Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Isolation and Genotypic Identification of Some Spoilage and Pathogenic Microbes from Yogurt

Mai, A. Fetouh[1*], Ekbal, M. Adel[2], Hend, A. ElBarbary[2], Ahmed, A. A. Maarouf[1]

[1.] *Animal Health Research Institute, ARC, Egypt*
[2.] *Food Hygiene and Control Department, Faculty of Veterinary Medicine, Benha University, Egypt*

ARTICLE INFO

Keywords

Yogurt
Bacillus cereus
E. coli,
Enterococcus faecalis
Fungi

Received 04/07/2022
Accepted 06/08/2022
Available On-Line 09/10/2022

ABSTRACT

This study was planned to evaluate the prevalence of *E. coli*, *E. faecalis*, *B.* cereus, mold and yeast in a total of 200 yogurt samples (Commercial and Balady yogurt types, 100 of each) which were be taken from different supermarkets and dairy shops at Qalyubia Governorate, Egypt. Bacteriological results revealed that, 27(13.5%) isolates were recovered from 200 yogurt samples, 8 (4.0%) *E. coli* (two strains of *E.* coli O86a H4, 3 strains of *E.* coli O119 H4, and 3 strains of *E.* coli O153 H2); 11(5.5%) *E. faecalis* and 8 (4.0%) *B. cereus.* Meanwhile, mycological results cleared that, 80 fungal isolates represented as: 37 molds (19 *Aspergillus niger*, 5 *Aspergillus fumigatus*, 5 Mucor spp., 3 *Aspergillus flavus*, 3 Penicillium spp. and 2 Rhizopus spp.) and 43 yeasts (23 *C. albicans*, 12 Rhodotorula spp. and 8 *C. tropicalis*) were isolated from both brand and balady yogurt. PCR results showed that, *pho*A virulence genes was detected in *E. coli* strains and *stx*1 gene was detected in one strain only, meanwhile, *stx*2 gene failed to be amplified in examined strains; the16SrRNA, *esp* and *gel*E genes were detected in all *E. faecalis* strains and the *gro*EL, *cyt*K and *nhe* genes were detected in all *B. cereus* strains. In conclusion, some of the examined yogurt samples have risky role in transmission of pathogenic bacteria (*E. coli*, *E. faecalis*, *B.* cereus), as well as mold and yeast to humans that considered as serious health hazard.

1. INTRODUCTION

Yogurt is a very versatile product that suits all palates and meal occasions and attractiveness has grown and used in most parts of the world. It is the most popular fermented dairy products that consumed in Egypt because of its high nutritive value as it has been considered a main source of high-quality fats, proteins, calcium, phosphorous, magnesium and potassium along with significant quantities of several vitamins as riboflavin, vitamin B6 and vitamin B12 (El Kholy *et al.*, 2014). Due to significance in human nutrition, health benefits, unique taste and aroma, yogurt consumption increase worldwide. Taste and aroma are related to production of volatile and non-volatile acids and carbonyl compounds (Papaioannou *et al.*, 2021).

Two types of yogurts are dominantly retailed in the Egyptian markets. One type has a firm, gel like structure together with a clean packaging (commercial natural set yogurt) (Robinson, 2002), while the other, and especially in Egypt, people still continue to make yogurt in their own homes by using the same yogurt repeatedly (Balady yogurt), which is more popular in the Egyptian market (Sadek and Koriem, 2020); the great popularity of balady yogurt is due to its refreshing and thirst-quenching in hot weather. The value of yogurt in human nutrition is based not only on the strict nutritive effect of milk from which it is made and increased digestibility due to changes of milk constituents during the fermentation period, but also on the beneficial effect of intestinal microflora, prophylactic and healing effects (Zedan *et al.*, 2001).

The quality of yogurt is governed by a number of factors. In fact, inferior milk quality, unhygienic conditions and the use of "wild home-made type" of starter culture give rise to poor grade yogurt, having lower shelf life. In addition, microbiological aspect is one of the most important factors (Ahmad *et al.*, 2013).

The microbial quality of yogurt reflects towards the quality and acceptability of the yogurt. Due to unhygienic conditions, there is possibility of microbial contamination, which may have serious impact on the health of consumers (Pal *et al.*, 2015). Yogurt is an ideal enrichment media for the multiplication of pathogenic foodborne bacteria such as *E. coli*, *E. faecalis* and *B. cereus* that may reach milk and milk products through some intrinsic and extrinsic factors that may found during dairy production, improper sanitation of production lines, contaminated equipment, production room air flow, and inadequate workers personal hygiene (El Biaa, 2018). The number of foodborne pathogenic and spoilage microorganisms, comprises bacteria and fungi, in yogurt depends not only on the animal health; but also, production conditions, storage facilities and technologies (Muehlhoff *et al.*, 2013).

