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# Antagonistic activity of potentially probiotic bacteria isolated from yoghurt with *Pseudomonas aeruginosa* in vitro

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**Key words:** *Pseudomonas aeruginosa*, antagonistic activity, probiotic, yoghurt, biofilm.

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**Abstract**

This study evaluated the ability of the probiotic organisms isolated from yoghurt to inhibit the pathogenic activity of *Pseudomonas aeruginosa* in vitro. A total of 8 bacterial strains isolated from samples were tested for antimicrobial activity. The antibacterial activity of the one strain was more potent than the other isolated strains that this isolate was identified as *Enterococcus faecium* strain by morphological, biochemical and 16S rDNA gene sequencing analysis. The various preparations (*Enterococcus* whole- cell culture containing $10^6$ CFU/ml, acid filtrate, neutralized filtrate and cells washed and resuspended in phosphate buffered saline) were tested in vitro for their effects on the production of the *P. aeruginosa* biofilm. No viable *P. aeruginosa* cells were detected after culture in the whole- cell culture and acid filtrate *E. faecium* preparations. Compared to *P. aeruginosa* grown in LB medium, the whole- cell culture and acid filtrate had a significant inhibitory effect on biofilm production.

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## Introduction

Antibacterial agents' resistance bacteria are the cause of numerous problems throughout the world. Increased resistance among pathogens is known to be related to the widespread use of antibiotics. *Pseudomonas* is a genus of Gram-negative aerobic gamma proteobacteria with functions of ecological, economic, and health-related importance. These bacteria demonstrate a great deal of metabolic diversity and consequently are able to colonize a wide range of niches (Barnali *et al*., 2010; Krieg, 1984). *P. aeruginosa* is an opportunistic bacterium, infects primarily immunocompromised individuals, such as patients with cystic fibrosis, cancer, AIDS, or patients with indwelling medical devices or burns (Vosahlikova *et al*., 2007; Banu *et al*., 2011; Andronescu *et al*., 2012). Probiotics are live microorganisms with different beneficiary characteristics that, when administered in adequate amounts, confer a health benefit on the host (Suzuki *et al*., 2011). These microorganisms produce various compounds such as enzymes, vitamins, antioxidants and bacteriocin (Knorr, 1998). With these properties, these bacteria constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990). Probiotic bacteria have been shown to possess antagonistic activity against food-borne disease agents such as *S. aureus, Salmonella* spp., *E. coli*, *L. monocytogenes* and *Cl. Perfringens* (Kasimoglu and Akgun, 2004; Karagozlu *et al*., 2007; Millette *et al*., 2007).

*Lactobacillus* and *Bifidobacterium* are the main probiotic groups; however, there are reports on the probiotic potential of *Enterococcus* (Mego *et al*., 2005). *Enterococci* are facultative anaerobic, Gram-positive cocci that form a part of the normal gastrointestinal tract flora in animals and humans. They are also frequently found in fermented food. Among *Enterococcus* species, *Enterococcus faecium* is the most used in commercial probiotics (Soccol1 *et al*., 2010). The mechanism of action varies from probiotic to probiotic. Many probiotics contribute to intestinal microflora balance. Some can inhibit replication of pathogenic bacteria by binding with the organisms, producing inhibitory substances and competing for essential nutrients or receptor sites (Soccol *et al*., 2010). However, studies relating to the antibacterial properties of probiotic organisms have been limited and not fully exploited for use. Therefore, this study attempts to evaluate the ability of the probiotic organisms isolated from yoghurt to inhibit the pathogenic activity of *P. aeruginosa*, in vitro.

## Materials and methods

*Collection of probiotic bacteria from yoghurt*

Four samples of yoghurt were provided from four different yoghurt producers in the Kerman Province of Iran. Five grams of each sample were homogenized with 10 ml of a sterile solution of Phosphate buffer saline (PBS), thus dilutions $10^{-1}$ - $10^{-6}$ were prepared and plated on the de Man Rogosa Sharpe agar (MRS agar) medium (Merck, Germany) to isolate the probiotic bacteria (De Man *et al*., 1960). After the incubation at 30 °C for 72 h, the plates were observed for any kind of growth on the media. The isolated and distinct colonies on the selective media were sub cultured and obtain in the form of pure culture. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain, cell morphology, catalase and oxidase reaction. One of the isolate was selected for further studies which exhibited strong inhibitory activity against *P. aeruginosa* CZO (Accession No: JX 441328) and identified on the basis of morphological, biochemical and 16S rDNA gene sequencing and phylogeny analysis.

