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ANTIMICROBIAL RESISTANCE, BIOFILM FORMATION PATTERNS AND VIRULENCE FACTORS PROFILES OF *ESCHERICHIA COLI* ISOLATED FROM YOGHURT AND KARIESH CHEESE IN ZAGAZIG CITY, EGYPT

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ABSTRACT

In the present study, Fifty random samples of each small-scale (plain yoghurt and kariesh cheese) were collected randomly from different dairy shops and markets in Zagazig city for isolation and identification of *Escherichia coli* which is considered a good indicator of faecal contamination and a major cause of food poisoning. Identification was done microscopically and biochemically by different biochemical tests (IMVIC).The incidence of *E. coli* in yoghurt and kariesh cheese samples were 36% and 50%, respectively. Also, the serological identification of *E. coli* isolates revealed that O26 is the most predominant serogroup by percentagesof27.77% and 28% in both yoghurt and kariesh cheese samples, respectively. Because of its rising resistance to antibiotics, *E.coli* represents a real threat to human health. Antimicrobial susceptibility testing (AST) was done by disc diffusion method against 10 antimicrobials and the results revealed that *E. coli* isolates were highly resistant to amoxicillin-clavulanate, ampicillin, Cefotaxime and Ceftazidime, and were highly sensitive to chloramphenicol, Ciprofloxacin and tetracycline. In addition, 83.72%of *E. coli* isolates showed multi drug resistance (MDR).Bacterial adhesion to food surfaces and the formation of biofilmisa source of food contamination that has an impact on food safety and industry. Micro titer plate assay was used for testing biofilm formation and revealed that 69.77% of *E. coli* isolates were non-biofilm producers, 9.30% were weak biofilm producers, 20.93%were moderate biofilm producers and none of isolates was strong biofilm producers.
Virulence genes of *E. coli* isolates were identified and characterized by a multiplex PCR assay. The results showed that among the target genes, stx1was most frequently detected by a percentage of81.82%.

Keywords: *E. coli*, Kariesh, Antimicrobial resistance, Biofilm, Multiplex PCR

Introduction

Kariesh is the most popular soft cheese in Egypt. It is an acid cheese made from skimmed cow's and buffalo's milk, apparently made only on farmsteads. Initially, it is made from Laban Khad (i.e. fermented buttermilk) or from sour defatted milk, Laban Rayeb. The latter is prepared from fresh whole milk placed in earthenware jars and left undisturbed, the fat rises to the surface and the partly skimmed milk beneath sours. After 24–36 h, the cream layer is skimmed off and the clotted skimmed milk (Laban Rayeb) is poured on to reed mats or into small cheese moulds. After a few hours, the ends of the mat are tied and some whey squeezed out. The pressed curd is permitted to drain further and the squeezing process repeated until the desired texture is obtained; the cured is then cut into pieces and salted (Phelan *et al*., 1993).

The traditional method of production under unsatisfactory conditions affords many opportunities for microbial contamination. Also, this product is sold uncovered without a container, thus the risk of contamination by different types of spoilage and pathogenic microorganisms is very high Therefore, it can be considered as a nice medium for the improvement of different sorts of pollution and pathogenic microorganisms (Yousef, 2007; Dawood *et al*., 2009).

Unfortunately, the majority of the population in Egypt's, especially in rural families, still consume raw dairy products without pasteurization including traditional Egyptian cheese as Kariesh (most popular soft white cheese) with a general believe that pasteurization would drastically affect the milk quality (Zeinhom and Abdel-Latef, 2014)

Kariesh cheese becomes very popular because of its remarkable health quality as the only known relatively fat-free cheese consumed by the Egyptians. It is often recommended for persons suffering from obesity, cholesterol and heart disease (Fayed *et al*., 2014).

Yoghurt is a functional dairy product consumed worldwide thanks to its positive effect on human health. However, if the selection of raw materials is not well controlled, if good production practices are not used, especially in small-scale production, or if storage conditions are unfavorable, yoghurt spoilage occurs in a short time, leading to an unacceptable product for consumers (Fleet, 1990, 1992; Mataragas *et al*., 2011; Gougouli *et al*., 2011).

It is possible that milk and dairy products can be contaminated with a variety of microorganisms from different sources (Oliver *et al*., 2005). *Escherichia coli* is one of these microorganisms, which is a normal inhabitant of large intestine in human and warm-blooded animals. The main source of *E. coli* in raw milk and milk products is fecal contamination during milking process along with poor hygienic practices. Therefore, *E.coli* is generally used as a reliable indicator of direct or indirect fecal contamination and

the possible presence of enteric pathogens in raw milk and raw dairy products (Kornaki and Johnson, 2001).

