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Full Length Research Paper

Cholesterol reduction *in vitro* by novel probiotic lactic acid bacterial strains of *Enterococcus* isolated from healthy infants' stool

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The present study aimed to isolate and evaluate the potentiality of some probiotic lactic acid bacteria from infants stool to degrade and reduce cholesterol level in fermentation medium. Out of 50 lactic acid bacterial isolates recovered from healthy infants (3 to 18 months old) stool, two bacterial isolates W7 and Y1 showed highest cholesterol reduction percentage (68.38 and 71.6%, respectively). The cholesterol reduction potentiality by the two isolates W7 and Y1 were optimized to 94 and 98%, respectively, by studying the different cultural conditions; inoculum size, inoculum age, pH, incubation temperature, incubation period and cholesterol concentration. The gas chromatography analysis of the fermentation extract revealed that the resulted end product of cholesterol was 5á-cholestane-3à,25-diol ($C_{27}H_{48}O_2$) of molecular weight 404. The selected bacterial isolate Y1 was identified phenotypically and genotypically as *Enterococcus faecium* and deposited in GenBank under the accession number KY788356.

Key words: Cholesterol, *Enterococcus*, optimization, probiotic, gas chromatography.

INTRODUCTION

Cholesterol is a waxy steroid compound formed in all cells of the animal and human body and required for formation of steroid hormones (Young, 2001, Rigotti et al., 2003), a precursor for biosynthesis of bile acids in the liver that help the body to digest fat (Russell, 2003). Longstanding increased serum cholesterol levels may lead to atherosclerosis and as a result lead to development of cardiovascular diseases (CVDs) (Ngongang et al., 2016; Tsai et al., 2016). CVDs were reported to be responsible for 30% of deaths worldwide and predicted to remain the leading causes of death (WHO, 2013). Both pharmacologic and non-pharmacologic approaches, including follow healthy diet, behavior alteration, and regular practice, are common strategies to lower serum cholesterol levels (Dunn-Emke et al., 2001), Although they record cholesterol-lowering ability, but in some cases unwanted side effects can be produced, such as gastrointestinal disorder (Davidson et

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al., 1999).

There are some microorganisms that live as normal flora in animal and human gut, known as probiotics. The presence of these microorganisms in healthy form have different healthy benefits such as protection against gastrointestinal disorder (Vanderhoof and Young, 1998; Niv et al., 2005; Saxelin et al., 2005), reduction of cholesterol level in blood and lipids (Gilliland and Walker, 1990; Naidu et al., 1999), synthesizing extracellular bacteriocins as antimicrobial metabolites (Gill, 2003) and enhancing the immune system by production and circulation of serum antibodies (Maassen et al., 2000; Ezendam and van Loveren, 2006). An adequate quantity administration about 10^9 CFU per day of external probiotics especially lactic acid bacteria will be appropriate to maximize and balance the human gastro-intestinal microbiota by replacing harmful pathogens and reinforcing the natural defence mechanisms (Casas and Dobrogosz, 2000; Ouwehand et al., 2002). Probiotics for human feeding are preferably isolated from the gastrointestinal (GI) tract of healthy human (Saarela et al., 2000).

Acid-producing probiotics have been extensively used for cholesterol reduction in human blood serum by applying theses bacterial strains in various fermented foods, milk products and beverages (Pereira and Gibson, 2002; Ouwehand and Vesterlund, 2004) and several studies reported the hypocholesterol ability of lactic acid probiotics *in vitro* or *in vivo*, especially the strains of genera *Lactobacillus, Enterococcus* and *Bifidobacterium* (Pan et al., 2011; Wang et al., 2012; Huang et al., 2013; Hu et al., 2013; Tsai et al., 2016). Lactic acid bacteria of cholesterol reduction potentiality are now available as probiotics to consumers in forms of milk products such as yoghurts and cheese, formulated tablets and lyophilized powders. However, long-term hypocholesterolemic effects requiring continuous research for novel health enhancing probiotic strains from healthy individual origin is still attracting area.

Different mechanisms for cholesterol removal by probiotics have been reported, such as deconjugation of bile salts by bile-salt hydrolase (BSH) (Ahn et al., 2003), cholesterol absorption into bacterial cell membranes (Kimoto et al., 2002), production of short-chain fatty acids (SCFAs) during the probiotics growth (Trautwein et al., 1998), and cholesterol transformation into coprostanol (Lye et al., 2010). Additionally, several cultural conditions such as media composition, pH, temperature, and inoculum size may change the metabolic pathways by altering pyruvate metabolism and external electron acceptors resulted in different end-products (Axelsson, 1998; Annuk et al., 2003).

The main objective of this study was to isolate new lactic acid probiotic strains of human origin with potential of highly cholesterol reduction and optimize the cultural condition for the cholesterol reduction process in addition to follow the resulted end products formed by the selected bacterial strain.

MATERIALS AND METHODS

Isolation of microorganisms

A total of 50 different healthy infants (3 to 18 months old) stool samples were obtained from different baby centers located at Ismailia and Suez Governorates of Egypt, transferred in sterilized plastic bottles to the laboratory and immediately used for isolation of lactic acid bacteria. Ten grams of each infant stool sample were aseptically homogenized in 90 ml of sterile saline solution and sequential decimal dilutions were done. One milliliter of each dilution was inoculated into sterilized plates and about 20 ml of melted de Man, Rogosa and Sharpe (MRS) agar medium of pH 6.2 were poured in each plate. The used MRS agar (De Man et al., 1960) medium contained (g/L distilled water): glucose 10, peptone 10, beef extract 10, yeast extract 5, K_2HPO_4 2, sodium acetate 5, tri-ammonium citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 4H_2O$ 0.2 and Tween 80 (1 ml). The plates were incubated for 3 days at 37°C under anaerobic conditions (GasPak System - Oxoid, Basingstoke Hampshire, England). The separated colonies were picked, subcultured, maintained on MRS slants and stored at 4°C for further experiments.

Preparation of bacterial inoculum

A loopful of refreshed bacterial culture was inoculated and grown in bottles containing 20 ml of MRS broth medium and incubated for 24 h at 37°C under anaerobic conditions in CO_2 incubator.

Cholesterol degradation in fermentation medium by the isolated bacteria

Cholesterol stock solution was prepared by dissolving 10 mg of cholesterol in 1 ml of 96% ethyl alcohol and filter sterilized. For each culture to be tested, 70 µl of cholesterol solution was added to 10 ml of MRS broth (final cholesterol concentration 70 µg/ml) and 1% (v/v) of freshly grown culture was added and incubated anaerobically at 37°C for 24 h. After incubation, the cells were removed by centrifugation at 10,000 *g* for 10 min at 4°C.

Determination of cholesterol concentration in fermentation medium

The cholesterol concentration was determined in the supernatant using a colorimetric method described by Rudel and Morris (1973). Three milliliters of supernatant, 2 ml of 33% (w/v) KOH and 3 ml 96% ethanol were placed in a capped test tube, vortexed for 20 s and incubated for 15 min at 60°C in a water bath. After incubation, the mixture was removed and cooled under tap water, then 5 ml of hexane and 3 ml of water were added and vortexed for one min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. One milliliter of cholesterol liquicolor enzymatic kit (Spinreact Kit, Spain) was added (Guo et al., 2011). The solution was mixed and left for 5 to 10 min at 37°C and absorbance was measured at 500 nm with a spectrophotometer. The cholesterol reduction was calculated as percentage from the following equation: A = 100 – (B/C)×100, where A = % of cholesterol removed, B = absorbance of the sample containing the cells and C = absorbance of the sample without

cells.

Characterization and identification of the selected bacterial isolate Y1

Morphological characterization

The morphological growth characters of the selected isolate Y1 was conducted on MRS agar medium and the colony color, shape and texture were recorded. The cell shape and arrangement were determined by microscopic examination after Gram staining technique in accordance with Collins and Lyne (1985).

Physiological and biochemical characterization

The physiological and biochemical characteristics were estimated according to the standard methods. The selected bacterial isolate Y1 was examined for Catalase production (Wittenberg, 1964), carbohydrate utilization (Pridham and Gottlieb, 1948), growth at 6.5% NaCl, growth at different temperatures (15 and 45°C), production of CO_2 from glucose and production of NH_3 from arginine.

Genotypic characterization

DNA extraction: The extraction of bacterial DNA was performed according to the method of Kozaki et al. (1992) using DNA preparation kit (Jena Bioscience).

Polymerase chain reaction (PCR) amplification: The PCR amplification was performed using the 16S primers: 16S F: 5′-GAGTTTGATCCTGGCTTAG-3′ and 16S R: 5′GGTTACCTTGTTACGACTT-3′ and Qiagen Proof-Start Tag Polymerase Kit (Qiagen, Hilden, Germany) according to standard PCR protocol. The following substrates were combined in a total volume of 25 µl containing about 20 ng of template DNA, 12.5 µl PCR Master Mix, 20 pmol (2 µl) of both forward and reverse primers and the reaction was completed by 8.5 µl of deionized water. The reaction conditions were: an initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s. A final extension was performed at 72°C for 5 min. PCR products were purified from gel with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Then analyzed by electrophoresis on 1.5% agarose gel in 1X TAE buffer and finally observed and pictured under UV light.

DNA Sequencing: Sequence similarity was recorded by searching the homology in the Genbank DNA database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). CLUSTALW program (http:// clustalw.ddbj. nig. ac.Jp/top- ehtml) was used for evaluating multiple sequence alignment and molecular phylogeny. The tree view program was applied for phylogenetic tree illustration.

Optimization of cholesterol reduction by the selected bacterial isolates

Effects of different cultural conditions on cholesterol reduction by the selected bacterial isolates W7 and Y1 were studied in MRS broth medium as follows; cholesterol concentration (50, 70, 100, 125 and 150 µg/ml), incubation temperature (25, 30, 37 and 45°C), pH (3, 5, 6.5, 7 and 9), incubation time (14, 24, 48, 62 and 72 h), inoculum size (0.5, 1.5, 2.0, 2.5, 3.0 and 4.0%) and inoculum age (4, 8, 10, 18, 24, 42 and 48 h).

Extraction of the remaining cholesterol and its derivatives from the bacterial culture

The LAB isolate (Y1) was grown in MRS broth medium supplemented with cholesterol (70 µg/ml) under the optimized cultural conditions and the obtained bacterial cells with the remaining broth medium were homogenized in chloroform (1:1, v/v) by sonication. The solvent-supernatant mixture was agitated and separated by separating funnel and filtered on sodium sulphate anhydrous to remove any water from the extract. All obtained chloroform extracts were dried using rotary evaporator at 50°C under vacuum and the crude extracts were then subjected to analysis by gas chromatography-mass spectra to clarify the structure of the resulted metabolites.