*In vitro Inhibition Test*

The antimicrobial activity of the isolated bacteria (cell free filtrate) against *P. aeruginosa* CZO was performed by the disk diffusion assay. The *P. aeruginosa* CZO was incubated in LB broth at 30°C for 24 h. Petri dishes containing 20 ml of Muller Hinton agar were prepared previously and inoculated with 0.1 ml of 24 h broth culture of *P. aeruginosa*. Four sterile paper blank disks were placed on the agar plate which was inoculated by different

concentrations like 25, 50, 75 and 100 µl of the filtered supernatant of isolated bacteria. The Petri dishes were incubated at 37°C for 24 h. After incubation diameter of the inhibition zone was measured in mm. The antibacterial activity was determined by measuring the clear zone around the disks.

*Identification of bacterial isolates*

Genomic DNA was extracted from isolated bacteria using DNA extraction kit (Cinnagen Cat No: DN8115C), according to the manufacturer's instructions. Bacterial 16S rDNA was amplified by using the universal forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). PCR amplification was performed in a total volume of 50µl mixture, containing 2 µl templates DNA (100 ng), 1 µl of F primer (10 pmol), 1 µl of R primer (10 pmol), 1 µl of 10 mM 4 dNTP mix, 2 µl of 50mM $MgCl_2$, 5 µl of 10X PCR buffer, 0.6 µl of Taq DNA polymerase (5 U $\mu l^{-1}$), 37.4 µl of sterile distilled water and microcentrifuged briefly. An initial denaturing step of 94°C for 5 min was followed by 30 cycles of amplification (1 min 94°C, 1 min 56°C, 2 min 72°C) and a final extension step at 72°C for 10 min. DNA amplification was checked by electrophoresis of 5 µl of PCR product in a 1% agarose gel for 2 h (Fig. 1) and visualization with ultraviolet illumination after staining with 0.5 µg $ml^{-1}$ ethidium bromide. The clean PCR product was subjected to cycle sequencing in both directions.

*Phylogenetic analysis*

The nucleotide sequence of 16S rDNA gene were edited using Bioedit V.5.0.9 (Hall, 1999). A BLAST search at the NCBI genome database server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to identify the nearest neighbors (Altschul *et al.*, 1990). Phylogenetic and molecular evolutionary analyses of 16S rDNA gene sequences were conducted using MEGA version 5 (Tamura *et al.*, 2011) after multiple alignment of data by CLUSTAL W (Thompson *et al.*, 1994). A bootstrap test and reconstruction was done 1,000 times to confirm the reliability of the phylogenetic tree (Felsenstein, 1985). Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The nucleotide sequences of 16S rDNA gene of bacterial strain (*E. faecium* Y-2) reported in this study has been deposited in GenBank under Accession No: KC529653.1.

*Evaluation of Antagonistic Activity*

*E. faecium* Y-2 was grown in MRS broth at 37 °C. The following preparations were used to study the in vitro interference of *E. faecium* with *P. aeruginosa* CZO: (1) a whole-cell culture containing $10^6$ *E. faecium* CFU/ml (T preparation); (2) culture supernatants recovered following centrifugation and filtration through 0.22 µm filters (acid filtrate; AF preparation); (3) aliquots of AF neutralised with 8 M NaOH to pH=7 (neutralised filtrate; NF preparation); and (4) *E. faecium* cells washed and resuspended in phosphate-buffered saline at a concentration of $10^6$ CFU/ml (EF preparation). To measure growth of *P. aeruginosa* CZO in the presence of *E. faecium*, 1 ml of an overnight culture of *P. aeruginosa* CZO, diluted 1:7 in LB medium, were grown in the presence of 1 ml of an *E. faecium* preparation (T, AF, NF or EF). After incubation for 16 h at 37°C, an aliquot of each mixture was spread on MacConkey agar plates for colony counting (*E. faecium* does not grow on MacConkey agar) (Valde´z *et al.*, 2005).

