Research Article

The Inhibitory Effect of Potassium Sorbate and Bifido-Bacterium on Shiga Toxin Producing *E. coli* in Kareish Cheese

Gamilat A. Elsaid[1], Weam Mohamed Baher[1*], Eman Shukry[1], Abeer E. Abd El. Ghafar[2], Marwa Shalaby[1]

[1]Food Hygiene Department, Animal Health Research Institute (AHRI), Mansoura branch, Egypt; [2]Bacteriology Department, Animal Health Research Institute (AHRI), Mansoura branch, Egypt.

Abstract | Kareish cheese is considered among the most important dairy products in Egypt. It is of a high nutritive value and supports part of the human needs of protein, vitamins, and minerals. This study aimed at estimation of most probable number (MPN) of coliforms and *E. coli* and investigation of the prevalence of shiga toxin producing *E. coli* in kareish cheese collected from grocery stores and street vendors at Mansoura city, Egypt. In addition, molecular confirmation, and detection of shiga toxin coding genes (*stx1*, and *stx2*) in the recovered *E. coli* isolates was done using PCR. Moreover, the inhibitory effects of potassium sorbate (KS) and *Bifidobacterium animalis subsp. lactis* on the growth of *E. coli O26* were screened. The estimated MPN of coliforms and *E. coli* in kareish cheese collected from grocery stores were 3.44 ± 0.12 and 2.22 ± 0.14. These values were 4.61 ± 0.11 and 3.60 ± 0.25 in samples collected from street vendors. The prevalence rates of *E. coli* were 20%, and 60% in kareish cheese samples collected from grocery stores and street vendors, respectively. The identified *E. coli* serotypes were *E. coli O2:H6, E. coli O26:H11, E. coli O55:H7, E. coli O78:H-, E. coli O111:H4,* and *E. coli O119: H.* All isolated harbored 16S rRNA specific gene for *E. coli*. The expression of shiga toxin coding genes indicated that *E. coli O2:H6, E. coli O111:H4,* and *E. coli O119:H* harbored only *stx2*. *E. coli O26:H11* and *E. coli O55:H7* harbored both *stx1*, and *stx2*; however, *E. coli O78:H-* did not harbor any of the tested genes. The use of potassium sorbate and *Bifidobacterium animalis subsp. lactis* during kareish cheese manufacture could significantly reduce *E. coli O26:H11* in a concentration-dependent manner. Therefore, strict hygienic measures should be adopted during all manufacture steps of kareish cheese. The use of KS and *Bifidobacterium animalis subsp. lactis* during kareish cheese manufacture is highly recommended.

Keywords | Kareish cheese, *E. coli*, Egypt, potassium sorbate, Bifido-bacterium

Received | July 10, 2022; **Accepted** | July 01, 2022; **Published** | August 01, 2022
***Correspondence** | Weam Mohamed Baher, Food Hygiene Department, Anima Health Research Institute, Mansoura Branch, Egypt **Email:** dr.weambaher2000@gmail.com
Citation | Elsaid GA, Baher WM, Shukry E, Ghafar AEA, Shalaby M (2022). The inhibitory effect of potassium sorbate and bifido-bacterium on shiga toxin producing *E. coli* in Kareish cheese. Adv. Anim. Vet. Sci. 10(8):1834-1840.
DOI | https://dx.doi.org/10.17582/journal.aavs/2022/10.8.1834.1840
ISSN (Online) | 2307-8316

INTRODUCTION

Cheese is one of the most consumed dairy products worldwide because of its specific aroma and flavor, and high nutritive values. Cheese is rich in protein, vitamins, trace elements and minerals such as calcium and magnesium, and (Gerosa and Skoet, 2013; Ma et al., 2020). In Egypt there are varieties of cheese types that are made from either raw or pasteurized milk such as kareish, Feta, Domiati, Rumy, and other cheese types. Kareish cheese is made from raw milk by the farmers at their homes, or at small processing plants with minimum hygiene, and being sold open to air with a high possibility for cross contamination because of poor personnel hygiene, contaminated equipment, and raw milk (Marcobal et al., 2012). Consumption of cheese was linked to many food

poisonings outbreaks worldwide. *Escherichia coli (E. coli)* is major bacteria responsible for many cases of food poisoning outbreaks (McSweeney, 2007).