E.coli includes a variety of different types that range from a virulent commensally strains that are present in the normal intestinal flora to highly virulent strains that cause a variety of severe infections in both humans and animals. The pathogenicity of *E.coli*is considered to be mainly determined by specific virulence factors such as adhesions, invasions, toxins and capsule *E. coli* associated with human diseases can be broadly divided into two categories, intestinal and extra intestinal infections, based on their distinct virulent properties and their clinical symptoms. E. coli causing intestinal infection is generally called diarrhea genic *E. coli* (DEC), which can further be subdivided into at least six categories, such as, entero pathogenic *E. coli* (EPEC), entero hemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), entero toxigenic E. coli (ETEC), entero invasive *E. coli* (EIEC), entero aggregative E. coli (EAEC), and diffusely adherent *E. coli* (DAEC) based on their distinct pathogenic mechanisms and presence of pathotype-specific virulence genes (Kaper *et al*., 2004).

Antibiotic resistance is one of the upcoming crucial concerns to global health care (Schjørring and Krogfelt, 2011). Moreover, the spread of antibiotic-resistant bacteria stands as one of the most dangerous global health care issues to human health (Paterson *et al*., 2004). This resistance among bacteria is now recognized to have a considerable effect in rising morbidity, mortality, and costs associated with major public health problems (Bunner *et al*., 2007). Increasing antimicrobial resistance problems do not only affect developed countries but also affect non-industrialized countries, where antibiotics resistance issues are more challenging, because of the lack of well-organized antimicrobial usage policies and the need for optimal hygiene situation and contagion, control practices (Rossolini and Thaller, 2010). The use of antimicrobials in animal farm does not only induce the resistance in the pathogens but also produce resistance in the commensal bacteria of individuals (Piddock, 1996; VanDenBogaardand Stobberingh, 1999). It is clear that genetic exchange system and the ability of *E. coli* to transfer and propagate genes between humans and animals may make it a significant vector for the spread of rapidly dispersed resistance genes (Eckburg *et al*., 2005; Wright, 2007).

A biofilm is defined as an organized collection of surface attached microbial communities of cells that are embedded into a self-produced exopolymeric matrix mainly composed of proteins, polysaccharides and sometimes DNA (Hall-Stoodley *et al*., 2004). The formation of biofilm is a result of different stress condition(s) where biofilms acts as a defense mechanism enhancing the survival rate of the microorganism. They play an important role in microbial pathogenesis and persistence as well as serve as grounds for genetic exchanges. It acts as as shield protecting the microbial community from action of various antimicrobial agents such as antibiotics, preservatives, chemical sanitizers, thermal treatment etc. that are traditionally used in food industry, thus making them robust and hard to eradicate (Monte *et al*., 2014). The biofilm formation for many bacterial species including *E. coli* occurs as early as two hours and they can survive up to ten years in food industries despite the regular cleaning and sanitation treatment (Corcoran *et al*., 2014).

Polymerase chain reaction (PCR) method, particularly multiplex PCR, being a rapid and effective gene detection assay, is increasingly popular in identification of various bacteria (Blanco *et al.*, 2005; Costa *et al.*, 2010). Multiplex PCR incorporates simultaneous amplification of more than one target sequence by including more than one set of primers in the same reaction mixture. It has been widely used in the differentiation of *E.coli*pathotypes, and various multiplexes have been developed (Pass *et al*., 2000; Vidal *et al*., 2005 and Müller *et al*., 2007).

Each *E. coli* subgroup has specific genes responsible for coding virulence factors that interfere with host physiology. Among the most important genes, stx has been associated with *E. coli* strains producing Shiga toxin (STEC).STEC are one of the most important strains, mainly due to their ability to cause an array of diseases (Peresi *et al*. 2016; Li *et al*. 2016). The genes st (enterotoxin heat-stable) and lt (enterotoxin heat-labile) have been associated with enterotoxigenic*E. coli* (ETEC), the most common cause of childhood diarrhea (Canizalez-Roman *et al*. 2013).

