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Original article

Cell free preparations of probiotics exerted antibacterial and antibiofilm activities against multidrug resistant *E. coli*

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ABSTRACT

The sharp increase in antibiotic resistance imposes a global threat to human health and the discovery of effective antimicrobial alternatives is needed. The use of probiotics to combat bacterial pathogens has gained a rising interest. Pathogenic *Escherichia coli* is causative of multiple clinical syndromes such as diarrheal diseases, meningitis and urinary tract infections. In this work, we evaluated the efficacy of probiotics to control multidrug-resistant *E. coli* and reduce their ability to form biofilms. Six *E. coli* resistant to at least five antibiotics (Ceftazidime, Ampicillin, Clarithromycin, Amoxicillin + Clavulanic Acid and Ceftriaxone) were isolated in this work. Preparations of cell-free spent media (CFSM) of six probiotics belonging to the genus *Bifidobacterium* and *Lactobacillus* which were grown in Man-Rogosa-Sharpe (MRS) broth exhibited strong antibacterial activity (inhibition zones of 11.77–23.10 mm) against all *E. coli* isolates. Two *E. coli* isolates, namely *E. coli* WW1 and IC2, which were most resistant to all antibiotics were subjected to antibiofilm experiments. Interestingly, the CFSM of MRS fermented by all probiotics resulted in inhibition of biofilm formation while *B. longum* caused highest inhibition (57.94%) in case of *E. coli* IC2 biofilms and *L. plantarum* was responsible for 64.57% reduction of *E. coli* WW1 biofilms. On the other hand, CFSM of skim milk fermented by *L. helveticus* and *L. rhamnosus* exhibited a slight inhibitory activity against IC2 isolate (inhibition percentage of 31.52 and 17. 68, respectively) while WW1 isolate biofilms was reduced by CFSM of milk fermented by *B. longum* and *L. helveticus* (70.81 and 69.49 reduction percentage, respectively). These results support the effective use of probiotics as antimicrobial alternatives and to eradicate biofilms formed by multidrug-resistant *E. coli*.

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1. Introduction

Escherichia coli is considered as a member of the dominant flora inhabiting the human colonic region. Although most of the members of this species are harmless to the intestinal lumen, some acquired virulence factors and can cause a wide range of human diseases (Nataro et al., 1998). The pathogenic *E. coli* is causative of three clinical syndromes: urinary tract infections, enteric/diarrheal diseases and meningitis (Kaper et al., 2004). The key mechanisms by which *E. coli* cause enteric diseases include attachment and colonization of the intestinal mucosa, manipulation of the host cell cytoskeleton or evading host immune defenses, and production of toxins (Torres, 2009). Six categories of pathogenic *E. coli* are well-studied and comprise enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enterohaemorrhagic *E. coli*, diffusely adherent *E. coli* and enteroinvasive *E. coli* (Croxen and Brett Finlay, 2012; Kaper et al., 2004). Diarrheal diseases caused by *E. coli* worldwide were estimated to be nearly 300–800 million clinical cases and 300,000–500,000 deaths every year (Torres, 2009); this highlights the significance of pathogenic *E. coli* in global health burden imposed by diarrheal diseases. Current interventions to inactivate/eliminate pathogenic *E. coli* involve the use of antibiotics. However, many pathogenic strains that are able to cause illness have become resistant to antibiotics (Collignon, 2009; Tadesse et al., 2012). The rise of antibiotic resistance has motivated research to find out antimicrobial alternatives of which probiotics have gained a growing interest.

The use of *Lactobacillus* spp. and *Bifidobacterium* spp. as probiotics to combat microbial infections and boosting human health inspired many studies. Probiotics have been associated with the treatment of gastroenteritis (Chai et al., 2013),

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antibiotic-associated diarrhoea (Friedman, 2012), necrotizing enterocolitis (Alfaleh et al., 2011), pouchitis (Wall et al., 2011), inflammatory bowel diseases (Schultz, 2008), allergic disorders and others (Minocha, 2009). The antimicrobial activity of a range of probiotics against pathogens including *E. coli* has been reported (Tejero-Sariñena et al., 2012). In addition, down-regulation of virulence genes expression in *E. coli* O157: H7 using bioactive molecules secreted by probiotics has been described (Medellin-Pena et al., 2007). Moreover, probiotics were capable of reduction of *E. coli* O157: H7 and *E. coli* O127: H6 adhesion to epithelial cells monolayers (Erdem et al., 2007). The ability of pathogenic *E. coli* to form biofilms that contribute to their pathogenicity was documented (Beloin et al., 2008; Martinez-Medina et al., 2009). The antibiofilm activity of probiotics against pathogenic *E. coli* is poorly studied. Here, we aimed to better utilize probiotics to combat multidrug-resistant *E. coli* and reduce their ability to form biofilms.