Determination of cholesterol degradation products using gas chromatography-mass spectra (GC-MS)

The GC-MS analysis of the crude extracts was carried out at the Central Laboratory, National Research Center of Egypt with the following specifications: Instrument, a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 m × 0.32 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min. Temperature program: 50°C for 3 min; rising at 5°C/min to 300°C and held for 5 min. The injector and detector were held at 220 and 200°C, respectively. Diluted samples (1:10 diethyl ether, v/v) of 1 µl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 50 to 500 (Said-AL Ahl and Omer, 2016).

Statistical analysis

Analysis of variance (ANOVA) was performed using CoStat V. 6.311 (CoHort software, Berkeley, CA94701). Cholesterol reduction mean values were compared at 5% significance level using Tukey's test. Least significant difference (LSD) test was used to test the significant differences between the whole means of different groups and compared with the critical difference at the 5% level.

RESULTS AND DISCUSSION

Fifty lactic acid bacterial isolates were recovered from the collected 50 infants stool samples based on criteria of lactic acid bacteria such as morphological shape, catalase negativity, Gram positive, and lactic acid formation. The selected bacterial isolates were tested for their potentiality to reduce or biodegrade cholesterol on basal cholesterol broth medium. A total of 10 out of 50 tested lactic acid bacterial isolates exhibited cholesterol reduction in high percentages (Table 1). Apparently, cholesterol reduction varied among the tested bacterial strains ranged from 48.22 to 71.6% reduction from the initial cholesterol concentration and the highest reduction mean values of 71.6 and 68.38% were obtained by the two isolates Y1 and W7, respectively. The results in the present study are in accordance with the experimental findings of Sieladie et al. (2011) who reported that about 11 lactobacilli strains isolated from raw cow milk exhibited cholesterol

Table 1. Mean values of cholesterol reduction percentage of the most active ten LAB isolates.

Isolate code	Cholesterol reduction* (%)
Y1	71.6 ± 0.05[a]
H3	48.22 ± 0.02[e]
W1	61.61 ± 0.04[cd]
W2	64.6 ± 0.02[c]
W4	65 ± 0.03b[c]
W6	65 ± 0.04[bc]
W7	68.38 ± 0.05[ab]
Mix	61.61 ± 0.06[cd]
YF- black	62.9 ± 0.09[c]
YF- green	58.06 ± 0.08[d]

*Values are means of three replicates ± standard deviation and values with the same letters are not significantly different.

degrading activity with cholesterol lowering potentials ranging from 42.88 to 97.20%. In the study conducted by Hassanein et al. (2013) the result revealed high cholesterol reduction (66.8%) by *Lactococcus lactis* KF 147. In this coincidence, Yehia et al. (2015) recorded maximum cholesterol reduction of 75.3% in liquid medium by *Enterococcus hirae* isolated from milk. Also, the highest cholesterol reduction by *L. lactis* subsp*. lactis* N7 was 97.0% as recorded by Kimoto et al. (2002). It has been reported that cholesterol removal by lactic acid bacteria appeared not to be the only strain specific but also growth dependent, as shown by studies that have evaluated cholesterol removal by probiotic cells during different growth conditions (Kimoto et al., 2002).

The two bacterial isolates W7 and Y1 were selected as highly cholesterol reducing lactic acid bacteria in the present study and subjected to identification and further experiments. The bacterial isolate W7 was identified as *Enterococcus faecalis* and published in another study for production of lactic acid by the same authors (Aboseidah et al., 2017), while the bacterial isolate Y1 was identified phenotypically and genotypically in the present study. The results of physiological and biochemical characterizations of the isolate Y1 are shown in Table 2. The obtained results revealed that the bacterial isolate was Gram positive and catalase negative. This isolate was able to tolerate NaCl (6.5%). Also, the bacterial isolate was able to grow at 15 and 45°C. The results also indicated that the isolate was unable to produce CO_2 from glucose and NH_3 from arginine. The tested isolate had the ability to ferment glucose, mannose, galactose, xylose, maltose, mannitol, lactose and arabinose but was unable to use sucrose and glycerol as a carbon source. Based on the taxonomic characteristics described earlier, the isolate Y1 was assigned to the genus *Enterococcus.*

The bacterial isolate Y1 was identified using phylogenetic analysis of 16S rRNA gene sequences. The partial 16S rRNA gene sequences of tested isolate was matched with previously published bacterial 16S rRNA gene sequences available in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Sequence analysis of the partial 16S rRNA gene sequence of 498 base pairs of the isolate Y1 revealed that this isolate had 96% similarity to *Enterococcus faecium.* A phylogenetic tree was constructed from a multiple sequences alignment of 16S rRNA gene sequences (Figure 1). The nucleotide sequences of the isolate Y1 was deposited in the Gen-Bank nucleotide sequence database under new accession number KY788356.

Figure 2 shows the effect of pH on cholesterol removal percentage by LAB isolates. The cholesterol reduction increased gradually with decreasing acidity of cultural medium until it reached maximum (65 and 78.5% for *E. faecalis* W7 and *E. faecium* Y1, respectively) at pH 6.5. At basic media the ability of bacteria to remove cholesterol decreased gradually with increasing alkalinity. Therefore, the optimum pH for maximum cholesterol reduction by the two selected isolates was pH 6.5. The study carried out by Kumar et al. (2013) suggested that a neutral pH is best for cholesterol reduction by *L. casei* LA-1. Also, Yazdi et al*.* (2001) reported that the optimal pH values for cholesterol decomposition are 7.2 for *Streptomyces fradiae* and 6.75 for *Rhodococcus erythropolis* ATCC 25544. The optimum production of enzymes required for metabolic process greatly affected by pH as enzymes, being proteins, contain ionizable groups; therefore, the pH of the culture medium affected their structure and function (Lekha and Lomane, 1997).

The effect of different incubation temperatures (25 to 45°C) on cholesterol reduction by the two tested bacterial isolates was studied and the obtained results are illustrated in Figure 3. The results revealed that the ability of bacterial isolates to remove cholesterol increased by increasing incubation temperature and reached to maximum values (80.7 and 85% for *E. faecalis* W7 and *E. faecium* Y1, respectively) at 37°C and then decreased by increasing temperature. Environmental and nutritional factors are known to influence the growth and decomposition of cholesterol by microorganisms. The optimum temperature for the decomposition of cholesterol in liquid medium by *E. hirae* was 37°C (Yehia et al., 2015). In contrast, several studies have reported other optimal incubation temperatures, such as 29°C for *R. erythropolis* ATCC 25544 (Sojo et al., 2002) and 30°C for *Bacillus subtilis* SFF34 (Lashkarian et al., 2010). The temperature range from 25 to 37°C is optimum for microbial growth and their enzymes activity and as a result maximum cholesterol removal was observed.

The effect of incubation time on cholesterol removal is observed in Figure 4. The maximum removal percentage was reported at 24 h of incubation. The bacterial isolate *E. faecium* Y1 removed 78.5% of cholesterol from the medium, while 65% of cholesterol was removed by isolate *E. faecalis* W7 at 24 h of growth. After that, the

Table 2. Morphological and biochemical characterizations of the selected bacterial isolate Y1.

Test	Observation
Colony morphology	White, circle, entire, convex
Gram stain	+
Cells shape	Cocci
Catalase production	-
Growth at 6.5% NaCl	+
Growth at 15°C	+
Growth at 45°C	+
Production of:	
CO_2 from glucose	-
NH_3 from arginine	-
Fermentation of:	
Xylose	+
Galactose	+
Arabinose	+
Maltose	+
Mannitol	+
Sucrose	-
Lactose	+
Gycerol	-
Mannose	+
Glucose	+

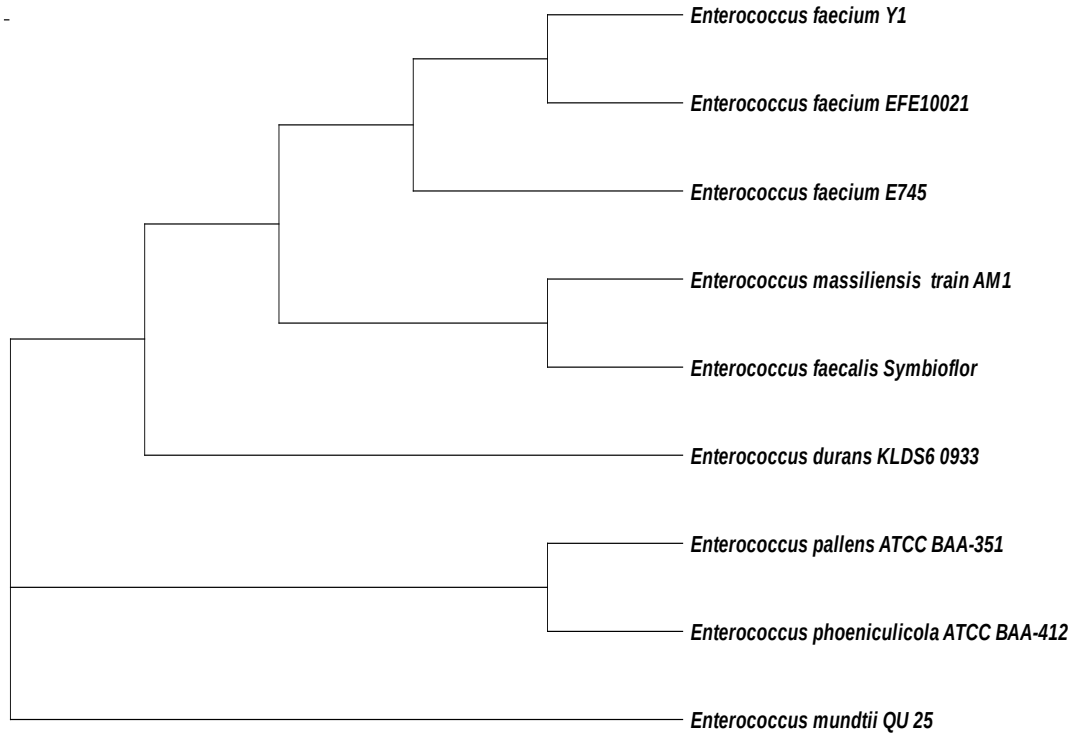

Figure 1. The neighbor-joining tree based on 16S rRNA gene sequences showing the position of *Enterococcus faecium* Y1 and related strains in GenBank.

