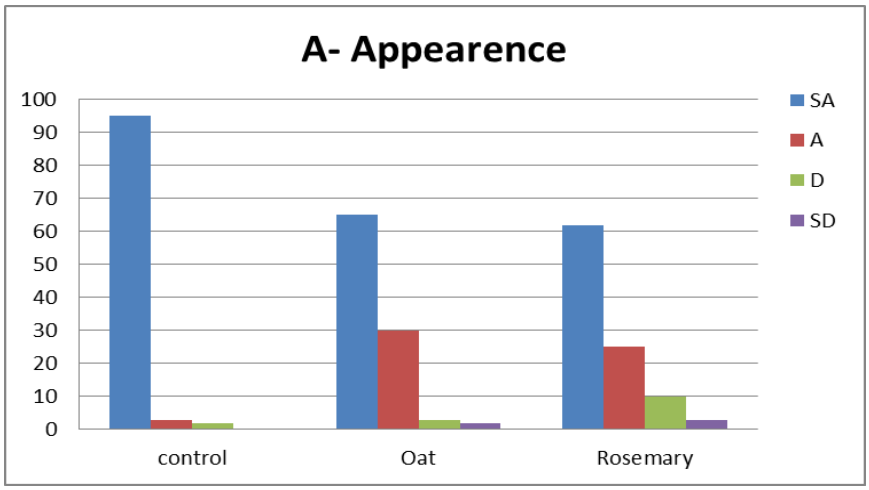


Fig. (5): Effect of oat and rosemary on *B. cereus* inoculated in Kariesh cheese during storage at refrigeration temperature.



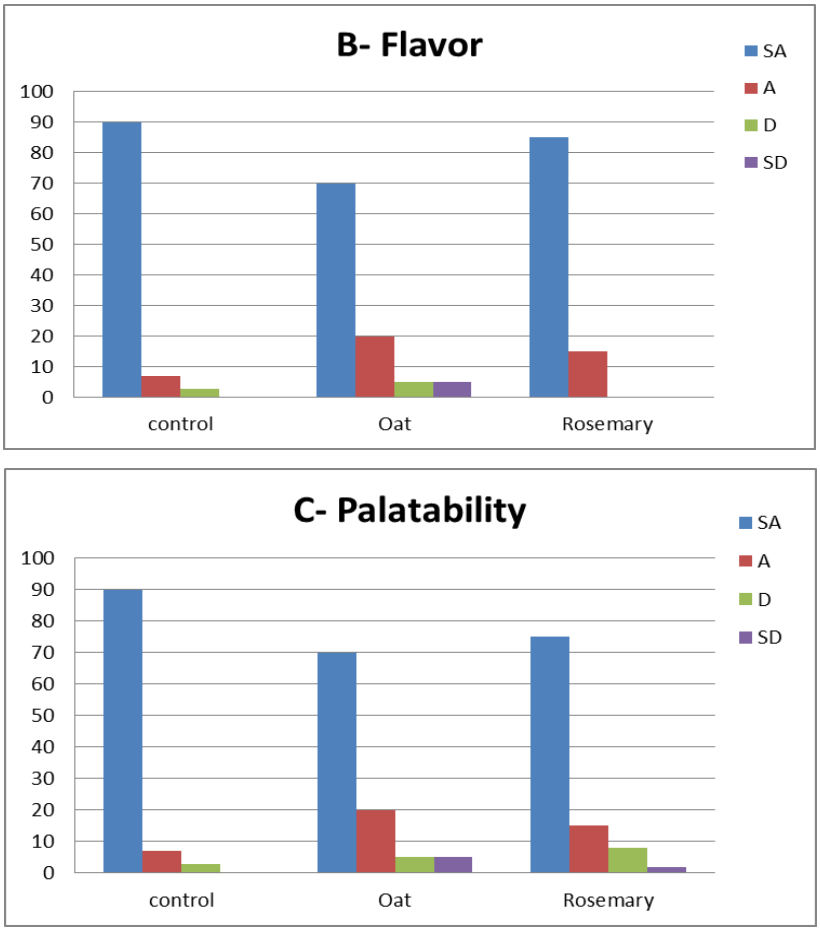


Fig. (6): Percentage of agreement for consumer acceptability to appearance (A), flavor (B), and palatability (C) of Kareish cheese manufactured with oat and rosemary.