Enteropathogenic *E.coli* (EPEC) is one of the major pathogens which is responsible for infantile diarrhea in developing countries. This pathotypeis determined by the presence of the locus of enterocyte effacement (LEE) region encoding for the intimin (*eae*gene) and the lack of *stx* genes.This last trait is also used to distinguish strains of EPEC from STEC. EPEC adherence factor (EAF) plasmid-encoded bundle-forming pilus (bfp) gene is an index for the classification of EPEC. BFP-positive isolates are termed typical EPEC (tEPEC), whereas BFP-negative ones are classified as atypical EPEC (aEPEC) (Canizalez-Roman *et al*., 2013). This protein called intimin is responsible for intimate attachment to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (Jerse*et al*., 1990).

Therefore, the objective of this study was to isolate *E.coli* of Kariesh cheese and yoghurt, as well as to evaluate their antimicrobial susceptibility, their capacity to form biofilms, to detect several genes related to their pathogenicity.

Materials and Methods

Samples collection and preparation

Fifty random samples of each small-scale (plain yoghurt and kariesh cheese) were collected from different dairy shops and markets in Zagazig city, Sharkia Governorate, Egypt under hygienic condition during the period from February to August 2020. Approximately not less than 100 gm. of kariesh cheese samples and retail packages of yoghurt samples were transferred to the laboratory of Food Control Department, Faculty of Veterinary Medicine, Zagazig University in an insulated icebox at 4°C with a minimum of delay to be examined microbiologically.

Isolation and identification of *E. coli*

For yoghurt samples, 11 ml of well-mixed samples were aseptically transferred into a sterile bottle containing 99 ml of sterile buffered peptone water (BPW) to make a dilution of 1:10 and incubated at 37 °C for 24hrs. and for kariesh samples,11 grams of each prepared samples were transferred to a sterile mortar warmed to 40-45°C and 99 ml of 2% sodium citrate solution warmed to 40°C were added

then thoroughly mixed for 2 minutes to emulsify the sample and make a dilution 1:10 and incubated at 37 °C for 24hr. A loopful of the BPW enrichment was streaked on Eosin Methylene Blue agar (EMBA; Oxoid) and then incubated at 37 °C for 24hrs. The agar plates were examined for growth of *E. coli*. To get pure cultures, a single colony was further sub-cultured on EMBA according to (Ngaywa *et al*., 2019)

Films of pure suspected cultures were stained with Gram's stain and examined microscopically. Cultures analyzed by using the following biochemical tests:

Indole Motility Test, Methyl Red, Voges- Proskauer Tests and Simmons Citrate Agar Test according to APHA. (2004)

Serological identification of isolated *E. coli*

The isolates were serologically identified by slide agglutination test according to Kok *et al*. (1996) using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) to identify O antigen.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was done using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid, Hampshire, England) as described by (CLSI, 2017). With an inoculum equivalent to 0.5 McFarland standards. Incubation was done at 35±2°C, ambient air, for 16–18 hrs. The following 10 antimicrobials were tested:

Amoxicillin/clavulanic acid, ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, kanamycin, Nalidixic acid, streptomycin and tetracycline

The sensitivity/resistance was interpreted based on the diameter zone of inhibition, inclusive of margins, following the Clinical Laboratory Standards Institute guidelines (CLSI, 2017). The isolates were classified as intermediate, susceptible or resistant (CLSI, 2017). In addition, we calculated the number of *E. coli* isolates that were considered multidrug resistant (MDR), that is, resistant to three or more antimicrobials of different classes (Frye and Fedorka-Cray, 2007) and the Multiple Antimicrobials Resistance index (MAR) for each *E. coli* isolate according to the methodology described by (Krumperman, 1983). This index shows the relationship between the number of resistant *E. coli* to each antimicrobial and the total number of classes tested.

Biofilm formation

The biofilm formation test was performed as described by Stepanovic *et al*. (2000), where *E. coli* strains were incubated in TSB broth at 37 ± 1 °C for 24 hrs. Then, aliquots were diluted in a new tube until reaching the turbidity of one on the McFarland scale. Subsequently, 200 µL of each suspension in triplicate were inoculated into a 96-well sterile polystyrene micro plates. In the first three wells, only sterile broth was added as a negative control. The plates were incubated without air circulation at 37 ± 1 °C for 24 h. Next, the bacterial suspensions were aspirated from each well and washed three times with 250 µL of sterile 0.9% sodium chloride solution. The bacterial cells were then fixed with 200 µL of methanol (PA) for 15 min and dried at room temperature. Later, they were stained with 200 µL of 2% Hucker crystal violet for 5 min, washed in running water and dried at room temperature. Re-solubilization was performed with 160 µL of 33% glacial acetic acid followed by reading using a spectrophotometer (Thermo Scientific® Multiskan GO) at 570 nm. After reading, the optical density value of each strain (OD) was obtained by calculating the arithmetic mean of the absorbance of the three wells, and this value was compared to three standard deviations above the mean of the absorbance of the negative control (ODC). The strains were classified in four different categories as follows: A) non-biofilm producer (OD ≤ ODC); B) weak biofilm producer (ODC < OD ≤ 2 × ODC); C) moderate biofilm producer (2 × ODC < OD ≤ 4 × ODC); and D) strong biofilm producer (4 × ODC < OD) (Stepanovic *et al*., 2000).