*Biofilm production assay by microtitre plate test*

To assess the biofilm formation potential of the *P. aeruginosa* CZO, an overnight culture was grown in LB broth (Merck, Darmstadt, Germany) for 18 – 20 h at 30°C. One ml of overnight culture was transferred to 10 ml of sterile LB. The suspensions were adjusted with LB to 0.5 on the McFarland turbidity standard corresponding to approximately $10^8$ CFU/ml. Then, 250 µl volumes were transferred into wells of a microtitre plate. Blank wells contained LB broth, only. Plates were made in duplicate, covered, and incubated for 24 h at 30°C. After 24 h, the planktonic suspension and nutrient solutions were aspirated and each well was washed three times with 300 µl of sterile physiological saline. The plates were vigorously

shaken in order to remove all nonadherent bacteria. The remaining attached bacteria were fixed with 250 µl of 96% ethanol per well and, after 15 min, plates were emptied and left to dry. Each well was then stained for 5 min with 0.2 ml of 1% crystal violet (CV Gram stain, Merck, Germany). Excess stain was rinsed off by placing the plates under running tap water (Shakeri *et al.*, 2007). Then the optical density (OD) of the stain was measured at 540 nm by an ELIZA reader (STAT-FAX 2100) (O'Toole and Kolter, 1998).

*Assessment of the potential of E. faecium to kill biofilm cells*

After preparation of overnight cultures of *P. aeruginosa* CZO, suspension of this isolate was diluted 1:100 in LB. The wells in a 96-well plate were filled with 100 µl of the culture and 100 µl of the T, AF, NF and EF preparations. Then the plates were covered and incubated for 24 h at 30°C. After incubation, the planktonic cells were aspirated and each well was washed three times with 300 µl of sterile PBS. Then 200 µl of a 1% solution of crystal violet was added to each well (this dye stains the bacterial cells but not the wells). The plates were incubated at room temperature for 15 min and rinsed thoroughly. Biofilm formation was quantified by the addition of 200 µl of 95% ethanol to each well and the absorbance was determined with an ELIZA reader (STAT-FAX 2100) (O'Toole and Kolter, 1998). A measure of *E. faecium* Y-2 preparations efficacy (i.e. the percentage reduction in stain) was calculated from the blank, control and treated absorbance values on a plate (Equation 1):

$$\text{Percentage reduction} = \left[\frac{(C-B)-(T-B)}{(C-B)}\right] \times 100$$

Where B denotes the average absorbance for blank wells, C denotes the average absorbance for control wells, and T denotes the average absorbance for treated wells (Shakeri *et al.*, 2007).

**Results**

*Isolation of bacteria*

A total of 8 bacterial strains were isolated from yoghurt**.** The isolated bacteria were gram positive, catalase negative that have yellowish, mucoid and rounded colonies.

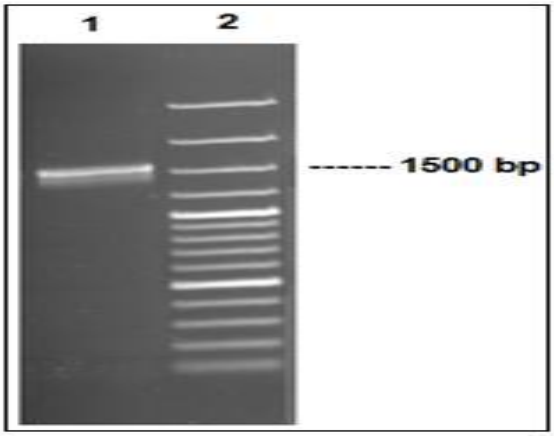


**Fig 1.** Agarose gel electrophoresis of 16S rDNA gene PCR product. Lane 1: 16S rDNA gene PCR product of *E. faecium* Y-2, Lane 2: DNA size maker (Gene Ruler 100bp DNA ladder plus, Fermentas)

*Antibacterial activity of isolated strains*

The isolated strains were tested for antibacterial activity. The antibacterial activity of the one strain was more potent than the other isolated strains (fig. 2). This isolate was identified as *Enterococcus faecium* strain by morphological, biochemical and 16S rDNA gene sequencing analysis.