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are summarized in Table 1. Six strains of probiotics belonging to the genera *Lactobacillus* and *Bifidobacterium* were grown in Man-Rogosa-Sharpe (MRS, Oxoid, Hampshire, England) agar medium for 24 h at 37 °C with 5% CO_2. A single colony from each strain was transferred into MRS broth under the same incubation conditions for 24 h for the preparation of the cell-free spent medium (CFSM). For pathogenic *E. coli* isolation and cultivation, clinical specimens from the intensive care unit (ICU) of Benha University Teaching hospital and sewage water samples from Benha city were collected. All samples were collected aseptically and transferred immediately to culture on MacConkey agar and Eosin methylene blue agar (Oxoid) followed by incubation aerobically at 37 °C for 24 h. The isolated colonies were further identified using Vitek 2 system (Biomerieux, USA). For the preparation of milk fermented by probiotics, a reconstituted skim milk powder (Nestle, Cairo, Egypt) was heated at 95 °C for 30 min and then cooled (4 °C) overnight. A 24 h fresh culture of the six probiotic strains was inoculated individually in the milk and then incubated under anaerobic conditions at 37 °C for 24 h.

2.2. Antibiotic susceptibility testing

Susceptibility testing was performed using the disc diffusion (modified Kirby Bauer) method (Biemer, 1973) for the following antibiotics (Oxoid, UK); Ampicillin (AM 10 μg), Cefotaxime (CTX 30 μg), Amikacin (AK 30 μg), Cefoxitin (FOX 30 μg), Amoxicillin + Clavulanic Acid (AMC 20 + 10 μg), Ceftriaxone (CRO 30 μg), Ciprofloxacin (CIP 5 μg), Clarithromycin (CL 15 μg), Ceftazidime (CAZ 30 μg). The results were inferred according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2013).

Table 1
Bacterial strains used in this study.

Bacterial Strain	Source
Lactobacillus acidophilus EMCC 1324 (La) *Lactobacillus helveticus* EMCC 1654 (Lh) *Lactobacillus plantarum* ss. *plantarum* EMCC 1027 (Lp) *Lactobacillus rhamnosus* EMCC 1105 (Lr) *Bifidobacterium longum* EMCC 1547 (BL) *Bifidobacterium bifidum* EMCC 1334 (Bb)	Egypt Microbial Culture Collection, Microbiological Resources Centre, Ain-Shams University, Cairo, Egypt
E. coli IC1 *E. coli* IC2 *E. coli* IC3 *E. coli* IC4 *E. coli* WW1 *E. coli* WW2	This study

2.3. Preparation of the cell-free spent medium (CFSM) of probiotics

The preparation of CFSM of each probiotic strain was performed as described previously (Bayoumi and Griffiths, 2012). Briefly; overnight cultures of the six probiotic strains grown in MRS broth at 37 °C were diluted 1:100 with fresh medium and allowed to grow under same conditions to an optical density at 600 nm of 1.6 ($\sim 1 \times 10^8$ cells/ml), the cells were then removed using centrifugation at 6000*g* at 4 °C for 10 min. The supernatant was filter-sterilized with 0.2 μm–pore-size filter, and referred to as cell-free spent medium (CFSM). In case of the milk fermented by probiotics, CFSM was obtained by centrifugation of the fermented milk at 10,000*g* at 15 °C for 15 min and then the supernatant was filter sterilized as mentioned above. The CFSM of all probiotic strains was stored at −20 °C until use for further assays.

2.4. Antibacterial activity of the probiotic CFSM

The *E. coli* test isolates were activated on trypticase soy agar (Oxoid) for 24 h at 37 °C. Suspension of each test bacteria of 10^9 CFU/ml was prepared by growing each bacterium in trypticase soy broth (Oxoid) for 24 h at 37 °C. The agar diffusion method was used to determine the inhibition zone of the test bacteria (Cruz et al., 2001). First, a bottom layer (10 ml) of TSA was prepared in the petri-dish. Secondly, a top layer of molten and cooled TSA (5 ml) mixed with each test bacteria suspension (10^9 CFU/ml) was poured on the bottom layer. Five 6 mm in diameter wells were prepared in each plate, and 100 μl of the probiotic CFSM was introduced in each well. The test bacteria were incubated for 24 h at 37 °C and the inhibition zones were measured in millimetres.