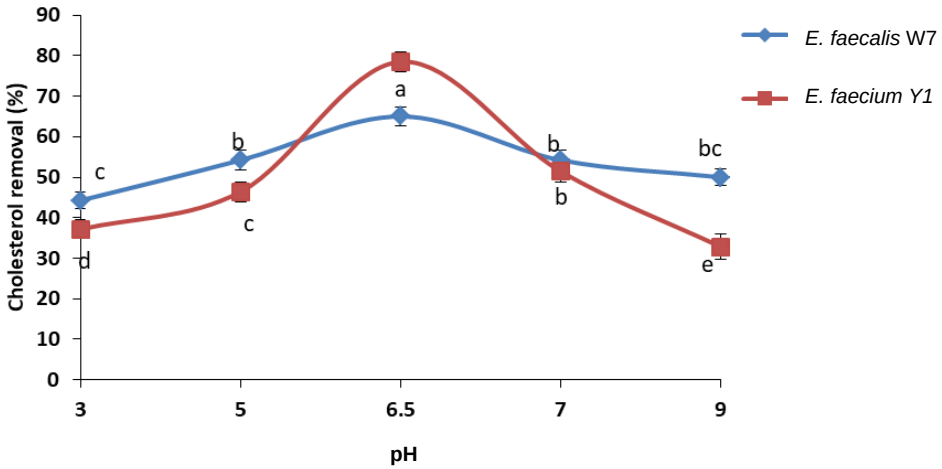

Figure 2. Effect of medium pH on cholesterol reduction percentage by *Enterococcus faecalis* W7 and *E. faecium* Y1.

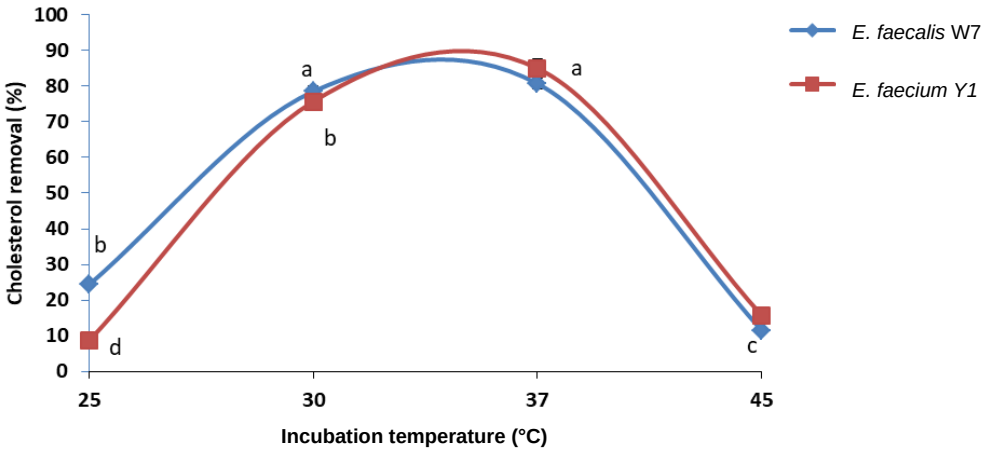

Figure 3. Effect of incubation temperature on cholesterol reduction percentage by *E. faecalis* W7 and *E. faecium* Y1.

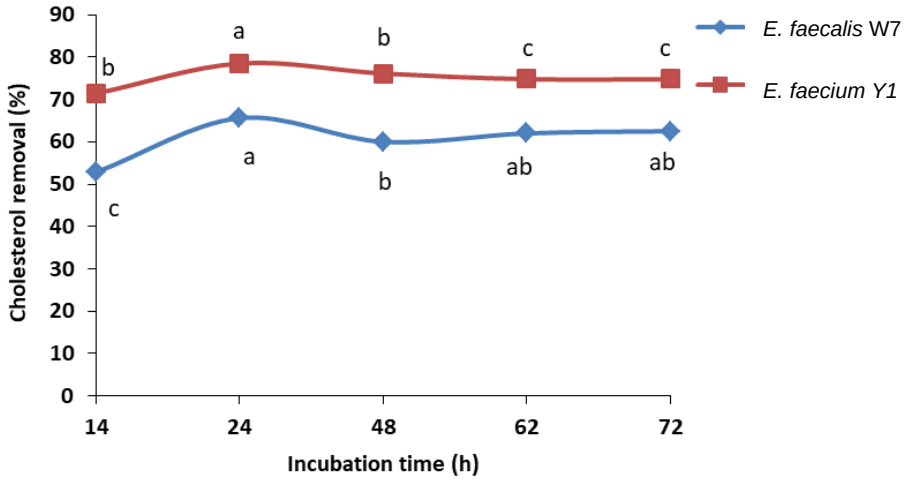

Figure 4. Effect of incubation time on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

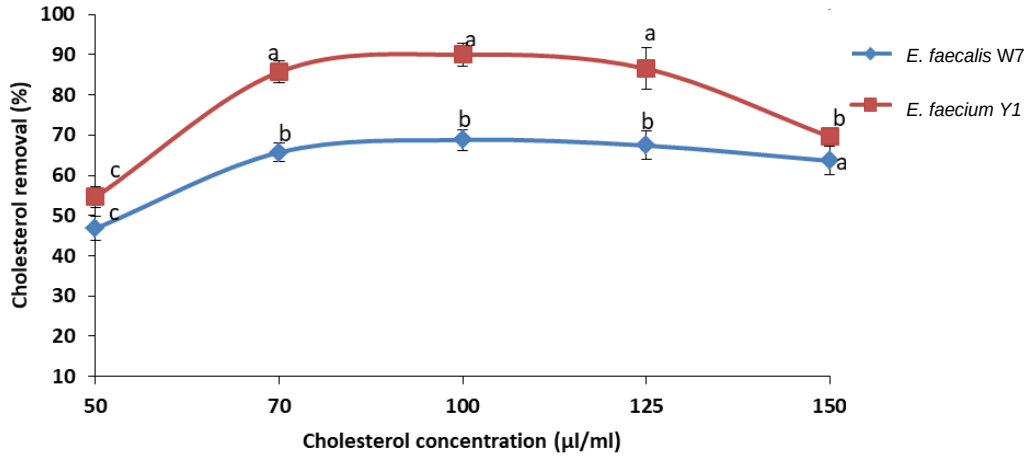

Figure 5. Effect of different cholesterol concentration on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

ability of both isolates to remove cholesterol decrease gradually and finally reached stability at 48 h for W7 and 62 h for *E. faecium* Y1. Our findings is in accordance with the study of Kumar et al. (2013) who suggested that the ability of *L. casei* LA-1 to assimilate cholesterol was maximum after an incubation of 24 h. Hassanein et al. (2013) also found that the maximum percentage of cholesterol reduction by *L. lactis* KF147 was achieved at 24 h. Additionally, Yehia et al. (2015) observed that the optimal cholesterol reduction (75.1%) in liquid medium was achieved after one day. However, Mahrous (2011) recorded maximum cholesterol reduction from liquid medium by probiotics bacteria (24.32 to 45.3%) at 20 h.

The cholesterol reduction by the two highly degradable bacterial isolates *E. faecalis* W7 and *E. faecium* Y1 were studied at different concentrations from 50 to 150 µg/ml of cholesterol in culture broth medium (Figure 5) and the obtained results indicated that the highest reduction of cholesterol by both tested isolates was recorded at 100 µg/ml. The reduction of cholesterol by *E. faecalis* W7 in the cultural medium increased gradually with increasing cholesterol concentration until it reached the highest value of 68.8% at cholesterol concentration of 100 µg/ml. However, the maximum percentage of cholesterol removal by *E. faecium* Y1 was 90% at 100 µg/ml, then decreased at higher cholesterol concentrations. In a similar study, Mahrous (2011) found that maximum cholesterol reduction by *Lactobacillus acidophilus* P106 in liquid medium was obtained at 70 µg/ml cholesterol. While Hassanein et al. (2013) reported that *L. lactis* KF147 was able to remove 66.8% of cholesterol from MRS media supplemented with 100 µg/ml. Also, Pereira and Gibson (2002) reported high cholesterol removal ability (47%) by lactic acid bacteria and bifidobacteria isolated from human gut in broth media containing 100 µg/ml cholesterol. Guo et al. (2016) reported cholesterol reduction (41.29 to 56.61%) from liquid media containing 100 µg/ml of cholesterol by *Enterococcus* strains. Excessive cholesterol concentration may suppress bacterial ability of cholesterol reduction. Above a critical substrate concentration, a decreased water activity and onset of plasmolysis combine to cause a decrease in the rates of assimilation (Roukas, 1993).

The percentage of cholesterol removal at different growth stages of LAB were recorded in Figure 6. The maximum removal ability of *E. faecalis* W7 (93.5%) was observed at 42 h inoculum age, but in case of *E. faecium* Y1, the maximum removal value (94%) was reached at 18 h. In general, the results revealed that maximum cholesterol reduction was observed during the exponential growth phase and maximum biomass production when the cultures attained the stationary phase in which the medium nutrients is soon depleted and enriched with inhibitory products leading to decrease in bacterial biomass and rate of cholesterol removal (Kumar et al., 2013).

The effect of inoculum size on the cholesterol removal percentage is as shown in Figure 7. The highest value of cholesterol removal percentage by the two bacterial isolates *E. faecalis* W7 and *E. faecium* Y1 were 94 and 98%, respectively. In the case of isolate Y1, the ability of bacteria to remove cholesterol increased gradually with increasing inoculum size to reach maximum value of 98% at 3% inoculum size, while the maximum removal of cholesterol was reached at 2% inoculum size of isolate *E. faecalis* W7 then decreased slightly and became stable. The maximum removal percentage of cholesterol was recorded with inoculum size ranging from 2 to 3%. Our findings is in accordance with the study conducted by Kumar et al. (2013) who suggested that the ability of *L. casei* LA-1 to assimilate cholesterol is highly dependent on its growth, perhaps reflecting the growth of the inoculum used. Mahrous (2011) and Pereira and Gibson (2002) reported optimum cholesterol reduction with 1%

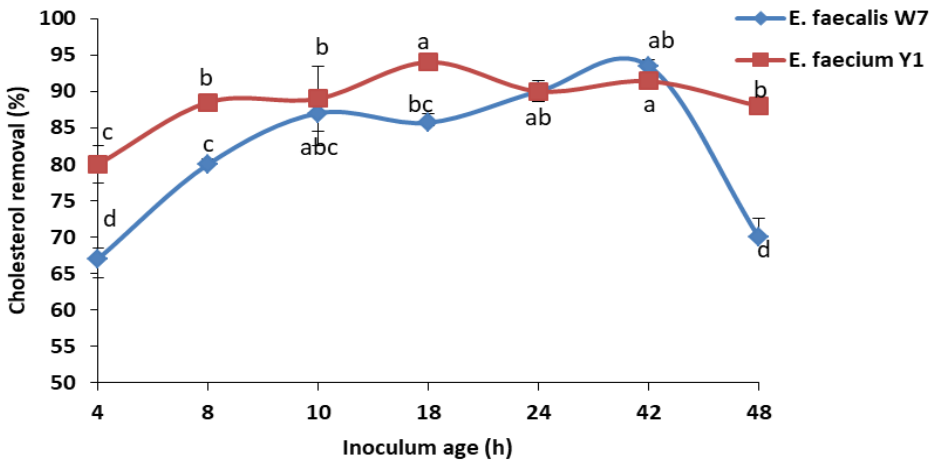

Figure 6. Effect of inoculum age on cholesterol removal percentage by *Enterococcus faecalis* W7 and *Enterococcus faecium* Y1.