PCR protocols

Bacterial DNA used for PCR analyses was prepared with a genomic DNA purification kit (Wizard® Genomic DNA Purification kit, Promega Corporation, USA) used according to the manufacturer's recommendations. Alternatively, bacterial DNA was extracted by a boiling procedure. Single colonies were cultured overnight at 37ºC in 1.5 ml tubes containing 1 ml LB broth. Each bacterial suspension was centrifuged for 15 min at 12,000 rpm, and the pellet was resuspended in 200µl double distilled water. After 10 min of boiling in a water bath and centrifugation for 15 min at 12,000 rpm, the supernatant was used as template DNA for PCR assays. All strains were evaluated by multiplex PCR for identification of six virulence genes. The PCR primers were presented in Table 1. We consulted the references about the conditions for DNA template amplification and further optimized for present multiplex PCR procedures. Bacterial DNA was amplified in the PCR reaction mixtures as follows. Each reaction system had a final volume of 50µl and contained 2.0µl of 10×*EX Taq* buffer, 4µl of 25 mM MgCl2, 4µl of dNTPs (each 2.5 mM), 1.25U of *EX Taq*DNA polymerase, 20-40µM of each primer, and 2µl of the DNA template. All reagents were the products of TaKaRa Biotechnology (Dalian) Co., Ltd,China). The mixtures were preheated at 94ºC for 1 min before submitted to recycling step. The amplification conditions for the multiplex PCR assays were 30 cycles at 94 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 1 min. A final extension step was performed at 72 ºC for 10 min. The PCR products were kept at 4 ºC until removed and were separated in agarose gel (2% w/v) electrophoresis and visualized under UV light after staining with a nucleic acid stain (GoldView TM, SBS Genetech, Beijing, China). According to (Huasai *et al*., 2012)

Primers: The PCR primers used in this study (Table 1) corresponded to sequences were synthesized by metabion international AG, (Germany).

Results and Discussion

Raw milk and cheese made from raw milk can be a major source of potentially harmful bacteria to human, such as pathogenic *Escherichia coli*(Oliver *et al*., 2005). Public health hazards associated with consumption of raw milk and raw milk products, and related foodborne disease outbreaks have been reported in the world (De Buyser *et al*., 2001; Oliver *et al*., 2005). Although raw milk and raw milk products have caused many illnesses and even deaths (FDA, 2012), their marketing and consumption widely exist in many countries including Egypt (Ayad *et al*., 2004; El Deeb *et al*., 2012). Kariesh cheese, an acid coagulated fresh unripened soft cheese. Normally made at home and sold from door-to-door or sold in local markets (FAO, 1990). Sodifferent reports highlighted the contamination of Kariesh cheese with

E. coli acting as a public health hazard (Ombarak *et al*., 2016).

Illness caused by pathogenic *E. coli* can range from self-limited watery diarrhea to life threatening manifestations such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombocytopenic purpura and may lead to death (Alexander and Prado, 2003).

The results in our study presented in Table (2) revealed that, the incidence of *E.coli* in yoghurt and kariesh cheese samples was (36%) and (50%) respectively. Nearly similar results were obtained by Darma *et al*. (2016) who found the incidence of *E.coli* in yoghurt samples was (26.67%). Lower results were obtained by Chaleshtori *et al*. (2017) whofound that the incidence of *E. coli* in yoghurt samples was (10%). The highest frequency distribution of *E. coli* in small-scale yoghurt samples was (46.7%) was obtained by El-Malt *et al*. (2013)

El-nahas *et al*. (2015) reported the same results where he found *E. coli* in kariesh samples by a percentage of 50%. Nearly similar results were obtained by Hassan and Elmalt (2008) where they isolated *E.coli* in 11 (47.8%) out of 23 Kariesh cheese samples. While lower results were reported by Hussien *et al*. (2019) where they found that, the incidence of *E.coli* in kariesh samples was (16%). Higher results were reported by Ombarak *et al*. (2016) where the prevalence of *E. coli* in Kariesh cheese was (74.5%).

