Original Article

Antagonstic Effect of Lactobacillus Fermentum Supernatant AgainstEnterococcus Faeciumand Enterococcus Faecalis In Vitro

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Summary:

Background: Lactobacillus fermentum selected as an alternative treatment to prevent or treat urogenital infection based on their probiotics properties and production of bacteriocins.

Objective: The present work was done to study the inhibition activity of L. fermentum cell free supernatant against urogenital pathogens Enterococcus faecium and Enterococcus faecalisinvitro.

Materials and methods: L.fermentum isolates have been collected from vaginal swabs . A supernatant of these isolates has been prepared and its antibacterial activity against 3 isolates of E.faecium and 3 isolates of E.faecalis has been studied.

Results: Different concentrations have been prepared and the most effective one was 1000µg/ml and the most affected isolate of E.faecalis was no. 3 which its MIC was 64µg/ml and MBC was 128 µg/ml, while the most affected isolate of E. faecium was no. 1 which its MIC was 128 µg/ml and MBC was 256 µg/ml .

Conclusion:L. fermentumCFS showed significant activity against both Enterococcus isolates and showed closely related results.

Keywords: Lactobacillus fermentum , Antibacterial activity , Antagonstic effect ,Enterococcus faecium , Enterococcus faecalis, vaginosis .

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Introduction:

Lactobacillus group are the dominant microorganisms in healthy pre-menopausal women and play an important protective role by limiting growth of pathogenic microorganisms (1). Lactobacillus are able to interfere with genitourinary pathogens by several mechanisms including competitive exclusion from the cell surface , production of adhesion inhibiting bio surfactant compounds , auto aggregation surface , hydrophobicity and co-aggregation with other bacterial species (2).Many reports show the usefulness of lactic acid bacteria (LAB) as probiotics for humans and animals (3). Appealing properties of probiotics include the ability to reduce antibiotic use, the apparently high index of safety, and the public>s positive perception about <natural> or <alternative> therapies. Potential probiotic bacteria are classified, and generally regarded as safe as opposed to antibiotics, which have a number of recognized adverse effects (4). They can be used as natural competitive micro biota or as specific starter cultures under controlled conditions (5). The promotion of immune system maturation and defense against infections as well as the anti-inflammatory properties are among the main healthy effects of these bacteria (6). Therefore, without education and good products, it is not surprising that family physicians barely use probiotics in their practices (7). So we aimed in this research to study the antagonistic effect of L.fermentumagainst urogenital pathogensE. faeciumandE. faecalis.

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Materials and methods:

Bacterial isolates: Nine L.fermentum isolates have been collected from vaginal swabs of healthy women their age ranged between 18-45 years old at AL-Yarmouk Hospital and the research was done at Ministry of Science and Technology and Department of Biology at College of Science / AL-MustansiriyahUniversity.The samples were inoculated in MRS broth and incubated at 37°C for 48 h. under anaerobic condition , and the growth cultures were plated on MRS agar at the same condition , the identity of the cultures was based on the characteristics of the lactobacilli such as cultural , microscopically and biochemical characters which included fermentation of different carbon sources , gas production from glucose , growth at different temperatures , tolerance to inhibiting substances such as bile (sigma) , phenol (merch) and sodium chloride(biotech) as described by(8,9), then the pure cultureswere maintained on MRS agar.Uropathogenic microorganisms included three isolates of E. faeciumandthree isolatesofE. faecalis isolated from urine were employed to study the antagonistic effect of L.fermentum against them.

Preparation of cell free supernatants (CFSs): An overnight culture of L. fermentum isolates were adjusted with MRS broth in accordance to McFarland turbidity standard solution no.5 as a measured by absorbance (0.08-0.1 at 625 nm) corresponding toapproximately 1.5-2 -10^8 CFU/ml. Afterword ,the cultures were propagated in the same broth at 37°Cfor 24h under anaerobic condition . Bacterial cells were removed by centrifugation of the cultures at 6000 rpm / 10min

at 4°C. The resulting supernatants filtered through sterilized 0.22 µm filter paper then cultured on MRS agar in order to confirm the absence of lactobacilli cells. The supernatants were concentrated by ammonium sulphate precipitation (700 g/L). After the mixtures had been stirred overnight at 4°C, the precipitates were pelleted by centrifugation at 10,000 rpm / 30min. Then , the collected precipitates were dissolved in 0,05 M sodium acetate buffer pH 5.0 and dialyzed against the same buffer at 4°C overnight (10) . Protein concentration after each purification step was determined(11) and the activity unit per ml (Au/mg) was assayed(12).The putative metabolites produced by L.fermentum isolates were lyophilized and dissolved in phosphate buffered saline (2mM Na_2HPO_4, o.5mM KH_2PO_4 , 1.3mM KCl , 135mM NaCl , pH 7.0) for prepare different concentrations (13) .

Antimicrobial activity assay:

1) Agar well diffusion method: This method was used to detect antimicrobial activity of CFS_s produced by L.fermentum against Enterococcus isolates at different concentrations (1000, 750, 500, 250) µg/ml according to Batdorjetal.(2006) (14) .

2) MIC and MBC: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were detected according toBatdorj et al. (2006)(14).