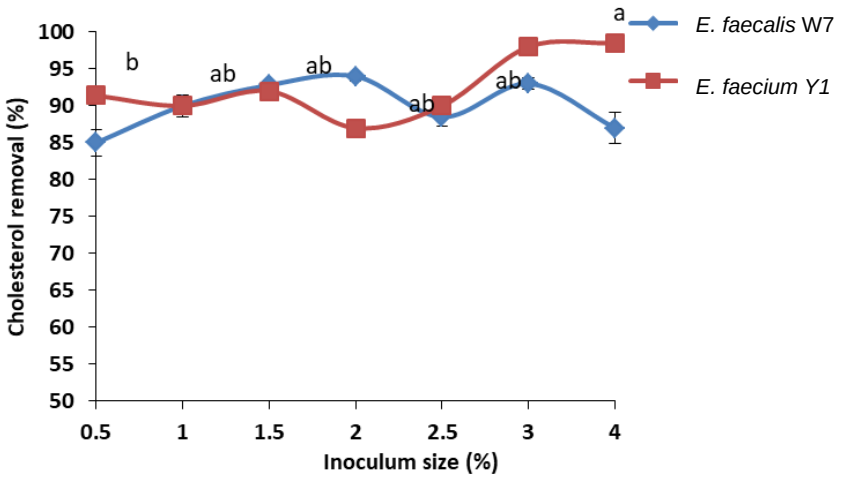

Figure 7. Effect of inoculum size on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

Table 3. Gas chromatography – Mass spectra analysis of cholesterol degradation products by *Enterococcus faecium* Y1.

Test (h)	Retention time (RT)	Compounds	Area (%)	Molecular weight	Molecular formula
0	51.08	Cholesterol	5.39	386	$C_{27}H_{46}O$
4	51.09	Cholest-5-ene-16,22-dione,3á,26-dihydroxy-, 3-acetate	1.48	562	$C_{40}H_{50}O_2$
8	51.08	5á-Cholestane-3à,25-diol	0.78	404	$C_{27}H_{48}O_2$
24	51.07	5á-Cholestane-3à,25-diol	0.24	404	$C_{27}H_{48}O_2$

inoculum size of probiotic bacteria.

The results of GC-mass spectra analysis of *E. faecium* Y1 extract at interval incubation time is shown in Table 3. At zero time, the prominent peak was cholesterol which was observed at 51.08 min of retention time, while at 4 h of incubation cholest-5-ene-16, 22-dione, 3á,26-dihydroxy-, 3-acetate, was reported at 51.09 min of retention time and the peak of cholesterol became less predominant. At incubation time of 8 h, 5á-cholestane-3à,25-diol resulted in the peak at retention time 51.08 and the same result was observed at 24 h of incubation but by less amount (Figure 8). The results indicated that the bacterial isolate

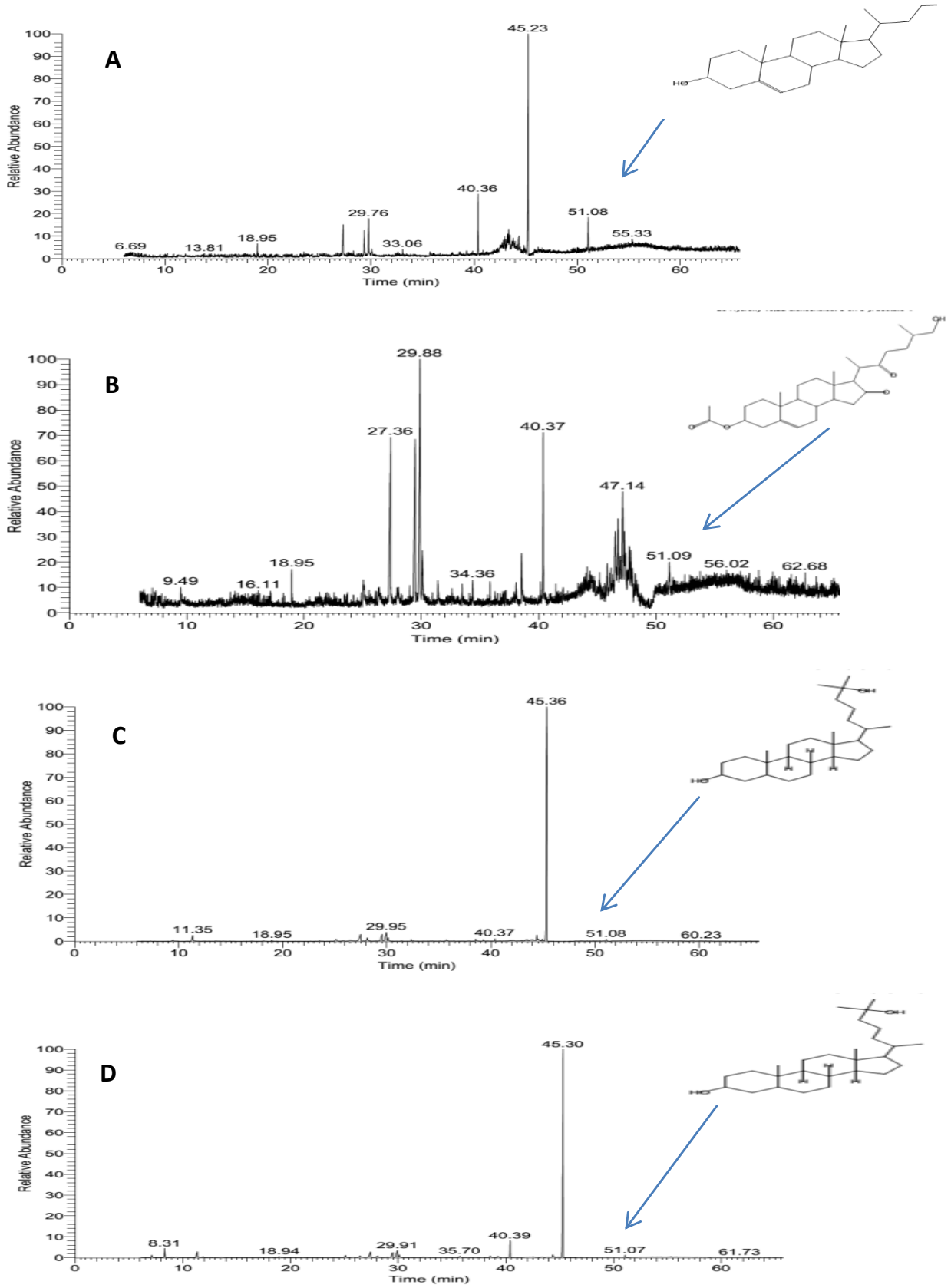

Figure 8. GC-mass analysis of cholesterol degradation by *Enterococcus faecium* Y1 at (A) zero time, (B) 4 h, (C) 8 h, and (D) 24 h of incubation period.

E. faecium Y1 transformed cholesterol to 5á-cholestane-3à,25-diol through oxidation and other chemical reactions whereas the reduced amount of cholesterol was utilized by the bacterial cells for its assimilation metabolism. The produced metabolites were ketonic derivatives of cholesterol and this due to transformation of some functional groups of cholesterol giving rise to ketones (Saranya, 2014). These results are in accordance with Suzuki et al. (1986), who reported that some intestinal bacteria such as *Bifidobacterium, Eubacterium, Lactobacillus, Enterobacteriaceae, Clostridium* and *Enterococcus* decompose cholesterol via the ChoX enzyme to cholest-4-en-3-one.

The reduction of cholesterol levels *in vitro* or *in vivo* by microorganisms may take place via enzymatic processes (Kovalenko et al., 2004) as the bacteria may decompose cholesterol due to their production of extracellular cholesterol oxidase (ChoX). ChOX attacks sterols at the 3β-hydroxyl positions to form 4-cholestenone and H_2O_2. This is the first step of microbial degradation of cholesterol in the oxidation of the 3β-hydroxyl group by ChOX (Sih et al., 1967) followed by the degradation of the side-chain (Shen et al., 1997; MacLachlan et al., 2000) by enzyme-complex possibly involving cytochrome P-450 and lyases leading to 4-androstene-3,17-dione, or 1,4-androstadiene-3 or 17-dione, and eventual breakdown of the steroid moiety with carbon dioxide and water being the final products. Also, the oxidation of cholesterol may results in the intermediate 3-ketosteroid as the final product (Motteran et al., 2001). Liu and Shan (2006) reported that cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one are produced from the oxidation of cholesterol degradation by bacteria. Doukyu (2009) reported that the ChoX enzyme from *Burkholderia cepacia* strain ST-200 produces 6-beta-hydroperoxycholest-4-en-3-one from cholesterol. Liu and Shan (2006) reported that cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one are produced through the oxidation of cholesterol. The end products resulted from cholesterol degradation by microorganisms and their quantity varies between the different microbial strains and depends on the incubation period of the tested strains.

Conclusion

The use of fermented dairy products containing probiotic lactic acid bacteria is considered as one of attractive and effective treatment for hypercholestermia. Therefore, obtaining a novel probiotic strains with cholesterol reducing abilities is required and will be safe alternative for clinical drugs. Consequently, in the present study a total of 50 different probiotic lactic acid bacterial strains were isolated from healthy infants' stool which showed various cholesterol lowering abilities in culture media. The most active isolates *E. faecalis* W7 and *E. faecium* Y1 were selected to optimize cholesterol removal process under different condition. About 90% of cholesterol reduction was achieved by *E. faecium* Y1 under the optimized cultural conditions in the broth medium. In addition, the end products which resulted from the degradation process were also followed to study their impact when this probiotic strain was applied as cholesterol lowering agent in the dairy products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of EDTA on biofilm formation and antibiotic susceptibility of multidrug resistant uropathogenic *Escherichia coli* clinical isolates in Egypt

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Urinary tract infections are mainly caused by uropathogenic *Escherichia coli* (UPEC). Biofilm-producer UPEC tends to have a high level of resistance to antibiotics and this leads to recurrent episodes of urinary tract infections. The study tested the effect of a non-antibiotic adjuvant, ethylenediaminetetraacetic acid (EDTA) on the bacterial susceptibility to antibiotics and biofilm formation by multidrug resistant (MDR) strong biofilm producer UPEC from Egypt. The ability for *in vitro* biofilm formation was detected in 88 MDR UPEC isolates in the absence and presence of two concentrations of EDTA (10 and 20 mM). The minimum inhibitory concentrations (MIC) of the tested antibiotics were detected in the presence and absence of sub-inhibitory concentration of EDTA (2 mM) by the two-fold broth microdilution method. The effect of polyvinylchloride gelatin-EDTA coat on biofilm formation by strong and moderate biofilm producers was tested. The addition of 2 mM EDTA to antibiotics resulted in a decrease in the antimicrobials MIC values with the highest effect recorded with Meropenem (81.6%) and Nitrofurantoin (61.4%). EDTA with concentrations (10 and 20 mM) and Gelatin-EDTA coat inhibited biofilm formation by strong and moderate biofilm producing UPEC by 45.8, 78.8, and 81.1%, respectively. The combination of Carbapenems with EDTA in parenteral preparations to treat life threatening infections could greatly improve the clinical outcome. There is a continuous need for the development of new strategies for treatment of MDR biofilm-producer UPEC. Novel approaches to control microbial biofilm are needed.