Therefore, the current study mainly targeted the prevalence and phenotypic characterization of *E. coli*, *E. faecalis*, *B. cereus*, mould and yeast in yogurt samples collected from Qalyubia governorate markets, besides studying the genotypic identification and detection of some virulence

[*] Correspondence to: maiabdallaphd@gmail.com

genes in some isolated *E. coli*, *E. faecalis* and *B. cereus* strains by PCR technique.

2. MATERIAL AND METHODS

2.1. Preparation of yogurt samples

A total of 200 random samples of plain yogurt, commercial and balady produced (100 of each), were collected from different markets at Qalyubia governorate. The yogurt cups were intact and within the valid date of consumption. Samples were prepared according to ISO (2017); 5g of sample was mixed with 45ml of sterile 0.1% peptone water, followed by tenfold serial dilution, and examined for the following microbiological criteria.

2.2. Isolation and identification of E. coli ISO (2001)

The prepared samples were culture in Tryptone Bile X-glucuronide agar (TBX agar) by pour-plating method, and incubated at 45^{O}C for 24h. suspected colonies went to further bacteriological identification. Serological identification was performed using *E. coli* antisera "SEIKEN" Set 1, consists of 8 polyvalent and 43 (OK) antisera of DENKA SEIKEN Co. LTD. Tokyo, Japan) according to Edward and Ewing (1972) as recorded in Table (1).

Table 1 Antisera used in serological identification of *E. coli*

Polyvalent Sera	Monovalent sera						
Polyvalent 1	O1	O26	O86a	O111	O119	O127a	O128
Polyvalent 2	O44	O55	O125	O126	O146	O166	
Polyvalent 3	O18	O114	O142	O151	O157	O158	
Polyvalent 4	O6	O27	O78	O148	O159	O168	
Polyvalent 5	O20	O25	O63	O153	O167		
Polyvalent 6	O8	O15	O115	O169			
Polyvalent 7	O28ac	O112ac	O124	O136	O144		
Polyvalent 8	O29	O143	O152	O164			

H-sera:H2, H4, H6, H7, H11, H18 and H21(Edward and Ewing ,1972)

2.3. Isolation and identification of Enterococcus faecalis

It was performed on Slantez and Bartly medium plates; KF Streptococcus agar and Bile Aesculin agar plates with sodium azide (Oxoid, Basingstoke, Hampshire, England, UK) and incubated at 37°C for 24-48 h; suspected colonies were picked up and identification was performed according to Domig *et al.* (2003).

2.4. Isolation and identification of Bacillus cereus

According to FDA (2001) the isolation of Bacillus cereus was performed on *Bacillus cereus* agar base supplemented with Polymyxin B and egg yolk. Following identification was performed according to Markey *et al.* (2013).

2.5. Isolation and identification of moulds and yeast species

Mycological examination was performed according to ISO (2008) after inoculation on modified sabouraud dextrose broth at 25°C ± 1 °C for 5-7 days, then was streaked on Dicloran Rose Bengal Chloramphenicol agar (DRBC), and incubated for 5-7 days at 25°C ± 1°C in an upright position. Fungal growth was picked up and kept on agar slopes for further macroscopical and microscopical characterization according to David *et al.* (2007).

2.6. Genotypic identification and detection of some virulence genes by PCR

Genotypic identification and detection of alkaline phosphatase (*pho*A**)** gene assessed commonly in the *E. coli* strains, shiga toxin1 gene (*stx*1) and shiga toxin 2 gene **(***stx*2**)** in three random isolated *E. coli*; species-specific gene of *E. faecalis* (16SrRNA); Enterococcal surface protein gene (*esp*) and gelatinase gene **(***gel*E**)** in five random isolated *E. faecalis*; beside, phylogenetic marker gene of *B. cereus* (*gro*EL); cytotoxic K gene (*cyt*K) and non-hemolytic enterotoxin (*nhe***)** gene in four random isolated *B. cereus* using uniplex and multiplex PCR techniques.

Preparation of samples, DNA extraction and thermocycling adjustment were performed following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR master mix (Takara, Japan) and 1. 5% agarose gel electrophoreses (Sambrook *et al.,* 1989) using the primer sequences, target genes, amplicons sizes and cycling conditions showed in Table (2).