**Fig 2.** Effect of *E. faecium* Y-2 preparations on the growth of *P. aeruginosa* CZO by disk diffusion method. 1: 25 µl, 2: 50 µl, 3: 75 µl, 4: 100 µl

*Phylogenetic analysis Based on 16S rRNA Gene*

Phylogenetic affiliation of the isolated strain (*E. faecium* Y-2) was ascertained by 16S rDNA gene sequence analysis. In order to find the most similar available sequences, a BLAST search was done in NCBI database. 16S rDNA sequence data of most closely related species of *Enterococcus* were retrieved

and used in tree construction to demonstrate the taxonomy of this isolate. Figure 3 show the inferred phylogenetic relationships derived from a neighbor-joining analysis of 16S rDNA gene sequence of the *E. faecium* Y-2 with most validly described species of the genus *Enterococcus*.

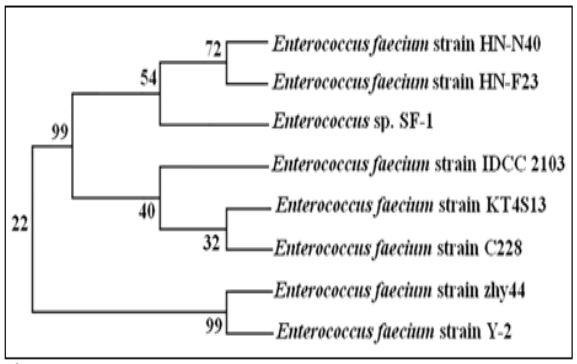


**Fig 3.** Neighbor-joining tree based on 16S rRNA gene sequences, showing relationships of *E. faecium*Y-2 with closely related members of the genus *Enterococcus*

*In-vitro effects of E. faecium Y-2 on P. aeruginosa CZO*

No viable *P. aeruginosa* CZO cells were detected after culture in the T and AF *E. faecium* preparations. Although NF showed a reduced growth-inhibiting capacity, it produced a significant reduction in the *P. aeruginosa* CZO viable count, compared with *P. aeruginosa* CZO cultured in LB medium. The EF preparation had no inhibitory effect.

*Effect of E. faecium Y-2 on P. aeruginosa CZO biofilm production*

Figures 4 and 5 show the effects of the various preparations on the formation of biofilm by *P. aeruginosa* CZO. Compared to *P. aeruginosa* grown in LB medium, the T and AF preparations had a significant inhibitory effect on biofilm production.

**Discussion**

*P. aeruginosa* infection is often difficult to eradicate because of remarkable resistance to many antibacterial agents, due to constitutive expression of b-lactamases and efflux pumps, combined with low permeability of the outer-membrane or acquisition of resistance genes (Valde´z *et al.*, 2005; Mesaros *et al.*, 2007). This organism has always been considered to be a difficult target for antimicrobial chemotherapy (Mesaros *et al.*, 2007). The probiotic microorganisms have been used mainly to treat gastrointestinal disorders. However, their ability to secret acids, bacteriocins and other by-products may neutralized infection caused by pathogens such as *P. aeruginosa*. The inhibitory activity of probiotics bacteria against some resistant clinical isolates of *P. aeruginosa* has been reported (Jamalifar *et al.*, 2011). This study investigated the in vitro inhibition exerted by *E. faecium* Y-2 on the virulence of *P. aeruginosa* CZO. Growth of *P. aeruginosa* CZO was inhibited fully by the *E. faecium* T and AF preparations. The finding of present study is very similar to the results of valdez *et al* (2005) in which it was determined that *L. plantarum* and its by-products are active against *P. aeruginosa*. Antimicrobial activity of *probiotic* strains against bacterial pathogens emerges to be multifactorial and to include the production of hydrogen peroxide, lactic acid, bacteriocin-like molecules and unknown heat-stable, non-lactic acid molecules (Servin, 2004).