2.5. Antibiofilm assay

2.5.1. Inoculum preparation

Initial bacterial inoculum for the biofilm experiment was prepared as mentioned previously (Arora and Kaur, 1999). A single colony of *E. coli* isolates was transferred into 5 ml of nutrient broth (Oxoid, UK) and incubated for 16 h at 37 °C to obtain the cells at exponential phase.

2.5.2. Evaluation of antibiofilm potential

The anti-biofilm formation activity of CFSM prepared from probiotics was assessed as previously described (Jadhav et al., 2013). Two groups labeled test agents (probiotic CFSM) along with their experimental control (broth medium) were prepared in the microtitre plate. Supplementation with 10% (vol/vol) of all probiotic CFSM was used as suggested previously (Medellin-Pena et al., 2007; Medellin-Peña and Griffiths, 2009). In each group, 40 μl of the test antibiofilm CFSM prepared was added in triplicate to the corresponding wells of sterile 96-well microtitre plate (Sigma Aldrich, USA) except for the negative controls. Then, 160 μl of *E. coli* cultures were added into wells in all the groups (the same volume of broth medium was added instead in case of experimental control) in a final volume of 200 μl per well. The microtitre plates were sealed and incubated for 16 h at 37 °C. The biofilm biomass compared to negative control was determined using crystal violet (CV) assay. The results were expressed as the percentage of inhibition.

After the treatment periods in preceding experiments, quantification of the biomass was carried out by CV assay (Djordjevic et al., 2002). The amount of biofilm formed on wells surface is reflected by the amount of stain absorbed. After incubation, the culture

medium was discarded from each well and then all wells were washed three times with sterile distilled water to remove any loosely adhered cells. The microtitre plate was allowed to be air-dried, then further dried in the oven at 60 °C for 35 min and finally stained with 150 µl of 0.1% crystal violet. After incubating the microtitre at room temperature for 20 min, the wells were washed two times with sterile distilled water to remove the excessive unabsorbed stain. For estimating biofilm density, the absorbance was determined at 595 nm using an automated microplate reader (Sun Rise –TECAN. Inc.® USA). The mean absorbance ($OD_{595\ nm}$) of test organisms was determined and the percentage inhibition was calculated using the following formula (Eq. (1)):

$$\text{Percentage inhibition} = 100 - ((OD_{595nm}\ \text{test for positive control well}/OD_{595nm}\ \text{negative control well}) \times 100) \quad (1)$$

3. Results

3.1. Coli isolation and antibiotic susceptibility profiling

In this work, six multidrug resistant *E. coli* were isolated. They were designated as *E. coli* IC1 to *E. coli* IC4, *E. coli* WW1 to *E. coli* WW2 as they were isolated from the intensive care unit and sewage water, respectively. All isolates were resistant to at least five antibiotics (Ceftazidime, Ampicillin, Clarithromycin, Amoxicillin + Clavulanic Acid and Ceftriaxone). Only one isolate showed no resistance to Cefoxitin. In addition, three isolates were intermediate in sensitivity to amikacin while two isolates exhibited intermediate sensitivity to cefoxitin and ciprofloxacin. These results demonstrated that the multidrug resistance pattern was exhibited by all isolates of which *E. coli* WW2 and *E. coli* IC2 were the most resistant to all antibiotics and were subjected to antibiofilm experiments. The antibiotic resistance patterns were summarized in Table 2.

3.2. Antibacterial activity of cell-free preparations of probiotics

The multidrug resistant *E. coli* isolates showed sensitivity to CFSM of all probiotics (Table 3). All the isolates were inhibited to a similar extent by the six probiotics (inhibition zones ~ 13–14 mm) while highest antibacterial activity was observed in case of *B. longum*, *L. acidophilus* and *B. bifidum* against *E. coli* IC3, *E. coli* IC1 and *E. coli* WW2 (inhibition zones of 17.10 mm, 17.10 mm and 23.10 mm, respectively), respectively.