Key words: Ethylenediaminetetraacetic acid (EDTA), *Escherichia coli*, biofilm, antibiotic resistance.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infectious diseases affecting all ages (Neupane et al., 2016). Catheter associated UTI (CAUTI) is common in patients with indwelling bladder catheter leading to an increase in the length of hospitalization and prolonging the antibiotic therapy period than non-catheterized

patients (Jacobsen et al., 2008). Uropathogenic *Escherichia coli* (UPEC) is responsible for more than 80% of UTI in healthy people and are the most common isolates in catheterized patients with UTI (Kumar et al., 2017). The multidrug resistant (MDR) UPEC strains are major public threat worldwide (Lee et al., 2016) and are highly prevalent in Egypt (El-Sokkary and Abdelmegeed, 2015; Abdel-Moaty et al., 2016).

Biofilms are the microbial communities of the surface attached to cells embedded in a self-produced extracellular polymeric matrix (Niveditha et al., 2012) and biofilm-producers show higher resistance to antimicrobial agent and this leads to recurrent episodes and persistence of UTI (Tayal et al., 2015). Biofilm-producer UPEC are also the most common cause of UTI (Bang et al., 2016), which are difficult to treat with a single antibiotic (Wu et al., 2015). Several strategies have been tested to inhibit biofilm formation on the indwelling urinary catheter (Cai et al., 2016), including coating catheters with natural products as green tea and Dandasa, fresh garlic extract, honey and Oregano essential oil (Sadekuzzaman et al., 2015); ethylenediaminetetraacetic acid (EDTA)-gallium gelatin coating (Zhu et al., 2013); and using levofloxacin and vitamin C (El-Gebaly et al., 2012).

EDTA is a polyamine carboxylic acid used as a metal chelator with established anticoagulant activity (Raad et al., 2003) and in low concentrations act as a food preservative and in combination with antibiotics (Lerma et al., 2014); ZOSYN® (Wyeth Pharmaceuticals Inc.) is a commercially available antibiotic combination (Piperacillin/Tazobactam) for intravenous use that contains EDTA in the formulation. It is used intravenously, in combinations with vitamins and minerals in treatment of various diseases including atherosclerotic vascular disease and renal ischemia. EDTA is shown to be safe up to 40 mg/kg/body weight when administrated intravenously to swiss albino mice (Chaudhary et al., 2012) and can be administered with a daily dosage of 50 mg/kg of body weight in humans (ENDRATE®, Hospira inc).

EDTA prevents curli production and inhibits bacterial adhesion which is required for biofilm development. EDTA chelates divalent ions present in lipopolysaccharide layer of biofilm (Chaudhary et al., 2013); potentiating the antibiotic effect by enhancing the drug penetration and disrupting the lipopolysaccharide present in the outer membrane, hence increasing the porosity of membrane and increasing the drug permeability (Abd et al., 2000; Chaudhary et al., 2013).

This study was conducted to evaluate the effect of a non-antibiotic adjuvant EDTA on *in vitro* biofilm formation and the antibiotic susceptibility of clinical MDR strong biofilm-producer UPEC from Egypt.

MATERIALS AND METHODS

Bacterial strains and identification

The study was performed on a total number of 88 MDR UPEC from inpatients and outpatients. Seventy seven (77) isolates were collected from Mansoura University Hospital (Dakhalia Governorate), and 11 isolates were collected from Misr University for Science and Technology (MUST) Hospital (Giza Governorate) in the period between January 2014 and December 2015. All experiments in this study were conducted in accordance with and approval of the ethical committee at Cairo University, Cairo, Egypt with approval number MI (1045).

Identification of the isolates was done by Gram staining and isolation on MacConkey agar (Oxoid, UK) and eosin methylene blue (Oxoid, UK) (Brenner, 1984). The molecular identification of *E. coli* was done by the PCR amplification of *uspA* gene (Chen and Griffiths, 1998). Multiplex PCR for detecting *gadA, chuA, yjaA* and *TspE4.C2* genes was used to determine the phylogenetic groups for each UPEC isolate (Doumith et al., 2012).

The antibiotic susceptibility testing of 88 MDR UPEC isolates was performed by using double disk diffusion using the following antibiotics (Cockerill et al., 2012): amikacin (30 µg), amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), cefixime (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), CO-trimoxazole (25 µg), gentamicin (10 µg), levofloxacin (5 µg), nalidixic acid (30 µg), nitrofurantion (300 µg), and norfloxacin (10 µg) all were supplied from Himedia, India; aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg), and tetracycline (30 µg) were supplied from (Oxoid, UK). *E. coli* strain ATCC 25922 was used as a reference strain and the result was interpreted according to CLSI guidelines (CLSI, 2012). The isolates were classified as MDR according to Magiorakos et al. (2012).

Effect of EDTA on the bacterial susceptibility to antibiotics

The MIC of EDTA (E. MERCK. Darmstadt, GERMANY) and the following antibiotics: ciprofloxacin, levofloxacin, amoxicillin/ clavulanic acid (Sedico Pharmaceutical Co., 6th of October city, Giza, Egypt), nalidixic acid, gentamicin (Memphis Pharmaceutical Co, Cairo, Egypt), nitrofurantoin (El-Kahera Pharmaceutical Co, Cairo, Egypt), cefotaxime and ceftazidime (EPICO, 10th of Ramadan), and meropenem (AstraZeneca Co, Cairo, Egypt) were performed using the microdilution broth method (Andrews, 2001). The antimicrobials MICs were determined in the absence of EDTA and in the presence of sub MIC of EDTA (2 mM EDTA).

Effect of EDTA on curli production

The presence of curli fibers were determined using Luria-Bertani agar (L.B.) (Difco Laboratories, U.S.A) without salts containing 40 mg/L congo red dye (Aldrich Chemical Co. Ltd. England) (Baugh et al., 2013). The effect of EDTA on curli production by curli positive strains was tested using two different concentrations of EDTA (5 and 10 mM) (Chaudhary et al., 2013).

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Effect of EDTA on *in vitro* biofilm formation

The ability of *in vitro* biofilm formation was determined using the microtiter plate assay (SarojGolia et al., 2012) in a 96-well microtiter plate (Greiner Bio-one, Stuttgart, Germany), in the absence and presence of EDTA (10 and 20 mM), in triplicates. The optical density was measured at 570 nm with ELISA reader (BioTek®, MQX 200, USA) and the degree of biofilm formation was estimated (SarojGolia et al., 2012).

Effect of coating polyvinyl chloride microtiter plate with 50 mM EDTA on *in vitro* biofilm formation

Coating of polyvinyl chloride (PVC) microtiter plates was performed using an EDTA-gelatin coating according to Zhu et al. (2013) with some modifications. The surface coat was developed by adding 150 µl of a mixture of 0.5% gelatin and 50 mM EDTA in triplicates to each well of a 96-well microtiter plate (Greiner Bio-one, Stuttgart, Germany) and drying overnight at 40°C. After drying, 125 µl of fresh Brain Heart Infusion broth (Difco Laboratories, U.S.A) supplemented with 2% sucrose (EL Naser Chemical Co. Egypt) (BHIS) was transferred to each well. Finally, these wells were inoculated with 25 µl bacterial suspension (10^8 CFU/ml) and incubated at 37°C for 24 h and then washed three times with sterile phosphate buffer saline (PBS) and air dried for 45 min (Chazotte, 2012). The wells were stained with 0.1% (w/v) crystal violet (Winlab, UK) for 15 min. The excess dye was removed by washing three times with bi-distilled water and then 200 µl of 95% ethanol was added for 1 h to release the attached dye and the optical density was measured at 595 nm using ELISA reader (BioTek®, MQX 200, USA). A negative control was performed (Rukayadi and Hwang, 2006). The extent of *in vitro* biofilm formation was also measured in PVC microtiter plates coated with gelatin only and in the absence of an EDTA-gelatin coat for comparison (SarojGolia et al., 2012).

Scanning electron microscope (SEM)

The biofilms produced by strong biofilm-producers MDR UPEC in the absence and presence of two concentrations of EDTA (10 and 20 mM) were scanned using SEM (JSM-840 SEM, JEOLE ltd., Tokyo, Japan). The biofilm was prepared in 6-well cell culture plate (Greiner Bio-one, Stuttgart, Germany) using BHI broth containing 5% sucrose. The biofilm produced was fixed with glutaldehyde 2.5% (v/v) in Dulebecco PBS (PH 7.2) for 1.5 h, rinsed with PBS and then dehydrated through ethanol series. The sample was dried and coated with gold-platinum coat (Soboh et al., 1995).

RESULTS

Bacterial strains, identification and antibiotic susceptibility

A total of 88 MDR UPEC isolates presumptively identified using the conventional culture methods and molecularly identified, were included in the study. The phylogenetic analysis of the 88 UPEC isolates revealed that a percentage of 62.5% (55/88), 18.2% (16/88), 13.6% (12/88) and 5.7% (5/88) belonged to the following phylogenetic groups B2, D, A and B1, respectively. High resistance levels were recorded with ampicillin (97%, 86/88) and cefuroxime (85.2%, 75/88), while high susceptibility was recorded with amikacin (12.5%, 11/88). Several patterns of antibiotic resistance were recorded as shown in Supplementary Table S1; patterns C, D and F were recorded each in 2 isolates from Mansoura hospital and all belonged to phylogenetic group B2, while pattern G was recorded in 3 isolates; all of them were isolated from Mansoura hospital and they all belonged to phylogenetic group A.

Effect of EDTA on bacterial susceptibility to antibiotics

The addition of a sub-MIC (2 mM EDTA) with antibiotics resulted in a decrease in the antimicrobials MIC values. The decrease in the fold of antimicrobials MIC in the presence of sub-MIC of EDTA is shown in Supplementary Table S2. The highest inhibitory effect of EDTA was observed with meropenem and nitrofurantoin rendering 81.6 and 61.4%, respectively of resistant UPEC to sensitive as shown in Figure 1.