Problems found in dairy products, like cheese and yoghurt, may originate from high microbial counting. It can be due raw milk, contamination in the started culture or curd and poor hygiene in the utensils, equipment and environment used in the production (Buzi *et al*., 2009; Rodrigues *et al*., 2010; Belli *et al*., 2013; Vasek *et al*., 2013). Furthermore, these results suggested a general lack of cleanliness in handling and improper storage (Shojaei and Yadollahi, 2008).

Table (3) illustrated the serological identification of isolated *E. coli*. The strains of *E. coli* isolated from examined yoghurt samples were O25, O26, O55, O86, O114, O125, O127, O158 and O168 by percentages of (5.56%), (27.77%), (11.11%), (5.56%), (5.56%), (16.66%), (11.11%), (11.11%) and (5.56%), respectively. While that of kariesh samples were O25, O26,O44, O55, O78, O114, O119, O125, O127, O158 and O168 by percentages of (8.00%), (28.00%), (4.00%), (8.00%), (4.00%), (8.00%), (4.00%), (16.00%), (4.00%), (8.00%) and (8.00%), respectively.

Abike *et al*. (2015) found that some *E. coli* strains isolated from raw milk, cheese and yoghurt were belonged to serogroups (O) include O26 (2), O55 (3), O86 (3), O114 (1), O119 (1), O127 (5). Serotyping of the isolated strains of *E. coli* from small scale yoghurt samples achieved by Hassan *et al*. (2021) revealed presence ofO55 (60%) and O125 (40%).

Serological identification of *E.coli* was carried out by Abd El-Maaboud (2014)who isolated *E. coli* O26 from Kariesh cheese samples. Gaffer *et al*. (2019) isolated *E. coli* O127 from kariesh cheese samples

El-nahas *et al*. (2015) found that the isolated strains of *E. coli* from Kariesh cheese samples were O26, O55, O114, O119 and O127.

The multi-drug resistance in pathogens has been recognized as a severe public health hazard globally (Interagency Coordination Group on Antimicrobial Resistance (IACG), 2019). A possible mechanism for being antibiotic resistant is the ability of bacterial subpopulation to transform into a biofilm-state with unique protective phenotype similar to spore-formation (Ito *et al*., 2009). The development of antimicrobial resistance among the pathogenic bacteria poses a problem of high concern. Table (4) showed that *E. coli* isolates were highly resistant to amoxicillin-clavulanate, ampicillin, cefotaxime and ceftazidime with percentages of 83.33%, 83.33%, 100.00% and 100.0%. 84.00%, 84.00%, 100.00% and 100.00% in yoghurt and kariesh cheese samples, respectively. In addition, the isolates were highly sensitive to chloramphenicol, ciprofloxacin and tetracycline with percentages of 100.00%, 100.00% and 88.89%.

100.00%, 100.00% and 84.00% in yoghurt and kariesh cheese samples, respectively.

Ombarak *et al*. (2018) found that (1.4%) of *E.coli* isolates from raw milk and raw milk cheese in Egypt were resistant to ciprofloxacin. Also Fallah *et al*. (2021) recorded very low resistance (12.1%) was observed for ciprofloxacin in the STEC group isolated from food products.

Hassan and Elmalt (2008) revealed that most frequent resistance of *E.coli* isolates from raw milk and kariesh cheese was observed to the following antimicrobials: nalidixic acid (42.9%), ampicillin (32.7%), tetracycline (22.4%) and ciprofloxacin (4.1%).

Dehkordi *et al.* (2014) reported that *E.coli* strains from fermented dairy product exhibited the highest level of resistance to tetracycline (84%), followed by ampicillin (38%) and streptomycin (36%).

El-Baz (2019) found that all detected *E.coli* isolates from fresh soft cheese showed the maximum resistance (100%) against streptomycin followed by Nalidixic acid (80%)

Chaleshtori*et al*. (2017) reported that STEC *E. coli* isolates from yoghurt, cheese and ice cream showed highest antibiotic sensitivity levels to chloramphenicol and ciprofloxacin respectively by percentages of (36.36%) and (18.18%). And all of the *E. coli* isolates were resistant to amoxicillin/clavulanic acid, ampicillin, tetracycline and kanamycin.