3.3. Antibiofilm activity of probiotics

The effect of CFSM of MRS fermented by probiotics on the initial attachment of *E. coli* IC2 and WW1 towards biofilm formation was observed and summarized in Table 4. In general, the two studied *E. coli* isolates showed a satisfactory ability to form biofilms while *E. coli* WW1 is a strong biofilm former as compared to IC2 strain. Interestingly, all probiotics resulted in inhibition of biofilm formation to a similar extent. *B. longum* caused the highest inhibition (57.94%) in case of *E. coli* IC2 while *L. plantarum* was responsible for 64.57% reduction of *E. coli* WW1 biofilms. *E. coli* WW1 biofilms were negatively influenced by CFSM of probiotics as compared to IC2 isolate. On the other hand, CFSM of skim milk fermented by probiotics exhibited a slight inhibitory activity against IC2 isolate using *L. helveticus* and *L. rhamnosus* (inhibition percentage of 31.52 and 17. 68, respectively). Unfortunately, the rest of probiotic strains had no inhibitory effect on IC2 biofilms (Table 5). In contrast, WW1 isolate was greatly affected by CFSM of fermented milk by all probiotics. *B. longum* and *L. helveticus* showed the strongest antibiofilm activity against the WW1 isolate (70.81 and 69.49 reduction percentages, respectively. These results demonstrated the promising inhibitory potential of CFSM of standard MRS broth medium and skim milk fermented by probiotics against biofilms formed by two multidrug resistant *E. coli* isolates.

Table 4
Antibiofilm potential mediated by CFSM of probiotics grown in MRS broth.

Probiotic strain	Mean of OD_{595nm} (% inhibition)	
	E. coli IC2	*E. coli* WW1
B. longum	0.066 ± 0.007 (57.94)	0.135 ± 0.020 (43.37)
L. acidophilus	0.067 ± 0.006 (56.90)	0.106 ± 0.007 (55.50)
B. bifidum	0.076 ± 0.010 (51.38)	0.139 ± 0.050 (41.56)
L. plantarum	0.069 ± 0.020 (55.63)	0.084 ± 0.070 (64.57)
L. helveticus	0.080 ± 0.005 (48.62)	0.113 ± 0.077 (52.44)
L. rhamnosus	0.072 ± 0.015 (54.14)	0.095 ± 0.089 (60.25)
Control	0.157 ± 0.010	0.239 ± 0.030

Table 2
Antibiotic susceptibility pattern of *E. coli* isolates to different antibiotics.

E. coli isolates	CAZ	AM	CL	AMC	CIP	FOX	CTX	AK	CRO
E. coli IC1	R*	R	R	R	I*	R	R	R	R
E. coli IC2	R	R	R	R	R	R	R	I	R
E. coli IC3	R	R	R	R	R	I	R	I	R
E. coli IC4	R	R	R	R	R	I	R	I	R
E. coli WW1	R	R	R	R	R	R	R	R	R
E. coli WW2	R	I	R	R	I	S*	R	R	R

* Denotes for Resistant (R), Intermediate (I) and Susceptible (S).

Table 3
Antibacterial activity of CFSM of six probiotics belonging to *Bifidobacterium* and *Lactobacillus* genera.

E. coli isolates	Inhibition zones (mm)					
	B. longum	*L. acidophilus*	*B. bifidum*	*L. plantarum*	*L. helveticus*	*L. rhamnosus*
E. coli IC1	14.77	17.10	16.43	15.77	16.10	14.10
E. coli IC2	14.10	14.77	15.10	15.10	16.43	13.43
E. coli IC3	17.10	16.77	15.43	12.77	–	13.43
E. coli IC4	14.77	15.77	13.77	13.77	11.77	14.43
E. coli WW1	13.43	13.43	13.77	12.77	14.10	16.10
E. coli WW2	14.77	13.10	23.10	15.43	13.10	11.43

Table 5
Antibiofilm potential mediated by CFSM of probiotics grown in skim milk.