E. coli is a natural inhabitant of the intestinal tract of the farm animals. Presence of *E. coli* in foods of animal origin such as milk, and dairies indicates fecal contamination. At the same time, *E. coli* is associated with several cases of human hospitalization (Darwish et al., 2015). *E. coli* strains are broadly classified into enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and shiga toxin-producing *E. coli* (STEC). This classification is dependent on *E. coli*-pathogenicity and virulence attributes (Xia et al., 2010). More than 100 serotypes of *E. coli* are currently classified as STEC. Of these, *E. coli O157* causes 50 to 90% of *E. coli*-related human food poisoning cases, with most of the remaining cases caused by *O26, O45, O103, O111, O121* and *O145* (Scallan et al., 2011).

Potassium sorbate (KS) is an unsaturated fatty acid salt of sorbic acid, which can easily be metabolized. It has been used since decades in the field of food technology and industry for its specific antifungal activities. Also, it can be used as a food additive for development of unique flavor of the food (Liang et al., 2019). However, the inhibitory effects of KS on shiga toxin producing *E. coli* are less informed.

Probiotics are living microorganisms that promote human health when administered in adequate amounts (Hill et al., 2014). In addition, probiotics were introduced to dairy industry in order to increase the shelf life of the final products and inhibit the growth and multiplication of spoilage and pathogenic microorganisms. Of particular, dairy strains of *bifidobacteria* which are known for their antimicrobial and health promoting activities (Fijan et al., 2018; Faghihi Shahrestani et al., 2021). However, the inhibitory effects of *Bifidobacterium animalis subsp. lactis,* on shiga toxin producing *E. coli* using kareish cheese as a food subject are less investigated.

The present study was conducted to firstly estimate most probable number of coliforms and *E. coli* in retailed kareish cheese in Mansoura markets, Egypt. Secondly, to investigate the prevalence of shiga toxin producing *E. coli* in the examined kareish cheese. Detection of shiga toxin coding genes (*stx1*, and *stx2*) was done using PCR. Besides, the inhibitory effects of KS, and *Bifidobacterium animalis subsp. lactis* on shiga toxin producing *E. coli O26* using kareish cheese as a food matrix were examined.

MATERIALS AND METHODS

Sample collection

A total of hundred kareish cheese samples were collected from grocery stores (n= 50 samples), and local vendors (n= 50 samples) at Mansoura city, Dakahlia Governorate, Egypt. Samples were transferred cooled without delay and under aseptic conditions to Animal Health Research Institute, Mansoura branch where bacteriological examination was conducted.

Sample preparation

Twenty-five grams were aseptically collected from each sample and homogenized in 225 mL sodium citrate 2% for 2 min at 2500 rpm to obtain a dilution of 10^{-1}, followed by preparation of decimal serial dilutions (APHA, 2001).

Estimation of MPN of coliforms

The three tubes method was used for the estimation of MPN of coliforms according to APHA (2001).

Estimation of MPN of *E. coli*

Loopfuls from the positive tubes (producing acid and gas) were aseptically injected into previously warmed (44.5°C) tubes containing 7 ml of *E. coli* (EC) broth (Himedia, Mumbai), and incubated for 24-48 hours at 44.5°C. According to the approved tables, the positive tubes were utilized to determine MPN of *E. coli*.

Isolation of *Escherichia coli*

A loopful from each positive EC broth (acid and gas) was streaked onto Eosin Methylene blue (EMB) agar plates tube, then incubated at 37°C for 24 hours (APHA, 2001). *E. coli* typical colonies are often greenish, metallic, and have a dark purple core. The identification of *E. coli* was based on staining and biochemical assays (APHA, 2001).

Serodiagnosis of *E. coli*

The confirmed *E. coli* isolates were serologically identified using the rapid diagnostic *E. coli* antisera sets (Hardy Diagnostics, Ohio, USA) (Kok et al., 1996).

DNA preparation

The DNA from the recovered *E. coli* isolates was extracted using the method previously described (Darwish et al, 2015).