3. RESULTS

The results of microbiological examination of yogurt samples, genotyping identification and detection of some virulence genes in *E. coli; B. cereus* and *E. faecalis* isolated strains were presented in Tables (3-5) and Figures (1-5).

Table (3) presents the prevalence of *E. coli*, *E. faecalis* and *B. cereus* in the examined yogurt samples, and revealed superiority of *E. faecalis* isolation in the overall examined samples (5.5%), followed by *E. coli* and *B. cereus* which came in the equal prevalence (4.0%). Furthermore, collected traditional yogurt samples had higher contamination levels than the commercial brand samples.

Serological typing of the isolated E. coli, as was tabulated in Table (4), revealed isolation of *E. coli* O86a H4, O119 H4 and O153 H2 in different prevalence depended mainly on the type of the collected samples; where, out of eight isolated strains, seven strains were isolated from the traditional made samples.

Table (5) presents the total number and percentage of the detected mould and yeast strains. Results revealed superior detection of *A. niger* and *C. albicans* in relation to the other detected strains of mould and yeast, respectively.

So, the overall incidences of bacteriological and mycological contamination were higher in traditional balady yogurt samples than commercial brand ones.

PCR results for genotypic identification and detection of virulence genes related to the isolated bacterial strains as were shown in Figs (1-5) revealed detection of *pho*A gene in all the examined *E. coli* strains; moreover, *stx*1 gene was detected in one strain only; while, *stx*2 gene failed to be amplified in any of the examined strains (Figs. 1-3). Moreover, for *E. faecalis* strains, the16SrRNA, *esp* and *gel*E genes were detected in all the studied strains (Fig. 4). Regarding with *B. cereus* strains, *gro*EL, *cyt*K and *nhe* genes were also detected in all the examined strains (Fig. 5).

Table 2 Primers sequences, target genes, amplicons sizes and cycling conditions

Target M.O.	Target gene		Primer sequence (5'-3')	Amplified segment (bp.)	Primary denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	References
E. coli	*pho*A	F	CGATTCTGGAAATGGCAAAAG	720 bp.	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Hu *et al.*, 2011
		R	CGTGATCAGCGGTGACTATGAC							
	stx1	F	ACACTGGATGATCTCAGTGG	614 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Dipineto *et al.*, 2006
		R	CTGAATCCCCCTCCATTATG							
	stx2	F	CCATGACAACGGACAGCAGTT	779 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
		R	CCTGTCAACTGAGCAGCACTTTG							
	16S Rrna	F	GTT TAT GCC GCA TGG CAT AAGAG	310 bp.	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	Zoletti *et al.*, 2006
		R	CCG TCA GGG GAC GTT CAG							
E. faecalis	*esp*	F	AGATTTCATCTTTGATTCTTGG	510 bp.	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Vankerckhoven *et al.*, 2004
		R	AATTGATTCTTTAGCATCTGG							
	*gel*E	F	TATGACAATGCTTTTTGGGAT	213 bp.	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	
		R	AGATGCACCCGAAATAATATA							
	*gro*EL	F.	TGCAACTGTATTAGCACAAGC T	533 bp.	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Das *et al.*, 2013
		R.	TACCACGAAGTTTGTTCACTACT							
B. cereus	*nhe*	F.	AAG CIG CTC TTC GIA TTC	766 bp.	94˚C 5 min.	94˚C 30 sec	49˚C 40 sec.	72˚C 45 sec.	72˚C 10 min	Ehling-Schulz *et al.*, 2006
		R.	ITI GTT GAA ATA AGC TGT GG							
	*cyt*K	F.	ACA GAT ATC GGI CAA AAT GC	421 bp.	94˚C 5 min.	94˚C 30 sec	49˚C 40 sec.	72˚C 45 sec.	72˚C 10min	
		R.	CAA GTI ACT TGA CCI GTT GC							

Table 3 Prevalence of *E. coli*; *E. faecalis* and *B. cereus* isolates in yoghurt examined samples

Samples	Commercial yoghurt		Baladi yoghurt		TOTAL	
Isolates	NO.	%*	NO.	%*	NO.	%**
E. coli	1	1.0	7	7.0	8	4.0
E. faecalis	3	3.0	8	8.0	11	5.5
B. cereus	3	3.0	5	5.0	8	4.0
Total	7	7.0	20	20.0	27	13.5

* Percentage in relation to total No. of each examined samples (100).
** Percentage in relation to total No. of samples (200).