Probiotic strain	Mean of OD_{595nm} (% inhibition)	
	E. coli IC2	*E. coli* WW1
B. longum	0.195 ± 0.077 (0)*	0.095 ± 0.028 (70.81)
L. acidophilus	0.157 ± 0.008 (0)	0.135 ± 0.017 (58.56)
B. bifidum	0.207 ± 0.090 (0)	0.201 ± 0.057 (38.34)
L. plantarum	0.213 ± 0.090 (0)	0.113 ± 0.079 (65.24)
L. helveticus	0.103 ± 0.038 (31.52)	0.099 ± 0.039 (69.49)
L. rhamnosus	0.124 ± 0.051 (17.68)	0.120 ± 0.069 (62.89)
Control	0.151 ± 0.029	0.326 ± 0.130

* Indicates no inhibition when biofilm biomass of treated *E. coli* is equal to or higher than the control.

4. Discussion

The emergence of multidrug-resistant *E. coli* has been increasingly reported (Paterson and Bonomo, 2005). Nearly, 63% of total *E. coli* isolates in many countries were able to produce extended-spectrum beta-lactamase and the majority belonged to *E. coli* isolated from the ICU patients (Magiorakos et al., 2012; Nakai et al., 2016). In this work, all *E. coli* isolates were resistant to at least six antibiotics of different classes. Multidrug resistance of clinical pathogens imposes a rising threat to the human health by increasing the disease burden and spread. In previous studies, the resistance of *E. coli* to multiple drugs such as co-trimoxazole, penicillin and nitrofurantoin was at high frequencies (Mubita et al., 2008). High resistance rates of 60.6% of *E. coli* strains to cefazolin, tetracycline, ampicillin and trimethoprim/sulfamethoxazole was reported by (Li et al., 2017). The continuous emergence of multidrug resistance and even resistance to antibiotics of the last resort led to developing alternative intervention strategies to combat bacteria pathogenesis. Probiotics have received a growing interest in the prevention and treatment of infectious and other human diseases such as gastrointestinal, urogenital, respiratory and even periodontal diseases (Vuotto et al., 2014). In this work, cell-free preparations of different probiotics belong to *Lactobacillus* and *Bifidobacterium* species were able to reduce the growth of drug-resistant *E. coli* when investigated using agar well diffusion method. In other words, the inhibitory activity of selenium-enriched probiotics against pathogenic *E. coli* under in vitro and in vivo conditions was well-documented (Yang et al., 2009). In addition, probiotics isolated from yoghurts exhibited antibacterial effects against some common pathogens including *E. coli* (Kaboosi, 2011). Moreover, fifteen strains of probiotics belonging to many genera among which *Lactobacillus* and *Bifidobacterium* had antibacterial properties against gram negative and gram positive bacteria (Tejero-Sariñena et al., 2013). In the same cell, free supernatants of probiotics were used and the evidence of antibacterial properties was due to produced organic acids lowering the pH. In addition, bioactive compounds released by probiotics such as bacteriocins and hydrogen peroxide were responsible for their antimicrobial properties (Drider et al., 2016; Noordiana et al., 2013; Yang et al., 2014). These results support the potential use of probiotics/their bioactive compounds as antimicrobials against multidrug-resistant pathogens such as *E. coli*.

Biofilm formation is a phenomenon of microorganisms which results in a persistent microbial mass resistant to antimicrobial agents and related to about 80% of bacterial infections to humans. Infections due to biofilms formed by *Staphylococcus* sp. and enterobacteria such as *E. coli* are difficult to diagnose and can contribute to high healthcare costs and morbidity rates (Römling and Balsalobre, 2012). The antibiofilm properties of probiotics against biofilm-forming enteropathogens have been investigated, although the results obtained so far are few and conflicting. In this study, CFSM of probiotics grown in MRS broth or skim milk reduced the biofilm formation of two multidrug resistant *E. coli*. In previous studies single strains of *L. acidophilus*, *L. plantarum*, *B. longum* and *B. lactis* were effective is displacing *Salmonella* Typhimurium and *E. coli* from Caco-2 cell layer (Candela et al., 2008). In addition, exopolysaccharides produced by *L. acidophilus* decreases enterohaemorrhagic *E. coli* biofilms on polyvinyl chloride and polystyrene surfaces by affecting curli production genes (Kim et al., 2009). In vitro investigations focusing on bacteriocins production, adhesion, growth inhibition and co-aggregation of probiotics support their potential role in modulating microbial biofilms (Vuotto et al., 2014).

In conclusion, the antibacterial effects of cell-free preparations obtained from probiotics against multidrug-resistant *E. coli* support their effective use as antimicrobial alternatives and widen their applications in medicine and food bio-preservation as well as the possibility to eradicate biofilms formed by pathogenic *E. coli*.

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