Discussion

Bacillus cereus strains are wide spread in the environment which found on inert as well as on living surfaces and contaminate persistently the production lines of the food industry (**Majed *et al*.2016).** The prevalence of *B. cereus* in some tested commercial dairy products was illustrated in Table (1). Among the examined products, *B. cereus* has been most frequently found in raw milk followed by Kariesh cheese and zabadi balady in percentages of 42, 30 and 18%, respectively. The highest contamination was found in raw milk and kariesh cheese (>4.0 log CFU g^{-1} or mL^{-1}). These results were in agreement with and **Kumari and Sarkar (2014)** who found that the percentage of *B. cereus* in cheese was 33 %. On the other hand higher results were detected by **EL-Shinawy, 2004 and Rezende-Lago *et al*., 2007** who their isolation rate of *B. cereus* was 62 % and 50% in raw milk, respectively. Presence of high count of *B. cereus* in raw milk and Kariesh cheese may be due to milk production of healthy cows could be considered free of bacteria, but farm and dairy environments may be a source of contamination especially during milking and cheese production **Christiansson *et al .,*(1999).** In particular, *B. cereus* was previously recognized as responsible for raw milk spoilage plus the ability to grow in these products **(Bartoszewicz *et al.,* 2008)**; on contrary that the low contaminant detected in zabadi balady returned to that the yogurt is not an optimal substrate for *B. cereus* growth, due to low pH as well as presence of Lactic Acid Bacteria (LAB) as natural inhabitant in yoghurt in high count **(Tirloni *et al.,* 2017).**

B. cereus was demonstrated to be able to cause disease with loads between 4 and 8 log cfu/g of food; nevertheless, this range may be even wider, due to possible differences in toxin production and specific growth rate **(Hassan *et***

***al.,* 2010).**
B.cereus produces a variety of biofilms which differ in their architecture and mechanism related to their environments. Moreover, *B.cereus* biofilm exists in different physiological state and generate highly resistant and adhesive spores, which themselves will increase the resistance of the bacterium to antimicrobials or to cleaning procedures (**Majed *et al.* 2016).** As shown in (Table 2) and Figure (1) detection of biofilm production by *Bacillus cereus* showed 28 isolates (62.2%) were positive using Congo Red agar (CRA) .This result was lower than that detected by **Asmaa *et al.* (2014)** who reported that 18 isolates (81.8%) of *Bacillus cereus* were positive using (CRA). Also as in (table 2) the highest positive results 71.4% for *B. cereus* isolated from raw milk followed by kariesh cheese 66.7% while lowest one for zabadi balady 33.3%. **Mathur *et al.* (2006)** recorded that CRA method is a simple qualitative method to detect biofilm production. The Congo red test is based on the ability of this dye to stain polysaccharides black. A black color interpreted as positive biofilm producing strains in contrast with red colonies which interpreted as negative biofilm producing. The phenotypic coloration on agar improved upon modification of agar ingredients. The reduction in the concentration of agar constituents resulted in permanent formation of intense black pigment in isolates with biofilm genes. (**Zuberi and Nadeem, 2017).**
Also, the ability of *B. cereus* to cause food poisoning (e.g., diarrheal syndrome) is strictly related to its ability to resist to low pH, as the production of enterotoxins is a consequence of the survival of *B. cereus* spores and vegetative cells ingested with food through the stomach reaching the small intestine alive **(Ceuppens *et al.,* 2012).** So, this study detect the emetic (cereulide) toxin gene (*ces*), the enterotoxigenic and cytotoxic gene (*hbl* and *Cytk*) in isolated strains and found that all the tested 10 isolates were positive for *Cytk* gene and some of them have *hbl* (6,9,10) or *ces (7,8,10)* genes and just one isolate harboring all three genes (Figure 2-4). Several studies reported that none of the virulence factor was able individually or in combination to fully explain the cytotoxic potential of *B. cereus* group bacteria **(Dietrich *et al.,* 2005; Jeßberger *et al.,* 2015; Castiaux *et al.,* 2016; Miller *et al.,* 2018).** Therefore, the combined and possibly synergistic action of multiple toxins can probably explain the diarrheal syndrome related to *B. cereus* group bacteria **(Gdoura-Ben *et al.,* 2018).** Indeed, **Fogele *et al.* (2018**) and **Frentzel *et al*. (2018)** detected that the rates of *ces* gene were significantly lower compared to the detection of the *Hb*l and *Nhe* complex genes. Emetic *B. cereus* group strains containing various enterotoxin genes such as those of the *Nhe* and *Hbl* complexes, *cytK* and/or *bceT* could have the potential to cause diarrheal and emetic food poisoning simultaneously (**Arslan *et al.,* 2014).**
The natural food additives have potent antioxidant and antimicrobial properties that could substitute synthetic additives with healthier benefits for the human body **(Brewer, 2011).** Regarding their chemical nature and origin, these compounds do there antimicrobial action by several mechanisms through disturbances in bacterial cell wall maintenance, protein synthesis and or inhibition of DNA replication **(Li, *et al.,* 2017).** In this study, the antimicrobial activity of oat and rosemary against *B.cereus* was evaluated. As the potential for the use of these plants as natural antimicrobial agents is insufficiently exploited, determination of the MIC is essential to measure the degree of their activities. Oat and rosemary proved to have antibacterial activity against *B. cereus* at the same concentration of 1.25% so we used it *in vivo* by inoculation in Kariesh cheese. *B. cereus* could not be detected in kariesh cheese treated with rosemary at first day after inoculation and at third day by using oat as in Fig. (5). In this study, environmental conditions during the storage period such as pH (5.2), and temperature (4ºC) slightly affected the count of *B. cereus* in control (non-treated kariesh cheese). Gross composition of the Kareish cheese was not significantly different throughout aging, indicating that addition of these plants to the cheese milk did not affect the composition of resultant cheese. The obtained results were in agreement with **Genena *et al.,* (2008)** who claimed that the count of *S. aureus* and *B. cereus* was reached to the quarter after 6 hours inoculation. **Jawad *et al.* (2019)** studied the importance of rosemary against food pathogens