Molecular confirmation and detection of shiga toxin coding genes in the identified isolates

PCR detection of the *16S rRNA* gene was used for molecular confirmation of the recovered *E. coli* serotypes. Furthermore, PCR was also used to detect the coding genes for shiga toxins (*stx1* and *stx2*) in the confirmed *E. coli* isolates. Table 1 lists the primer sequences as well as the sizes of the amplified products. A Thermal Cycler was used to perform the amplification (Master cycler, Eppendorf, Germany). PCR experiments were performed using the

Table 1: Oligonucleotides' sequences used in the present study.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
16S rRNA	F 5′ CTTTCAGCGGGGAGGAAGG ′3	390	Abd-El Aal *et al.* (2021)
	R 5′ TCAACCTCCAAGTCGACATCGT ′3		
stx1	F 5′ ACACTGGATGATCTCAGTGG ′3		Dhanashree and Mallya (2008)
	R 5′ CTGAATCCCCCTCCATTATG ′3	614	
stx2	F 5′ CCATGACAACGGACAGCAGTT ′3		
	R 5′ CCTGTCAACTGAGCAGCACTTTG ′3	779	

AmpliTaq DNA polymerase kit according to Dhanashree and Mallya (2008) technique. *E. coli O157:H7* Sakai was used as a positive control. While *E. coli K12DH5* was used as a negative control. The DNA fragments were visualized using agarose gel electrophoresis at 2% in 1x TBE buffer. A 100 bp plus DNA Ladder was used as the experimental marker.

Reduction trial for *E. coli O26* using KS and *Bifidobacterium animalis subsp. lactis*

Preparation of *E. coli O26*

E. coli O26 isolated in the present study was refreshed in tryptic soy broth (TSB) (Oxoid, UK) overnight at 37°C. Then, a loopful was streaked onto tryptic soy agar (Oxoid, UK) and incubated at 37°C for 24 h. A single purified colony was inoculated into a sterile TSB and incubated at 37°C /24 h to obtain a final concentration of approximately 10^9 cfu/mL (Elafify et al., 2022).

Preparation of bacteriocins' suspensions

Pure probiotic bacterial strain (*Bifidobacterium animalis subsp. lactis*) was kindly gifted from Animal Health Research Institute, Egypt. Cell-free supernatant (CFS) was prepared according to Ibarra-Martínez *et al.* (2022) as follows: One ml of *Bifidobacterium animalis subsp. lactis* was cultured overnight in 20 ml M17 broth (HiMedia, Mumbai. India), then 1 ml of the obtained culture was sub-cultured overnight in 20 ml M17 broth. Once the probiotic strain reached 1 × 10^8 cells / ml, the medium was centrifuged at 5000 x g for 15 min at 4°C and the supernatant was recovered and filtered. The supernatants were stored at −20°C until use.

Experimental groups

Three liters of milk free from pathogens were inoculated with *E. coli O26* at a concentration of 10^6 cfu/mL (10^9 cfu/mL was diluted into 1 L of milk to obtain 10^6 cfu/mL). Then the milk was divided into 6 groups as following: The first group contained *E. coli O26*-treated milk only and served as a control group. The second group received *E. coli O26*-treated milk + KS (Merck KGaA), Darmstadt, Germany) 0.15%. The third group received *E. coli O26*-treated milk + KS 0.3%. The fourth group received *E. coli O26*-treated milk + KS 0.15% + *Bifidobacterium animalis subsp. lactis*. The fifth group received *E. coli O26*-treated milk + KS 0.3% + *Bifidobacterium animalis subsp. lactis*. The six-group received *E. coli O26*-treated milk + *Bifidobacterium animalis subsp. lactis*. Then kareish cheese was manufactured from such milk groups in the laboratory according to Fayed et al. (2014). Kareish cheese made from each group was cut into five pieces (each piece = 100 g). The formed kareish cheese was stored at 4°C for 21 days and examined for *E. coli* count on a weekly basis at zero, 7th, 14th and 21st days of the chilling storage to evaluate the effect of the above-mentioned treatment on *E. coli* growth. *E. coli* counts were recorded, calculated, and expressed as log10 cfu/g. Antibacterial effects of potassium sorbate and *Bifidobacterium* were calculated and expressed as inhibitory rates (%).

Statistical analysis

Statistical analysis was done using analysis of variance (ANOVA) followed by Tukey–Kramer HSD test where p <0.05 indicated statistical differences.