Effect of EDTA on *in vitro* biofilm formation and curli production

The degree of biofilm formation in the tested MDR UPEC clinical isolates revealed that 85.2 (75/88), 11.3 (10/88) and 3.4% (3/88) of the isolates were strong, moderate and weak biofilm producers, respectively.

The degree of *in vitro* biofilm formation was determined for strong and moderate biofilm producers (85 isolates) in the presence of two different concentrations of EDTA (10 and 20 mM). The ability of *in vitro* biofilm formation decreased with the increase in EDTA concentration as shown in Supplementary Table S3 and Figure 2; where 45.8 (39/85), 43.5 (37/85), 3.5 (3/85) and 7% (6/85) were rendered negative, weak, moderate and still strong (no effect) biofilm-producers, respectively after the addition of 10 mM EDTA. Also, 78.8 (67/85), 17.6 (15/85) and 3.5% (3/85) were rendered negative, weak and still strong (no effect) biofilm producers, respectively after the addition of 20 mM EDTA.

The curli production was detected in 67% (59/88) of tested isolates; they showed bright red colonies on congo red agar plate (CRA) and were confirmed to be curli producers. The ability for curli production was tested in the presence of two different concentrations of EDTA (5 and 10 mM), where the ability of curli production decreased by increasing the concentration of EDTA; 69.4% (41/59) and 89.8% (53/59) of curli producing isolates were negative producers after the addition of 5 and 10 mM EDTA, respectively.

SEM analysis showed reduction in biofilm formation following treatment with EDTA at both tested concentrations, with the highest reduction following the addition of 20 mM EDTA, as shown in Figure 3.

Effect of coating polyvinyl chloride microtiter plate with 50 mM EDTA on *in vitro* biofilm formation

Gelatin coating alone had no effect on biofilm formation.

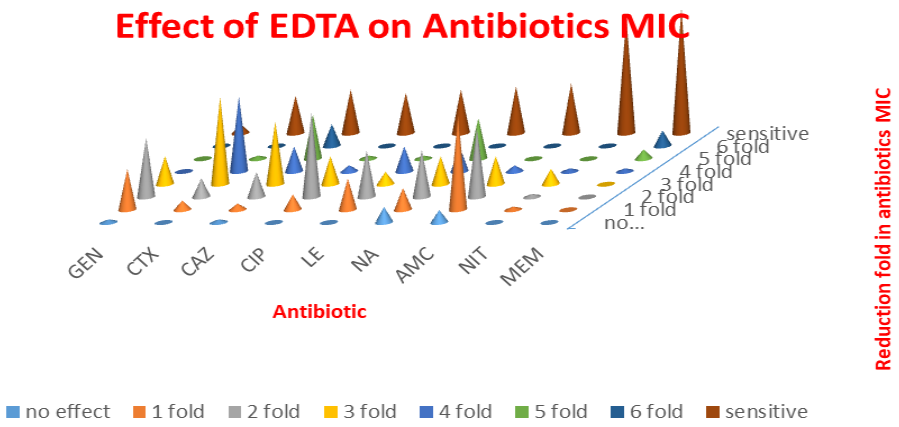

Figure 1. The effect of EDTA on bacterial susceptibility to antibiotics. GEN, Gentamicin; MEM, meropenem; CFM, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; LE, levofloxacin; NA, nalidixic acid; AMC, amoxycillin/clavulanic acid; NIT, nitrofurantoin.

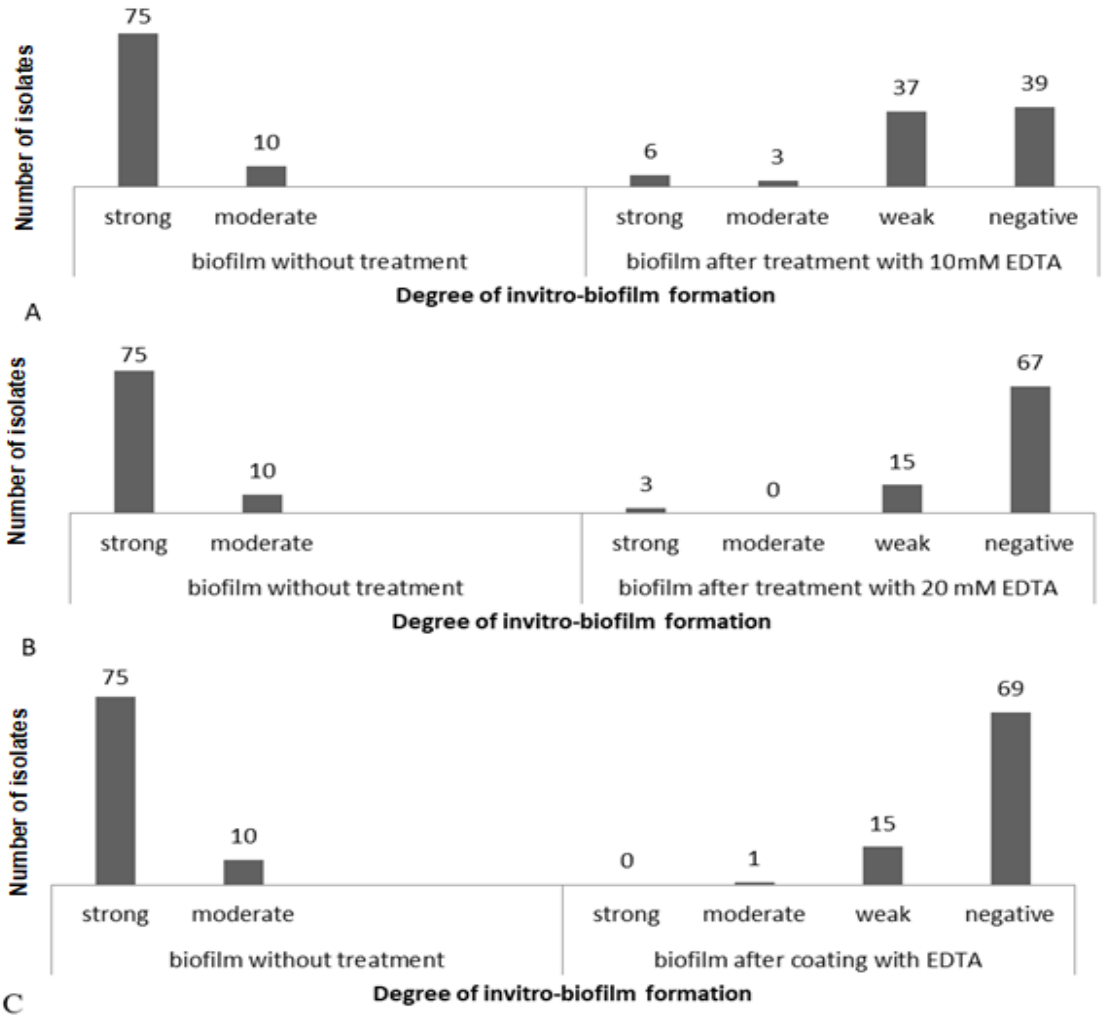

Figure 2. (A) The effect of addition of 10 mM EDTA on biofilm formation by MDR strong and moderate biofilm producing UPEC. **(B)** The effect of addition of 20 mM EDTA on biofilm formation by MDR strong and moderate biofilm producing UPEC. **(C)** The effect of coating of microtiter plates with Gelatin-EDTA coat on biofilm formation by MDR strong and moderate biofilm producing UPEC.

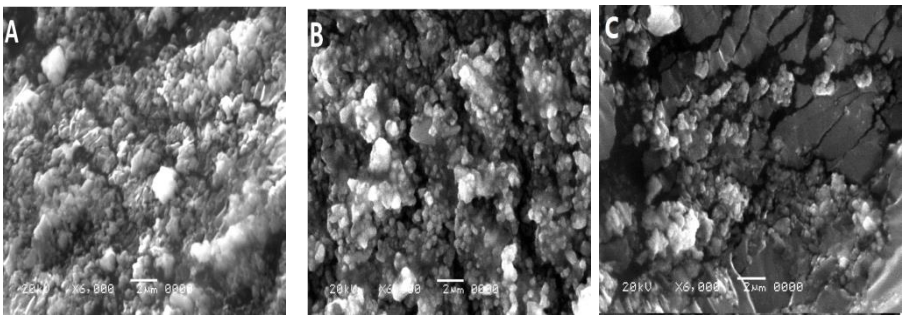

Figure 3. Scanning electron micrographs of a strong biofilm producer MDR UPEC isolate; where (A) is biofilm without treatment with EDTA, (B) biofilm in the presence of 10 mM EDTA and (C) Biofilm in presence of 20 mM EDTA.

EDTA at concentration 50 mM in gelatin coat effectively inhibited biofilm formation, where 81.1 (69/85), 17.6 (15/85) and 1.17% (1/85) were negative, weak and moderate biofilm-producers, respectively as shown in Figure 2.

DISCUSSION

UTI is a major cause of morbidity and may sometimes lead to mortality (Tajbakhsh et al., 2016) and represents a major health threat due to antibiotic resistance and high recurrence rate (Ponnusamy and Nagappan, 2013). Microbial biofilms in CAUTIs play an important role in antibiotic resistance and limits the therapeutic options (Deotale et al., 2015), so the effect of a non-antibiotic adjuvant EDTA on *in vitro* biofilm formation and antibiotic susceptibility of MDR strong biofilm producing UPEC clinical isolates from Egypt was studied.

The results revealed that using EDTA with concentrations 5 and 10 mM inhibited curli production, the first step in biofilm production. A similar study in India showed that EDTA at concentrations 4 and 5 mM can inhibit curli production (Chaudhary et al., 2013). It was also shown that EDTA with concentrations of 10 and 20 mM inhibited biofilm formation in UPEC biofilm producers by 45.8 and 78.8%, respectively and Chaudhary and colaboraters (2013) showed a decrease in biofilm formation by increasing EDTA concentrations.

In the present study, a novel approach was used to eradicate *in vitro* biofilm production and further evaluated the effect of EDTA coating of PVC microtiter plates, the material is often used for medical implants such as urinary catheter, on biofilm production. The results indicated that EDTA-gelatin coat was effective in inhibiting biofilm formation in 81.1% of tested isolates. Another study in China used EDTA and gallium coat in gelatin to inhibit the bacterial biofilms (Zhu et al., 2013). Trials to sustain the release of EDTA in wound dressings and contact lenses were done, using the therapeutic polymer of chitosan-EDTA (Netsomboon et al., 2017) and polylactic-glycolic acid disc containing 10% EDTA (Nishi et al., 1996). From the results, the coating of urinary catheters using combinations of EDTA with other anti-biofilm agents could greatly improve the clinical outcome.