Table 4 Serological typing of *E. coli* strains isolated from yoghurt examined samples

Samples	Commercial yoghurt	Baladi yoghurt	TOTAL
E. coli serotype	NO.	NO.	NO.
O86a H4	0	2	2
O119 H4	1	2	3
O153 H2	0	3	3
TOTAL	1	7	8

Table 5 Total number and percentage of fungi (moulds and yeasts) isolated from yoghurt examined samples

Samples	Commercial yoghurt		Baladi yoghurt		TOTAL	
Mould strains	NO.	%*	NO.	%*	NO.	%**
Aspergillus fumigatus	1	1.0	4	4.0	5	2.5
Aspergillus flavus	0	0.0	3	3.0	3	1.5
Aspergillus niger	4	4.0	15	15.0	19	9.5
Rhizopus spp.	0	0.0	2	2.0	2	1.0
Mucor spp.	2	2.0	3	3.0	5	2.5
Penicillium spp.	1	1.0	2	2.0	3	1.5
Total	8	8.0	29	29.0	37	18.5
Yeast strains	NO.	%*	NO.	%*	NO.	%**
Candida albicans	7	7.0	16	16.0	23	11.5
Candida tropicalis	2	2.0	6	6.0	8	4.0
Rhodotorula spp.	4	4.0	8	8.0	12	6.0
Total	13	13.0	30	30.0	43	21.5
Grand total	21	21.0	59	59.0	80	40.0

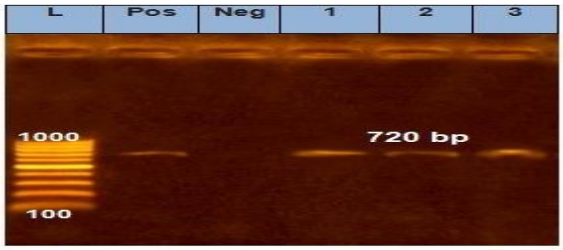

Figure (1): PCR amplification of *pho*A gene of *E. coli* strain on agarose gel 1 %. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*S. aureus* reference: ATCC25923). Pos.: Positive control (*E. coli*: ATCC14028 at 720 bp.). Lane 1; 2 &3: *E. coli* (Positive at 720 bp.)

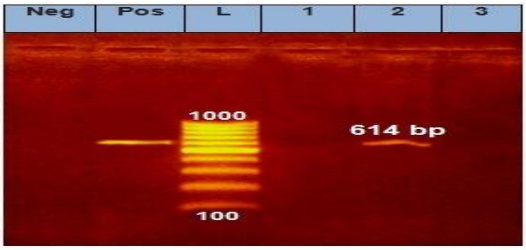

Figure (2): PCR amplification of stx1 genes of E. coli samples on agarose gel 1 %. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (S. aureus reference: ATCC25923). Pos.: Pos.: Positive control (E. coli: ATCC14028 at 614 bp.). Lane 2: E. coli (Positive at 614 bp.). Lane 1& 3: (Negative) E. coli strain for stx1 gene

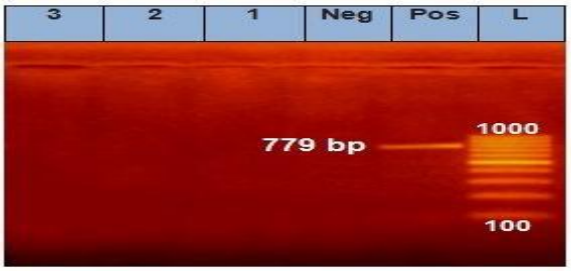

Figure (3): PCR amplification of stx2 genes of E. coli samples on agarose gel 1 %. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (S. aureus reference: ATCC25923). Pos.: Pos.: Positive control (E. coli: ATCC14028 at 779 bp.). Lane 1- 3: (Negative) E. coli strains for stx2 gene

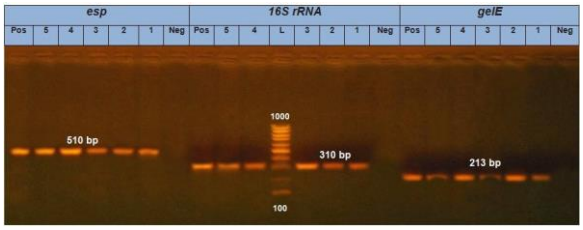

Figure (4): PCR amplification of16SrRNA; esp and gelE genes of E. faecalis samples on agarose gel 1 %.