RESULTS AND DISCUSSION

Kareish cheese is considered as one of the most important dairy products retailed in Egypt because of its high nutritive values, unique aroma, and flavors. In the present study, all collected cheese samples had normal sensory characteristics including fresh odor, cheesy taste, and whitish color (data are not shown). Estimation of MPN of coliforms and *E. coli* is regarded as a bioindicator for the hygienic status adopted during the manufacture process, and marketing of the end products (APHA, 2001). The obtained results of the bacteriological examination indicated that the estimated MPN of coliforms and *E. coli* were 3.44 ± 0.12 and 2.22 ± 0.14 in kareish cheese samples collected from grocery stores. These values were 4.61 ± 0.11 and 3.60 ± 0.25 in samples collected from street vendors (Figure 1). It is clear from the recorded results that cheese purchased from street vendors had poor hygienic measures compared to that from grocery stores. This result looks reasonable as street vendors sell their kareish cheese open to air and transfer it at the variable atmospheric conditions with clear fluctuations in the temperature of the final products making the cheese liable for microbial contamination (Mossel

et al., 1995). Likely, unsatisfactory hygienic measures of the retailed fresh cheese were recorded in Mexico (de la Rosa-Hernández et al., 2018), and in kareish cheese, tallaga cheese, Ras cheese retailed in Beni-Suef city, Egypt (Hassan et al., 2019).

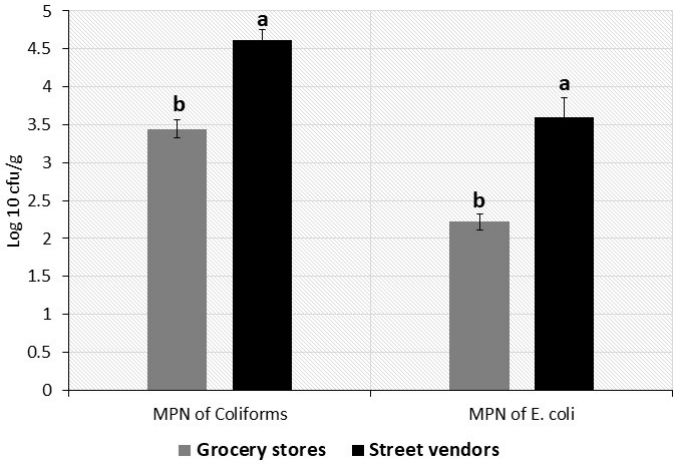

Figure 1: Most probable number of coliforms and *E. coli* in kareish cheese collected from grocery stores, and street vendors in Mansura city city, Egypt (n = 50). Values represent means ± SD (Log 10 cfu/g). Columns carrying different letter (a, b) are significantly different at $p < 0.05$.

E. coli is a major foodborne pathogen that has been linked to numerous hospitalizations and deaths, particularly among children and the elderly. Between October 2002 and February 2003, a cluster of *E. coli O157:H7* hemorrhagic colitis was discovered in Canada. This outbreak was caused by the ingestion of unpasteurized Gouda cheese (Honish et al., 2005). Furthermore, *E. coli O104:H4* was responsible for an outbreak in Germany in May 2011 that infected over 3000 people and resulted in 50 deaths (Frank et al., 2011). In addition, 19 people in six states in the United States were infected with the shiga toxin-producing *E. coli O121* (CDC, 2014). In the present study, the prevalence rates of *E. coli* were 20%, and 60% in kareish cheese samples collected from grocery stores street vendors, respectively (Figure 2). Likely, Hassan and Elmalt (2008) identified toxigenic *E. coli* from retailed kareish cheese in Qena city at 47.8%. Furthermore, Ombarak et al. (2016) found enteropathogenic and enterohemorrhagic *E. coli* in 74.5% of kareish cheese retailed in Egypt. In addition, Hussein et al. (2019) found *E. coli* in 16% of kareish cheese sold in Menoufia Governorate, Egypt. Globally, De Campos et al. (2018) showed that 19.05% of Minas cheese, which is commonly consumed in Brazil, was contaminated with *E. coli*.

Further serological identification of the recovered *E. coli* isolates revealed that *E. coli O26:H11* had the highest prevalence at 30% (12 out of 40 recovered isolates), followed by *E. coli O2:H6* at 25% (10 out of 40 recovered isolates), *E. coli O55:H7* at 20% (8 out of 40 recovered isolates), *E. coli O78:H-* at 12.5% (5 out of 40 recovered isolates), *E. coli O111:H4* at 7.5% (3 out of 40 recovered isolates), and *E. coli O119:H* at 5% (2 out of 40 recovered isolates) (Figure 3). Similarly, de Campos et al. (2018) could identify *E. coli O127, O73:H12,* and *O64474:H8* from Minas cheese in Brazil. In Egypt, Hussein et al. (2019) identified eight *E. coli* serotypes from Kariesh, namely, *E. coli O26: H11, O91: H21, O111: H2, O103: H2, O125: H21, O171: H2, O86:H-, and O119: H6*. Furthermore, Elafify et al. (2022) could recover nine *E. coli* serotypes from Kareish cheese retailed in Egypt. The recovered *E. coli* serotypes were *O17:H18, O26:H11, O55:H7, O111:H2, O114:H4, O119:H6, O121:H7, O128:H2,* and *O159*.