Very low concentrations of EDTA (2 mM) was found to reduce the antimicrobials' MIC of MDR UPEC in the findings; the reduction of antimicrobials' MIC in the presence of EDTA was highly observed with Meropenem 81.6%, Nitrofurantoin 61.4%, Levofloxacin 26.4%, Ciprofloxacin 23.2% and Nalidixic acid 20.3%, Amoxicillin Clavulanic acid 18.8%, Ceftazidime 18.7%, Cefotaxime 15.8% and Gentamycin 6.5%.

Carbapenems are broad spectrum antimicrobial agent used as last resort treatment for Gram-negative bacteria. Emergence of resistance to carbapenems is a major threat and started to increase in the Middle East, and in this study, 55.6% (49/88) of isolates were resistant to Meropenem. In similar studies from Egypt, 44% of tested Gram negative bacteria were Carbapenem resistant (Khalifa et al., 2017). High prevalence of Carbapenem resistance among Gram negative bacteria was recorded worldwide, where similar studies in North Lebanon recorded Carbapenem resistance among 24.4% of tested Enterobacteriacea (Christophy et al., 2017) and in Germany, 16% of Carbapenem resistance organisms were detected among MDR Gram negative organisms (Maechler et al., 2015). EDTA is an inhibitor of metallo β-lactamases (MBLs) activity (Franklin et al., 2006), and in the present study, the addition of 2 mM EDTA to meropenem rendered 82.7% of meropenem resistant isolates to completely sensitive ones. Yet, no pharmaceutical preparations are available in the market that combines carbapenems with EDTA in parenteral preparations to treat life threatening infections.

Conclusions

The high prevalence of MDR phenotype among strong biofilm producers UPEC from Egypt is recorded and the combination of carbapenems with EDTA in parenteral

preparations to treat life threatening infections could greatly improve the clinical outcome. There is a continuous need for the development of new strategies for treatment of biofilm-producing UPEC with MDR profile and novel approaches to control microbial biofilm are needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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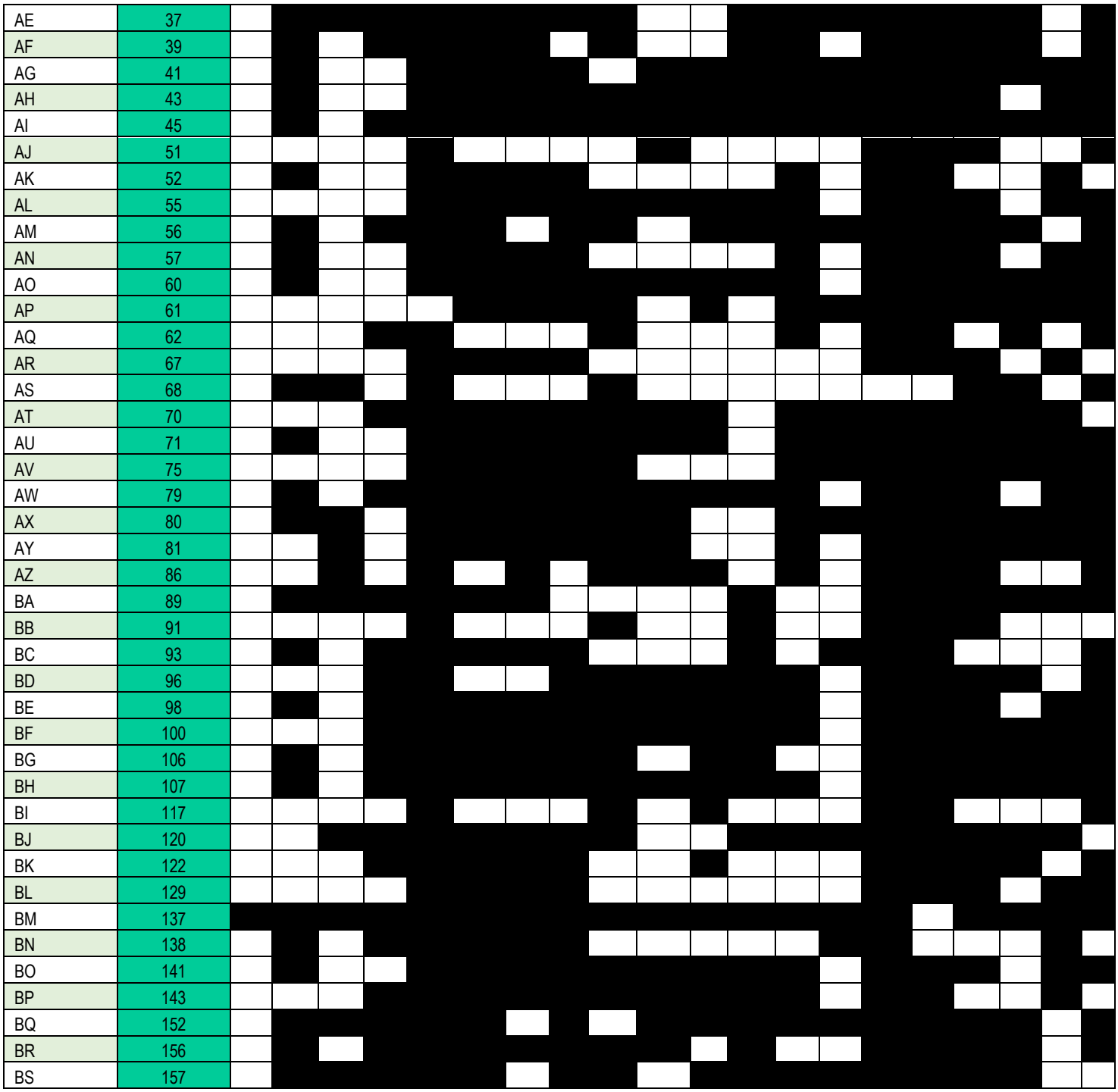
Supplementary Table S1. Antibiotic susceptibility patterns of tested isolates.

Pattern number	Sample number	AK	GEN	IPM	MEM	CXM	CFM	CTX	CAZ	COT	CIP	NX	LE	NA	NIT	AMP	AMC	A/S	TZP	ATM	TE
A	2																				
A	6																				
A	10																				
A	65																				
B	72																				
B	73																				
B	78																				
B	84																				
B	97																				
B	113																				
B	160																				
B	171																				
C	7																				
C	144																				
D	8																				
D	83																				
E	69																				
E	85																				
E	102																				
F	16																				
F	119																				
G	87																				
G	88																				
G	121																				
H	1																				
I	3																				
J	4																				
K	5																				
L	9																				
M	12																				
N	14																				
O	15																				
P	17																				
Q	18																				
R	19																				
S	20																				
T	22																				
U	23																				
V	27																				
W	28																				
X	29																				
Y	30																				
Z	32																				
AA	33																				
AB	34																				
AC	35																				
AD	36																				

Supplementary Table S1. Contd.



AK, Amikacin ; AMC, amoxicillin/clavulanic acid ; AMP, Ampicillin ; A/S, ampicillin/sulbactam; CFM, cefixime; CTX, cefotaxime; CAZ, ceftazidime; CXM, cefuroxime; CIP, ciprofloxacin (), CO-Trimoxazole (COT), Gentamicin (GEN), Levofloxacin (LE), Nalidixic Acid (NA), Nitrofurantion (NIT), Norfloxacin (NX), Aztreonam (ATM), Imipenem (IMP), Meropenem (MEM), Piperacillin/Tazobactam (TZP), and Tetracycline (TE). Black filled cells represent resistant results while white cells represent susceptible results.

Supplementary Table S2. Antibiotics MIC in the absence and presence of subMIC of EDTA.

Isolates number	NIT MIC		NA MIC		GEN MIC		MEM MIC		CIP MIC		LE MIC		AMC MIC		CAZ MIC		CTX MIC	
	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA	- EDTA	+ EDTA
1	<2	<2	>512	32	<2	<2	<2	<2	<2	<2	<2	<2	512	128	<2	<2	<2	<2
2	<2	<2	>512	32	<2	<2	<2	<2	128	<2	64	16	512	<2	>512	32	>512	<2
3	<2	<2	512	32	<2	<2	<2	<2	128	16	64	16	256	32	>512	16	>512	64
4	64	4	>512	32	<2	<2	<2	<2	<2	<2	<2	<2	128	64	>512	32	256	64
5	256	4	>512	8	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	512	32	512	64
6	<2	<2	256	<2	<2	<2	<2	<2	128	<2	64	16	512	<2	512	4	>512	<2
7	<2	<2	>512	32	>512	256	<2	<2	128	4	64	16	256	128	>512	128	>512	64
8	256	4	512	32	>512	256	<2	<2	128	32	64	16	256	128	>512	16	512	64
9	128	8	>512	512	<2	<2	<2	<2	128	32	<2	<2	512	256	<2	<2	>512	64
10	<2	<2	>512	128	<2	<2	<2	<2	64	16	32	16	256	128	>512	32	512	32
12	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	128	128	<2	<2	<2	<2
14	<2	<2	>512	8	<2	<2	512	<2	128	32	<2	<2	256	128	>512	32	512	128
15	512	64	512	32	<2	<2	512	8	<2	<2	<2	<2	256	128	512	16	512	256
16	128	4	>512	128	>512	128	512	<2	128	16	128	16	128	64	>512	16	512	64
17	<2	<2	512	128	<2	<2	8	<2	<2	<2	<2	<2	64	32	>512	4	>512	128
18	256	32	>512	512	<2	<2	512	<2	<2	<2	<2	<2	128	64	>512	16	>512	128
19	256	64	512	256	<2	<2	64	<2	8	<2	16	<2	128	64	128	8	128	<2
20	128	32	>512	>512	>512	256	512	<2	128	32	64	16	256	16	128	8	256	32
22	8	4	>512	256	<2	<2	16	<2	128	8	<2	<2	32	16	<2	<2	<2	<2
23	16	<2	>512	32	<2	<2	512	<2	64	<2	<2	<2	64	8	32	4	512	64
27	128	32	>512	256	<2	<2	8	<2	128	8	64	16	128	8	512	128	512	64
28	8	4	>512	256	<2	<2	<2	<2	64	8	<2	<2	16	8	<2	<2	<2	<2
29	128	4	512	32	<2	<2	<2	<2	128	16	256	16	64	8	>512	128	>512	64
30	128	4	>512	256	<2	<2	<2	<2	128	<2	<2	<2	64	64	>512	32	>512	64
32	64	8	>512	32	>512	512	32	<2	64	<2	<2	<2	256	128	>512	32	512	64
33	<2	<2	<2	<2	<2	<2	64	<2	<2	<2	<2	<2	128	64	>512	4	512	128
34	128	8	512	16	<2	<2	16	<2	<2	<2	<2	<2	16	4	>512	16	>512	64
35	<2	<2	>512	<2	>512	512	<2	<2	128	32	256	32	128	128	16	<2	>512	64
36	<2	<2	>512	<2	<2	<2	32	<2	128	64	<2	<2	128	64	<2	<2	512	64
37	128	4	>512	256	>512	128	64	<2	<2	<2	128	8	256	32	256	8	512	64
39	<2	<2	512	32	>512	128	64	<2	<2	<2	64	4	128	64	<2	<2	512	64
41	64	8	>512	256	512	128	<2	<2	128	16	256	16	512	128	>512	64	>512	128
43	256	4	512	64	256	64	<2	<2	128	16	64	8	64	8	>512	4	512	<2
45	128	4	>512	32	16	<2	512	<2	64	16	128	16	512	128	>512	32	512	64
51	<2	<2	<2	<2	<2	<2	<2	<2	64	<2	<2	<2	128	4	<2	<2	<2	<2
52	<2	<2	>512	256	64	4	<2	<2	<2	<2	<2	<2	64	<2	>512	4	256	4
55	16	4	>512	64	<2	<2	<2	<2	128	32	32	4	128	64	128	16	>512	128
56	256	4	>512	32	>512	256	512	8	<2	<2	128	4	256	32	128	16	<2	<2

Supplementary Table S2. Contd.