(*B. cereus, E. coli and Pseudomonas*). Rosemary extract give a large inhibitory zone against *B. cereus* about 14-26 mm. The authors added the essential oils of Rosemary can be used in the pharmaceutical industry for the production of new synthetic agents in the treatment of bacterial disease caused by *Bacillus cereus, Escherichia coli,* and *Pseudomonas.*

Many studies have shown that oat β-glucan has physiological antitumor, anticancer, antiviral, antibacterial, and antiparasitic effects. These may be attributed to its strong immune-enhancing activity. Because the oat β-glucan has significant physiological activity to enhance immunity, it has been used in immune-adjuvant therapy for tumors and cancers **(Ross *et al.,* 2004 and Daou and Zhang, 2012).** Our results (Fig.4) showed that oat is lower antibacterial action than rosemary this was in agreement with **Roaaya Alrahmany (2012)** who concluded that oat was higher antioxidants but lower in antibacterial action.

Manufacturers have strived to produce high-quality food with superior texture, color, flavor, and nutritional values in the shelf life **(Zhang *et al.,* 2006).** There is limited information on the antimicrobial activities of the plants in combination with consumer acceptability. In this study, the perception of consumers toward treated cheese was studied with respect to three different attributes (flavor, appearance, and palatability). Generally, rosemary was considered the most preferable and strongly accepted treatments than oat as in Fig. (6). These results were nearly in agreement with **Tayel *et al.* (2015)** who concluded that plant extracts could be recommended as effective, safe, and ecofriendly antimicrobial agents for the protection of processed cheese from foodborne pathogens and enhancing the sensory attributes of flavoured cheese. These aromatic plants are defined as plant species with characteristic aromas and/or tastes, whose importance lies in having volatile components compounds which are secondary metabolites that can accumulate in leaves, roots, flowers, and seeds (**Forlin, 2012).**

This study concluded that *B. cereus* could be detected in raw milk, zabadi balady and Kariesh cheese from the retail market. Some isolated strains harbor more than one type of virulence genes as well as biofilm production which may cause a public health hazard. Rosemary not only enhances the preservation properties of kariesh cheese through its powerful antibacterial action but also has a strong acceptable flavor to consumers. Thereby, it may contribute to the development of new variety of safer and good flavor of Kariesh cheese. Further efforts are needed to evaluate the effect of rosemary and other aromatic plants on the local produced dairy products.

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