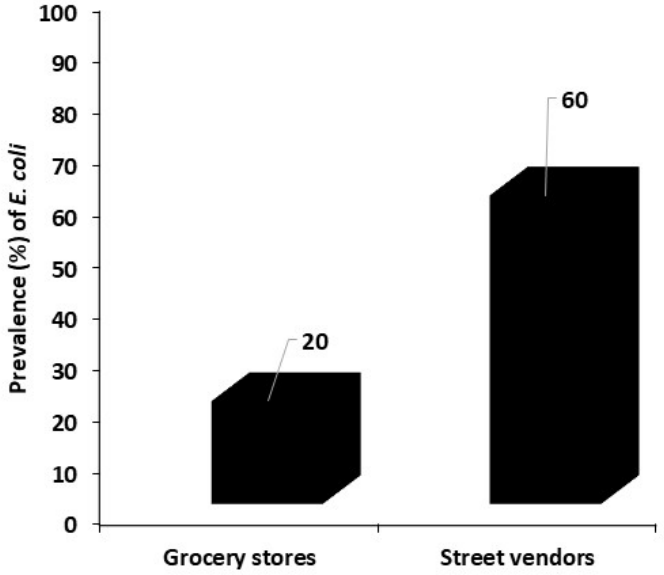

Figure 2: Prevalence rates (%) of *E. coli* in the examined kareish cheese in the present study.

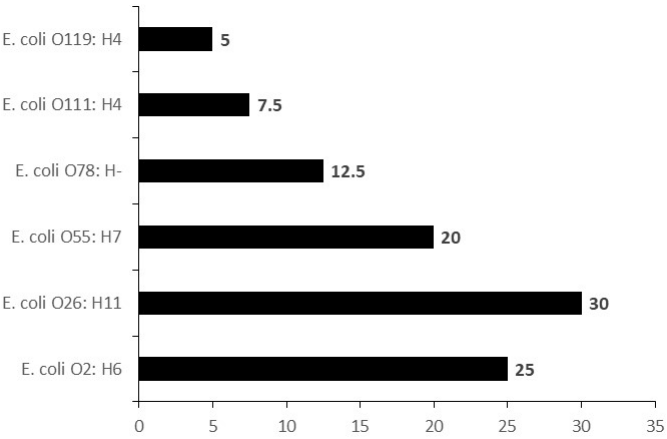

Figure 3: Prevalence rates (%) of different *E. coli* serotypes.

Shiga toxin producing *E. coli* (STEC) strains have been implicated in a number of foodborne diseases with deadly consequences in recent years (Chaleshtori et al., 2017). Their virulence is linked to their ability to produce shiga toxins (*stx1* and *stx2*), which play a key role in bacterial

adhesion to the intestinal epithelium of host cells, resulting in clinical signs (Assumpção et al., 2015). All isolated *E. coli* in the present study harbored *16S rRNA* specific gene for *E. coli*. The expression of shiga toxin coding genes indicated that *E. coli O2:H6, E. coli O111:H4,* and *E. coli O119:H* harbored only *stx2*. *E. coli O26:H11* and *E. coli O55:H7* harbored both *stx1*, and *stx2*; however, *E. coli O78:H-* did not harbor any of the tested genes (Figure 4). Non-*O157 E. coli* serogroups such as *O26, O103*, and *O111* have been found to be the most common food poisoning pathogens, particularly *O26*, which can cause a wide spectrum of illnesses in humans (Dambrosio et al., 2007). *E. coli* carrying shiga toxin coding genes was also isolated from a raw ewe's milk cheese from Spain (Caro and García-Armesto, 2007). Besides, Elhadidy and Mohammed (2013) isolated shiga toxin producing *E. coli* from kareish cheese sold in Egypt, including serotypes *O22:H8, O26:H11, O86:H21, O103:H2, O113:H21,* and *O146:H21*. Additionally, Hussein et al. (2019) identified eight *E. coli* serotypes that produce shiga toxins (*stx1*, and *stx2*) in kareish cheese. Many cases of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura have been linked to STEC (Karch et al., 2005).

