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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*.

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

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