Fig. (4): (Left)	Fig. (4): (Middle)	Fig. (4): (Right)
Enterococcal surface protein (*esp*) gene	species-specific gene of *E. faecalis* (16SrRNA)	gelatinase (*gel*E) gene
Lane L: 100-1000 bp. DNA Ladder	Lane L: 100-1000 bp. DNA Ladder	Lane L: 100-1000 bp. DNA Ladder
Neg.: Negative control (*E. coli* AJ413986).	Neg.: Negative control (*E. coli* AJ413986).	Neg.: Negative control (*E. coli* AJ413986).
Pos.: Positive control (*E. faecalis* form AHRI at 510 bp.)	Pos.: Positive control (*E. faecalis* form AHRI at 310 bp.)	Pos.: Positive control (*E. faecalis* form AHRI at 213 bp.)
Lane 1- 5: *E. faecalis* (Positive).	Lane 1- 5: *E. faecalis* (Positive).	Lane 1- 5: *E. faecalis* (Positive).

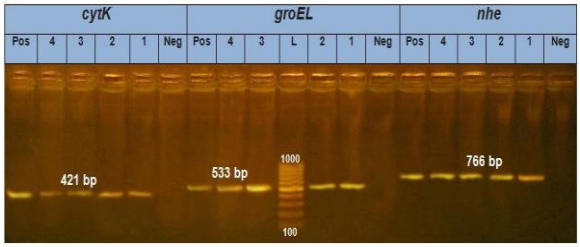

Figure (5): PCR amplification of groEL; cytK and nhe genes of B. cereus samples on agarose gel 1 %

Fig. (5): (Left)	Fig. (5): (Middle)	Fig. (5): (Right)
cytotoxic K (*cyt*K) gene	diagnostic, phylogenetic marker gene (*gro*EL) gene	non-hemolytic enterotoxin (*nhe*) gene
Lane L: 100-1000 bp. DNA Ladder	Lane L: 100-1000 bp. DNA Ladder	Lane L: 100-1000 bp. DNA Ladder
Neg.: Negative control (*E. coli* AJ413986).	Neg.: Negative control (*E. coli* AJ413986)	Neg.: Negative control (*E. coli* AJ413986)
Pos.: Positive control (*B. cereus* form AHRI at 421 bp.)	Pos.: Positive control (*B. cereus* form AHRI at 533 bp.)	Pos.: Positive control (*B. cereus* form AHRI at 766 bp.)
Lane 1- 4: *B. cereus* (Positive).	Lane 1- 4: *B. cereus* (Positive).	Lane 1- 4: *B. cereus* (Positive).

4. DISCUSSION

Escherichia coli frequently contaminates different food items and is considered a good indicator of fecal pollution; so, its presence in dairy products may indicate contamination with other enteropathogenic microorganisms which constitute public health hazard; especially Shiga toxin-producing *E. coli* (STEC) which can be transmitted to humans via consumption of contaminated food. Therefore, the incidence of STEC in raw dairy products depend mainly on the hygiene and effective control programs (Velázquez-Ordoñez et al., 2019). Our results of the incidence of *E. coli* in the examined yogurt samples were nearly similar to those reported by Ahmad et al. (2013); while higher incidences were recorded by El-Toukhy et al. (2021).

Presence of *E. coli* in yogurt samples declared the level of sanitary measures that reflecting the using of poor-quality raw milk, insufficient preheating process, as well as presence of other enteric pathogens. Moreover, *E. coli* as an indicator of post processing contamination in yogurt manufacture has been established and recommended by public health authorities worldwide (El Bakri and El Zubeir, 2009).

Enterococci and mainly *E. faecalis* are food poisoning bacteria; where its presence indicate severe poor factory sanitation because of their relatively high resistance to drying, detergents, as well as freezing temperature; furthermore, *E. faecalis* can also survive wide pH ranges, low as 4.8 and high as 9.6; in addition, it can resist bile salts about 40 % (w/v); so, enterococci have been proposed for inspection of overall hygiene in the production lines, and their presence in various dairy products always indicate inadequate production and processing sanitation (Carroll *et al.*, 2019). The *Enterococcus faecalis* has been associated with various infections, including nosocomial infections, bacteremia, meningitis, surgical wound infection, endocarditis, and urinary tract infection (Xia *et al.*, 2013).