Table (5) showed the multiple antibiotic resistance (MAR) index, an index describing the resistance of the isolates to different antibiotics; It was calculated for each isolate as described in the materials and methods section. Values for the MAR index were 0.2 (i.e., an isolate being resistant to two out of the 10 antibiotics tested), 0.4 (resistance to 4 out of the 10 antibiotics tested), 0.5 (resistance to 5 out of the 10 antibiotics tested) and 0.6 (resistance to 6 out of the 10 antibiotics tested) with percentages of 16.28%, 37.21%, 30.23% and 16.28%, respectively.

According to Newell *et al*. (2010), the use of antimicrobials for human therapy, animal health or plant health purposes can select for the emergence of resistance and promote the dissemination of resistant bacteria and resistance genes. Furthermore, commensal *E. coli* can be a source of resistance genes for pathogenic *E. coli* strains.

The increasing prevalence of MDR *E. coli* among community isolates is challenging because those isolates can occupy multiple niches, including human and animal hosts, thereby acquiring or transmitting antimicrobial resistance genes horizontally and vertically (Levy and Marshall, 2004)

In the present study, (83.72%) of *E. coli* isolates showed resistance to more than three classes of antimicrobials (Table 5).

Many of the resistances in MDR *E. coli* are located on plasmids, which increases the possibility of clonal dissemination of these resistance classes in the community (van der Donk *et al*., 2012; Williamson *et al*., 2013; Chen *et al*., 2014)

Biofilm formation is one of the most important virulence factors that protect microbes from antimicrobial drugs and treatments (Olsen, 2015). The ability of spoilage and pathogenic bacteria to adhere onto food surfaces and form biofilms serve as a persistent source of food contamination threatens food safety and causes huge losses to the food industry (Tezel and Şanlıbaba, 2018). Therefore combating microbial biofilms is a major challenge to food industry (Hall and Mah, 2017). *E. coli* is one of many bacteria that can switch between planktonic form and biofilm form. Several reasons can explain the need for bacteria to create biofilm, in this way bacteria can avoid being washed away by water flow, cells in biofilms are about 1000 times more resistant than their planktonic (Jefferson, 2004).

Table (6) revealed that (69.77%) of *E.coli* isolates were non-biofilm producers, (9.30%) were weak biofilm producers, (20.93%) were moderate biofilm producers and none of isolates was strong biofilm producers.

Nearly similar studies of Milanov *et al*. (2015) recorded that 19 (76%) isolates of *E.coli* did not produce biofilm, and 6 (24%) were classified as weak biofilm producers.

Bhardwaj *et al*. (2021) reported that out of 32*E.coli* isolates tested, 4 were strong formers, 11 were moderate, 15 were weak producers and 2 non-producers.

Numerous studies have shown that *E. coli* possess an ability to attach and form biofilm on varied surfaces including food contact surfaces such as stainless steel, PVC, polystyrene, polypropylene, glass etc. and resist chemical, pressure and heat treatments used in food industries (Galie *et al*., 2018; Carter *et al*., 2016). The high degree of persistence and resistance facilitates proliferation of biofilm-forming *E. coli* into food product thereby making them a leading cause of food-borne outbreaks globally (Galie*et al*., 2018).

However, most sectors of food industries are vulnerable to adverse effect of biofilms, animal food sector encompassing the dairy and meat, takes the hardest hit. The heavy usage of antibiotics in animal industry for disease prevention has resulted in development of multi-drug resistant (MDR) strains of different pathogenic bacteria which are hard to eradicate and difficult to alleviate clinically (Casburn-Jones and Farthing, 2004).

Enterotoxigenic *Escherichia coli* (ETEC) are a common cause of acute diarrheal disease in both humans and farm animals (Kotloff *et al*., 2013; Steffen *et al*., 2015; Liu *et al*., 2016) Children and travelers within ETEC endemic regions are the main populations that suffer from acute diarrheal illnesses (Field, 2003; Thapar and Sanderson, 2004).

ETEC release heat-labile (LT) and/or heat-stable enterotoxins, that act upon intestinal enterocytes by disrupting the electrolyte homeostasis, resulting in fluid loss and eventually secretory diarrhea (Kaper *et al*., 2004).

In the present study, Table (8) showed that only 3 isolates were positive to lt gene by a percentage of (13.64%) and none of them was positive to st gene.

Stx is one of the most important virulence factors in *E. coli* because shiga toxin producing *E.coli* (STEC) may cause a life-threatening sequel such as hemolytic uremic syndrome or HUS and neurological disorder (Kaper *et al*., 2004).