57	8	4	>512	32	512	128	<2	<2	<2	<2	<2	<2	128	16	>512	32	512	<2
60	<2	<2	>512	128	>512	128	<2	<2	128	32	64	16	256	64	512	<2	512	128
61	512	64	256	128	<2	<2	<2	<2	<2	<2	<2	<2	256	128	256	16	128	64
62	<2	<2	256	32	<2	<2	>512	<2	64	32	<2	<2	128	8	<2	<2	<2	<2
65	<2	<2	>512	128	<2	<2	<2	<2	128	<2	32	4	128	8	128	64	>512	64
67	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	64	16	32	4	>512	64
68	<2	<2	<2	<2	64	16	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
69	<2	<2	>512	32	<2	<2	<2	<2	128	32	128	16	64	16	512	128	>512	64
70	128	16	>512	32	<2	<2	64	4	64	16	<2	<2	128	64	512	64	256	64
71	128	64	>512	>512	>512	512	<2	<2	128	32	<2	<2	512	128	>512	128	512	<2
72	32	2	512	32	512	128	16	<2	128	32	256	8	512	128	>512	64	>512	64
73	512	4	512	4	>512	128	16	<2	64	16	256	4	>512	128	>512	128	512	<2
75	32	4	256	<2	<2	<2	<2	<2	<2	<2	<2	<2	256	128	16	<2	512	<2
78	512	64	>512	256	128	64	16	<2	128	32	256	16	>512	128	>512	128	>512	64
79	<2	<2	>512	16	256	64	16	<2	64	32	32	4	>512	64	512	32	256	<2
80	64	8	32	32	128	64	<2	<2	32	8	<2	<2	256	64	>512	128	>512	64
81	256	4	>512	>512	<2	<2	4	<2	64	32	<2	<2	64	16	>512	16	256	64
83	32	4	256	16	32	8	<2	<2	64	32	8	<2	128	32	>512	128	>512	64
84	128	4	>512	>512	128	64	32	<2	64	16	64	16	512	128	>512	32	>512	64
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86	<2	<2	128	16	<2	<2	<2	<2	128	<2	<2	<2	128	64	<2	<2	64	<2
87	64	<2	>512	256	>512	512	16	<2	<2	<2	256	16	512	128	>512	32	512	64
88	256	4	>512	256	128	32	32	<2	<2	<2	256	16	>512	128	>512	16	>512	64
89	<2	<2	<2	<2	256	128	64	<2	<2	<2	256	16	256	32	<2	<2	>512	64
91	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	16	<2	64	16	<2	<2	<2	<2
93	64	4	<2	<2	256	64	16	<2	<2	<2	64	16	128	64	128	16	>512	64
96	<2	<2	>512	128	<2	<2	32	<2	128	32	64	16	128	64	64	<2	<2	<2
97	64	<2	>512	128	>512	512	256	8	128	32	256	64	128	64	>512	128	>512	64
98	<2	<2	>512	16	>512	512	16	<2	128	32	64	16	128	8	>512	128	512	64
100	16	4	>512	512	<2	<2	8	<2	128	32	32	16	128	64	128	128	128	64
102	<2	<2	>512	512	<2	<2	<2	<2	256	32	32	16	64	64	128	32	512	64
106	<2	<2	<2	<2	>512	256	512	8	<2	<2	32	<2	64	16	256	32	512	64
107	<2	<2	>512	<2	>512	512	64	<2	128	32	256	16	256	128	>512	128	>512	64
113	512	4	>512	32	>512	512	64	<2	128	16	64	16	256	128	>512	128	>512	64
117	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	128	8	<2	<2	<2	<2
119	256	64	>512	512	>512	512	512	8	128	<2	64	4	128	64	>512	32	512	64
120	128	4	>512	32	<2	<2	16	<2	<2	<2	32	16	512	128	512	64	512	64
121	128	4	32	8	256	64	16	<2	32	8	64	4	512	128	>512	32	>512	64
122	<2	<2	<2	<2	<2	<2	64	<2	<2	<2	<2	<2	64	16	256	64	512	64
129	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	256	128	128	32	256	<2
137	128	4	>512	256	>512	256	16	<2	128	32	32	16	<2	<2	256	128	>512	64

Supplementary Table S2. Contd.

138	512	64	4	<2	512	128	64	<2	<2	<2	<2	<2	<2	<2	128	32	256	32
141	<2	<2	>512	256	<2	<2	<2	<2	128	<2	32	16	256	32	128	16	512	64
143	<2	<2	>512	512	<2	<2	16	<2	128	<2	32	16	128	8	>512	64	512	64
144	<2	<2	512	<2	128	128	<2	<2	128	16	32	16	32	8	>512	64	512	64
152	256	4	512	32	512	128	512	8	128	16	64	16	512	128	256	64	<2	<2
156	<2	<2	<2	<2	>512	128	128	4	128	32	32	16	64	16	>512	64	256	32
157	128	4	256	32	>512	256	16	<2	<2	<2	32	4	128	64	>512	32	<2	<2
160	256	8	512	128	>512	128	32	<2	128	32	128	64	512	128	512	64	512	64
171	128	4	>512	128	>512	128	64	<2	128	32	16	4	256	64	16	4	>512	64

GEN, Gentamicin; MEM, meropenem; CFM, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; LE, levofloxacin; NA, nalidixic acid; AMC, amoxycillin/clavulanic acid; NIT, nitrofurantoin.

Supplementary Table S3. The ability of *in vitro* biofilm formation by strong and moderate biofilm-producers MDR UPEC in the presence of increased concentrations of EDTA.

Isolates number	Biofilm without treatment	Biofilm after 10 mM EDTA	Biofilm after 20 mM EDTA	Biofilm after coating with 50 mM EDTA
1	Strong	Negative	Negative	Negative
2	Strong	Negative	Negative	Negative
3	Strong	Weak	Negative	Negative
4	Moderate	Negative	Negative	Negative
5	Strong	Weak	Negative	Weak
6	Moderate	Negative	Negative	Negative
7	Strong	Negative	Negative	Negative
8	Strong	Negative	Negative	Negative
9	Strong	Negative	Negative	Weak
10	Weak	Negative	Negative	Negative
12	Moderate	Negative	Negative	Negative
14	Strong	Negative	Negative	Negative
15	Strong	Weak	Negative	Negative
16	Strong	Weak	Negative	Negative
17	Strong	Negative	Negative	Negative
18	Strong	Weak	Negative	Negative
19	Strong	Weak	Negative	Negative
20	Strong	Weak	Negative	Negative
22	Moderate	Negative	Negative	Negative
23	Weak	Negative	Negative	Negative
27	Strong	Weak	Negative	Negative
28	Strong	Weak	Negative	Weak

Supplementary Table S3. Contd.

29	Strong	Weak	Negative	Weak
30	Strong	Negative	Negative	Moderate
32	Strong	Weak	Negative	Weak
33	Strong	Weak	Negative	Weak
34	Strong	Weak	Weak	Negative
35	Strong	Weak	Negative	Negative
36	Strong	Negative	Negative	Negative
37	Moderate	Negative	Negative	Negative
39	Strong	Weak	Negative	Negative
41	Moderate	Negative	Negative	Negative
43	Strong	Negative	Negative	Negative
45	Strong	Negative	Negative	Negative
51	Strong	Weak	Negative	Negative
52	Strong	Negative	Negative	Negative
55	Strong	Weak	Negative	Negative
56	Strong	Weak	Negative	Negative
57	Strong	Strong	Strong	Negative
60	Strong	Weak	Negative	Negative
61	Strong	Negative	Negative	Negative
62	Moderate	Moderate	Weak	Negative
65	Strong	Negative	Negative	Negative
67	Strong	Moderate	Weak	Negative
68	Strong	Weak	Negative	Negative
69	Strong	Weak	Negative	Negative
70	Strong	Negative	Negative	Negative
71	Strong	Negative	Negative	Negative
72	Strong	Weak	Negative	Negative
73	Strong	Negative	Negative	Negative
75	Strong	Weak	Weak	Weak
78	Strong	Negative	Negative	Negative
79	Strong	Weak	Weak	Negative
80	Strong	Weak	Weak	Weak
81	Strong	Weak	Negative	Negative
83	Strong	Negative	Negative	Negative
84	Strong	Strong	Strong	Weak
85	Strong	Strong	Strong	Weak
86	Strong	Weak	Weak	Weak

Supplementary Table S3. Contd.

87	Strong	Weak	Weak	Negative
88	Strong	Strong	Weak	Weak
89	Strong	Moderate	Weak	Weak
91	Strong	Strong	Weak	Weak
93	Strong	Weak	Weak	Weak
96	Strong	Negative	Negative	Negative
97	Strong	Negative	Negative	Negative
98	Strong	Weak	Weak	Negative
100	Strong	Weak	Negative	Negative
102	Moderate	Negative	Negative	Negative
106	Strong	Weak	Negative	Negative
107	Strong	Negative	Negative	Negative
113	Strong	Strong	Weak	Negative
117	Strong	Weak	Weak	Negative
119	Strong	Weak	Negative	Negative
120	Strong	Negative	Negative	Negative
121	Strong	Negative	Negative	Negative
122	Moderate	Negative	Negative	Negative
129	Strong	Negative	Negative	Negative
137	Strong	Negative	Negative	Negative
138	Strong	Negative	Negative	Negative
141	Weak	Negative	Negative	Negative
143	Strong	Weak	Negative	Negative
144	Moderate	Negative	Negative	Negative
152	Strong	Negative	Negative	Negative
156	Strong	Negative	Negative	Negative
157	Strong	Negative	Negative	Negative
160	Strong	Weak	Negative	Weak
171	Strong	Weak	Negative	Negative


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