Presence of *E. faecalis* in yogurt samples may be attributed to their comparatively heat resistance and growth at wide range of pH (Abd El Tawab *et al.*, 2016). The existence of Enterococci especially *E. faecalis* in yogurt is considered an indication of neglected sanitary control measures during production. Other studies recorded detection of *E. faecalis* in their examined dairy products such as El-Ansary (2014) and Abd El Tawab *et al.* (2016) in nearly similar prevalence, but higher results were reported by El-Malt *et al.* (2013).

Bacillus cereus is a heat resistant spore-forming bacterium, which is commensally proliferates in the environment, and their presence in food products usually causing worldwide severe food poisoning outbreaks represented by severe diarrhea because of, at least, three known enterotoxins named non-hemolytic (*nhe*) enterotoxin, *hbl* enterotoxin and cytotoxin K, which is characterized by two forms described encoded by *cyt*K-1 and *cyt*K-2 genes (Ceuppens *et al.*, 2013).

Bacillus cereus considered as one of the most important causes of food poisoning in the industrialized world because of its enterotoxins (Per and Terje, 2006). The recorded findings reported by Hassan *et al.* (2010) were nearly similar to our current findings; but lower than those reported by Ayoub *et al*. (2003) as he found that 20% of examined yogurt samples were contaminated with *B. cereus*; and disagreed with those recorded by Tirloni *et al.* (2017) who failed to detect *B. cereus* in yogurt samples.

Regarding with foodborne fungal contamination, they responsible for physical and chemical changes appeared as unpleasant odors and undesirable flavor leading to wastes and economic losses. Mould and yeast growing in yogurt metabolize some acids, and consequently rise the overall pH, which may favour the growth of putrefactive bacteria. In addition, some mould sp. like Aspergillus, Fusarium, and Penicillium have public health hazard owing to their ability to produce mycotoxins that are harmful to human health (Garnier *et al.*, 2017; Keta *et al.*, 2019).

The presence of mold and yeast in milk and dairy products is not acceptable even if it is found in a few numbers because they result in inferior changes that perilously undermine the quality of the product (Garnier *et al.*, 2017). Table (5) clarified that, yogurt samples were contaminated with moulds and yeasts. These results came in harmony with El-Ansary (2014) and Salim *et al.* (2020); but disagreed with those reported by Ahmad *et al.* (2013) and El-Malt *et al.* (2013) who recorded higher results; while, El-Shinawy *et al.* (2018) who failed to detect fungal growth on yogurt samples. The isolated and identified moulds and yeast species from yogurt samples were previously reported by Keta *et al.* (2019) and Salim *et al.* (2020).

These recorded results indicated inadequate sanitary measures either during processing or from the milk itself which may be contaminated from the surrounding environment, equipment, handling, and transportation. Based on the previous indications, variation between different authors may be attributed to the difference in the locality of collection, raw milk quality, processing technique and the hygienic measures of the production lines.

Referring to the obtained results of PCR examination, in regard with *E. coli* isolates, the strains producing phoA, stx1 and stx2 are associated with haemorrhagic enteritis in humans (Markey et al., 2013). PCR results cleared detection of phoA gene in all studied strains which came in harmony with those of Abd El-Badiea et al. (2019). Moreover, stx1 was amplified in one E. coli strain only, while stx2 failed to be amplified in all the studied E. coli strains. Similar findings were recorded by Abd El-Badiea et al. (2019), but disagreed with those obtained by El-Shora (2019) who failed to detect stx1 and stx2 in examined E. coli isolates.

Referring to the obtained PCR amplifications of the examined E. faecalis isolates, it came in agreement with the results of previous reports for E. faecalis strains isolated from yogurt and dairy products (Vidana et al., 2016).

Compared to the obtained results of PCR examination of the isolated B. cereus strains, similar detection of groEL gene was previously recorded by Lim et al. (2011). Moreover, all of them were Enterotoxigenic strains that involved in food poisoning, as the two virulence genes cytotoxic K (cytK) and non-hemolytic enterotoxin (nhe) were amplified in all the examined isolates; results came in harmony with those of El-Toukhy et al. (2021).

5. CONCLUSION

Afterall, E. coli, E. faecalis, B. cereus A. niger and C. albicans, as foodborne and spoilage pathogens, were significantly present in the traditional balady samples higher than the commercial brand ones. Moreover, each molecularly examined isolates harbored at least one of the enterotoxin genes indicating their pathogenic nature, which considered as serious health hazard and there is a high probability of the potential transmission of such enterotoxigenic strains to humans.

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