We found that 18 isolates were positive to stx1 by a percentage of (81.82%) and none of them was positive to stx2 gene. (Table 8)

The eae gene is well-known virulence factor not only for EPEC and EHEC but also atypical EPEC in which eaeA gene alone but not bfp and EAF genes are present (Kaper *et al*., 2004). In addition, we found that eae gene was negative in all *E.coli* isolates. (Table 8)

Hussien *et al*. (2019) reported that 14 (87.5%), 11 (86%) and 4 (25%) of isolates contains stx1, stx2 and eaeA and virulence genes, respectively.

Frank *et al*. (1984) reported the presence of 3.2% of ETEC strains in milk and milk products. Results were obtained by Hassan and Elmalt (2008) showed that only one of the tested *E.coli*strains carried LT gene. Paneto *et al*. (2007) showed that only one *E.coli*isolate carried the lt gene while the st gene was not found.

Conclusion

Results obtained in this study indicated that Kariesh cheese and yoghurt represent health risks to consumers due to contamination by strains of *E. coli*, some of which have biofilm forming ability, some pathogenic genes and multidrug resistant strains. These risks explained due to the transmission of pathogenic or biofilm forming or multi resistance genes to other pathogenic microorganisms and/or commensal residents of the human gut, resulting in difficulties in the selection and use of appropriate therapeutic treatments. Monitoring the antimicrobial resistance, MAR indexing and evaluation of *E.coli* pathotypes is very important. Additionally, proper hygienic practices and the adoption of GMPs are needed to constrain strain dissemination throughout the entire food chain.

Table 1 : Primer sequence used in this study

Target gene	Primer name	Nucleotide sequence 5`-3	Amplicon Size (bp)	primer (pMol) in mix	Reference
lt	F R	´GGC GAC AGA TTA TAC CGT GC´ ´CGG TCT CTA TAT TCC CTG TT	450	5	Stacy-Phipps *et al*., 1995
st	F R	TT TTT CTT TCT GTA TTG TCT T ´CAC CCG GTA CAA GCA GGA TT	190	6.47	Stacy-Phipps *et al*.,1995
*stx*1	F R	CTG GAT TTA ATG TCG CAT AGT ´AGA ACG CCC ACT GAG ATC ATC	150	3.88	Paton *et al*.,1998
eae	F R	GAC CCG GCA CAA GCA TAA GC´ ´CCA CCT GCA GCA ACA AGA GG	384	3.88	Paton *et al*.,1998
*stx*2	F R	´GGC ACT GTC TGA AAC TGC TCC ´TCG CCA GTT ATC TGA CAT TCT G	255	2.5	Paton *et al*.,1998

Table 2 : Incidence of *E. coli* in examined yoghurt and kariesh cheese samples

Dairy products	No. of samples	Positive samples	
		No.	% of total
Yoghurt	50	18	36%
Kariesh	50	25	50%

Table 3 : Serological identification of *E. coli* strains isolated from yoghurt and kariesh cheese samples

Serogroup	Yoghurt		Kariesh	
	No.	%	No.	%
O25	1	5.56%	2	8.00%
O26	5	27.77%	7	28.00%
O44	0	0.00%	1	4.00%
O55	2	11.11%	2	8.00%
O78	0	0.00%	1	4.00%
O86	1	5.56%	0	0.00%
O114	1	5.56%	2	8.00%
O119	0	0.00%	1	4.00%
O125	3	16.66%	4	16.00%
O127	2	11.11%	1	4.00%
O158	2	11.11%	2	8.00%
O168	1	5.56%	2	8.00%
Total	18	100.00%	25	100.00%

Table 4 : Antibiogram pattern of identified *E. coli* isolates from yoghurt and kariesh cheese samples

Class of antimicrobial	Antimicrobial	Conc. (µg)	No. of isolates (%)					
			Resistant		Intermediate		Susceptible	
			Yoghurt	Kariesh	Yoghurt	Kariesh	Yoghurt	Kariesh
β - Lactams	Amoxicillin-clavulanate	20/10 µg	15 (83.33%)	21 (84.00%)	0 (0.00%)	0 (0.00%)	3 (16.67%)	4 (16.00%)
Penicillins	Ampicillin	10 µg	15 (83.33%)	21 (84.00%)	0 (0.00%)	0 (0.00%)	3 (16.67%)	4 (16.00%)
Cephalosporins	Cefotaxime	30 µg	18 (100.00%)	25 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
	Ceftazidime	30 µg	18 (100.00%)	25 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Phenicols	Chloramphenicol	30 µg	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	18 (100.00%)	25 (100.00%)
Fluoroquinolones	Ciprofloxacin	5 µg	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	18 (100.00%)	25 (100.00%)
Quinolones	Nalidixic acid	30 µg	0 (0.00%)	0 (0.00%)	15 (83.33%)	22 (88.00%)	3 (16.67%)	3 (12.00%)
Aminoglycosides	Kanamycin	30 µg	6 (33.33%)	8 (32.00%)	10 (55.56%)	15 (60.00%)	2 (11.11%)	2 (8.00%)
	Streptomycin	10 µg	3 (16.67%)	4 (16.00%)	10 (55.56%)	17 (68.00%)	5 (27.78%)	4 (16.00%)
Tetracyclines	Tetracycline	30 µg	2 (11.11%)	4 (16.00%)	0 (0.00%)	0 (0.00%)	16 (88.89%)	21 (84.00%)