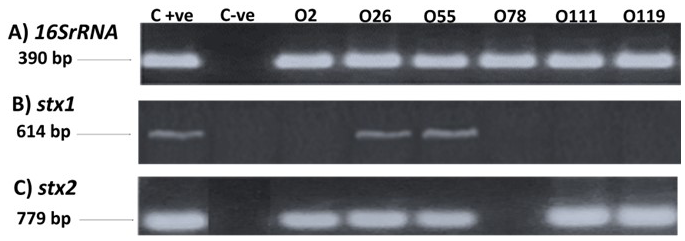

Figure 4: DNA expression of 16S rRNA, and shiga toxins coding genes (*stx1* and *stx2*) in the identified *E. coli* serotypes isolated from retailed Kareish cheese in the present study.

The inhibitory activities of KS and *Bifidobacterium animalis subsp. lactis* on the growth of *E. coli O26* using kareish cheese as a food substrate over three weeks of preservation at 4°C were further screened. The obtained results showed that KS at 0.15% had achieved reduction rates of 30.53% on the 7th day, 25.94% on the 14th day, and 22.86% on the 21st days of refrigeration, respectively. While, these rates were 35.04%, 30.42%, and 25.02% when KS was used at 0.3%. *Bifidobacterium animalis subsp. lactis* alone caused reduction on *E. coli O26* count at of 24.42% on the 7th day, 20.33% on the 14th day, and 18.23% on the 21st days of refrigeration, respectively. Combined effects of KS 0.15%, and *Bifidobacterium animalis subsp. lactis* caused comparatively higher reduction rates on *E. coli O26* count reaching to 40.88% on the 7th day, 32.85% on the 14th day, and 27.41% on the 21st days of refrigeration, respectively. Interestingly, the highest reduction rates were 55.66%, 44.94%, and 32.86% when KS 0.3% was combined with *Bifidobacterium animalis subsp. lactis* (Table 2). All examined samples were apparently normal with no visible sensory alterations (data are not shown). In agreement with the obtained results of the present study. In agreement with the obtained results of the present study, Abu-Ghazaleh (2010) reported that a combination of low pH and potassium sorbate significantly reduced growth and caseinase production by *E. coli O28*. In addition, *Bifidobacterium animalis subsp. lactis* had strong antibacterial activity against *Pseudomonas aeruginosa, Listeria innocua,* and *Salmonella Enteritidis* using whey cheese as a substrate (Madureira et al., 2011). To the best of our knowledge, this is the first report to investigate the inhibitory effects of a combination made from KS, and *Bifidobacterium animalis subsp. lactis* against *E. coli* using kareish cheese as a substrate.

Table 2: Inhibitory effects of potassium sorbate and *Bifidobacterium* on *E. coli O26*-artificially inoculated to kareish cheese and preserved for 3 weeks at 4°C.

Day	Control	KS 0.15%	KS 0.3%	KS 0.15% + Bifidobacterium	KS 0.3% + Bifidobacterium	Bifidobacterium
7	0	30.53	35.04	40.88	55.66	24.42
14	0	25.94	30.42	32.85	44.94	20.33
21	0	22.86	25.02	27.41	32.86	18.23

CONCLUSIONS AND RECOMMENDATION

The obtained results of the present study demonstrated occurrence of shiga toxin *E. coli* contamination in retailed kariesh cheese at Mansoura city, Egypt. Kareish cheese purchased from street vendors had higher *E. coli* contamination compared with that obtained from grocery store. Interestingly, the use of potassium sorbate and *Bifidobacterium animalis subsp. lactis* had clear antibacterial activities against *E. coli O26* as demonstrated in an experimental trial. Therefore, strict hygienic measures should be adopted during processing and handling of kariesh cheese with selection of raw milk with high bacterial quality. The use of probiotics such as *Bifidobacterium animalis subsp. lactis* with KS in dairy industry is also highly recommended.

ACKNOWLEDGEMENT

We would like to thank all members at Animal Health Research institute, Mansoura branch for their kind support, and valuable guidance during offered during conducting this work.

NOVELITY STATEMENT

To the best of our knowledge, this is the first report to

investigate the inhibitory effects of a combination made from potassium sorbate, and *Bifidobacterium animalis subsp. lactis* against *E. coli* using kareish cheese as a substrate.

AUTHOR'S CONTRIBUTION

All authors contributed equally.

Conflict of interest

The authors have declared no conflict of interest.

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