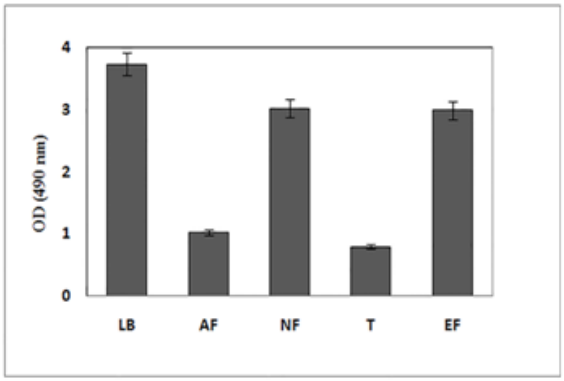


**Fig 4.** Effect of different preparations of *E. faecium* Y-2 on *P. aeruginosa* CZO biofilm production. LB, *P. aeruginosa* in Luria-bertani medium; AF, *P. aeruginosa* in the presence of *E. faecium* acid filtrate; NF, *P. aeruginosa* in the presence of *E. faecium* neutralised filtrate; T, *P. aeruginosa* in the presence of $10^6$ CFU *E. faecium*/ml; EF, *P. aeruginosa* in the presence of *E. faecium* cells washed and resuspended in PBS at a concentration of $10^6$ CFU/ml.

A bacteriocin producing strain, *E. faecium*, previously isolated from honey, was used to produce bacteriocin. Gupta and Malik (2007) also isolated *enterococci* from a total number of 68 dairy products

and were screened for bacteriocin production. The bacteriocin produced by *Enterococcus* strain was checked for its antimicrobial potential by using agar well diffusion assay. It produced 14mm zone of inhibition against indicator strain of *S. aureus* (Gupta and Malik, 2007). Achemchem *et al.* (2005) revealed that the bacteriocin was active against many Gram positive bacteria but have no antimicrobial activity against Gram negative bacteria. The results in this experiment revealed that the bacteriocin isolated from *E. faecium* has great potential to inhibit pathogenic bacteria like *S. aureus*. Enterocins, the antibacterial peptides produced by *Enterococcus* species are small, hydrophobic and thermostable with activity over a wide range of pH. Enterocins are active against many Gram-positive bacteria encompassing undesirable and pathogenic microbes, such as *Clostridia*, *Bacillus*, *Staphylococcus* and *Listeria*, as well as some Gram-negative bacteria (Franz *et al.*, 1996, Munoz *et al.*, 2004). *Enterococci* have drawn research interest because of their presence almost everywhere in the food chain, as well as in the environment, and also because of their use in the production of probiotics and other fermented foods. Besides this, bacteriocin production is also a common characteristic among *enterococci* and is responsible for the inhibition of food spoilage and pathogenic organisms (De Vuyst *et al.*, 2003, Franz *et al.*, 2003).

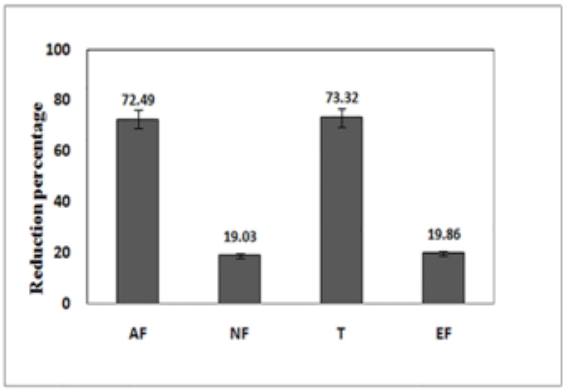


**Fig. 5.** Biofilm treatment with different preparations of *E. faecium* Y-2

## Conclusion

*E. faecium* Y-2 is able to inhibit the growth of *P. aeruginosa* CZO. These friendly bacteria might be good candidate to overcome the growing challenge of nosocomial infections due to antibacterial agent's resistant strains of *P. aeruginosa*.

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