Table 5 : Multi drug resistance pattern and multiple antibiotic resistance index of identified *E. coli* isolates

Dairy products (No. of isolates)	Multi drug resistance (MDR)		Multiple antibiotic resistance (MAR)							
			0.2		0.4		0.5		0.6	
	No.	%	No.	%	No.	%	No.	%	No.	%
Yoghurt (18)	15	83.33%	3	16.67%	7	38.89%	5	27.78%	3	16.67%
Kariesh (25)	21	84.00%	4	16.00%	9	36.00%	8	32.00%	4	16.00%
Total (43)	36	83.72%	7	16.28%	16	37.21%	13	30.23%	7	16.28%

Table 6 : Ability of *E. coli* strains isolated from yoghurt and kariesh samples to form biofilm

Dairy products (No. of isolates)	Degree of biofilm formation							
	None		Weak		Moderate		Strong	
	No.	%	No.	%	No.	%	No.	%
Yoghurt (18)	12	66.67%	2	11.11%	4	22.22%	0	0.00%
Kariesh (25)	18	72.00%	2	8.00%	5	20.00%	0	0.00%
Total (43)	30	69.77%	4	9.30%	9	20.93%	0	0.00%

Table 7 : Virulence genes profiles of positive *E. coli* isolates (n=22)

Product / Gene	Yoghurt											Kariesh										
	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
stx1	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
stx2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
eae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
lt	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	
st	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 8 : Distribution of target virulence genes of positive *E. coli* isolates (n=22)

Gene / Product	No. of *E.coli* isolates	stx1 positive isolates		stx2 positive isolates		eae positive isolates		lt positive isolates		st positive isolates	
		No.	%	No.	%	No.	%	No.	%	No.	%
Yoghurt	11	9	81.82%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Kariesh	11	9	81.82%	0	0.00%	0	0.00%	3	27.27%	0	0.00%
Total	22	18	81.82%	0	0.00%	0	0.00%	3	13.64%	0	0.00%

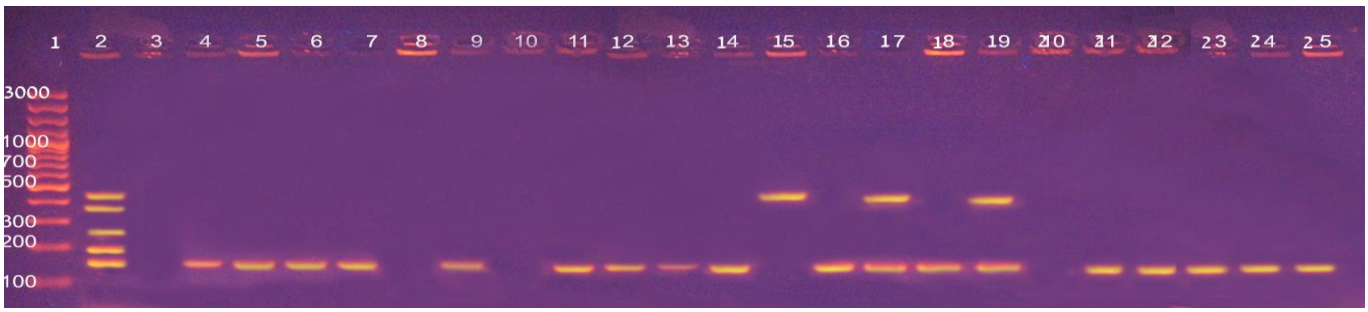

Photo 1 : polymerase chain reaction (PCR) product Multiplex

Lane M: 100bp DNA ladder Lane +ve: Control Positive Lane-ve: Control Negative Lane 4-25: sample

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