Results:

Lactic acid bacteria (LAB) are characterized as Gram-positive,short, single and paired square bacilli. The colonies on MRS agar were smooth and convex , catalase negative , produced gas from glucose and $NH_3$from arginine , grew at 45°C but poorly at 15°C , the isolates tolerated 0.3% and 10% bile , 0.3% and 0.4% phenol and 4% NaCl but not 8% NaCl, non-aerobic but aero tolerant, able to ferment carbohydrates included lactose , xylose , maltose , fructose , dextrose , galactose , raffinose , trehalose , mellibiose , sucrose , mannose for energy and lactic acid production but could not ferment L-arabinose , inuline , sodium gluconate , salicin , glucose amine , ribose , cellobiose and esculin. CFS of L.fermentum no. 5 was recovered with an increase in specific activity from 280-297.5 Au/mg after precipitation with ammonium sulphate as shown in table (1) Antimicrobial activity assay of L.fermentum CFS_s was done on 3 isolates of E. faeciumand3 isolates ofE. faecalis. Among 9 L. fermentumCFS_s only no. 5 was able to inhibit the growth of all Enterococcusisolates. All of L.fermentumCFSconcentrations showed closely related results and the most effective one was 1000 µg/ml as shown in table (2) , and the most affected isolate of E. faecalis was no. 3 as shown in figure (1 , 2) , which its MIC was 64µg/ml and MBC was 128µg/ml as shown in table (3) . The most affected isolate E. faecium was no. 1 as shown in figure (3 , 4) , which its MIC was 128µg/ml and MBC was 256 µg/ml as shown in table (3).The results indicate that L. fermentum CFS in all concentrations (250, 500, 750, 1000) µg/ml possesses significant antibacterial activity against all E. faecalis and E. faecium isolates contrast with control $P<0.05$ and the antibacterial activity of CFS in concentration 1000 µg/ml was significantly higher than other concentrations (250, 500, 750) µg/ml, $P<0.05$.

Table (1): The purification strategy and results obtained for L.fermentum CFS no. 5 .

Purification strategy	Volume (ml)	Activity	Total activity(Au)	Total protein(mg)	Specific Activity(Au/mg)	Yield (%)
Culture supernatant	28	18	504	1.8	280	100
Amoniumsulphate precipitate	7	34	238	0.8	297.5	47

Table (2): Inhibition zones of L.fermentum CFS no. 5 against Enterococcus isolates at concentrations (1000, 750, 500, 250) µg/ml.

Isolates	Inhibition zone (mm) [mean ± SD]				
	Concentration of L. fermentum CFS (µg/ml)				Control D. W.
	250	500	750	1000	
E. faecalis	19.33±1.82 p_2 p_1	21.33±2.1 p_2 p_1	27±0.86 p_2 p_1	32.66±2.11 p_1	0±0
E. faecium	26.6±1.69 p_2 p_1	29±1.33 p_2 p_1	31.2±1.23 p_2 p_1	33.33±1.79 p_1	0±0

p_1: probability compared to control $P<0.05$

p_2: probability compared to 1000µg/ml $P<0.05$

Table (3): MIC and MBC of L.fermentum CFS no. 5 against Enterococcus isolates .

Enterococcus isolates	MIC	MBC
E. faecalis no. 1 E. faecalis no. 2 E. faecalis no. 3	512 µg/ml 128 µg/ml 64 µg/ml	1024 µg/ml 256 µg/ml 128 µg/ml
E. faecium no. 1 E. faeciumno. 2 E. faecium no. 3	128 µg/ml 256 µg/ml 256 µg/ml	256 µg/ml 512 µg/ml 512 µg/ml

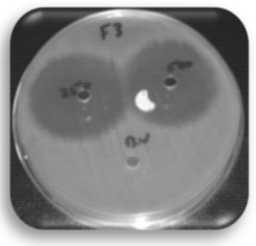

Figure (1): Antibacterial activity of L.fermentum CFS no. 5 againstE. faecalis no. 3 at concentrations (250, 500) µg/ml.

Figure (2): Antibacterial activity of L.fermentumCFS no. 5 againstE. faecalis no. 3 at concentrations (750, 1000) µg/ml.

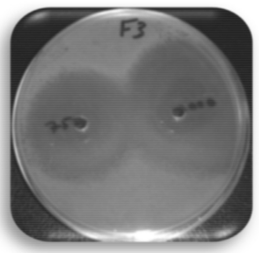

Figure (3): Antibacterial activity of L.fermentumCFS no. 5 againstE. faecium no. 1 at concentrations (250, 500) µg/ml.

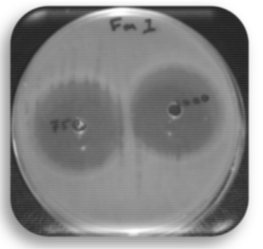

Figure (4): Antibacterial activity of L.fermentum CFS no. 5 againstE. faecium no. 1 at concentrations (750, 1000) µg/ml.

Discussion:

To date, the main anti- infective properties described for lactobacilli are their ability to (i) adhere to surfaces and inhibit the adhesion of pathogens, (ii) inhibit the growth of pathogens, (iii) deplete nutrients otherwise available to pathogens, and (iv) modulate the host immune response and microenvironment, such that risk of infection is reduced (15). In vitroand clinical studies have provided evidence which supports the notion that lactobacilli may protect their hosts and keep them from acquiring urinary tract infection (16). Lactobacillus and Streptococcus species have been shown to be able to displace adhering uropathogenic E. faecalis strains from hydrophobic and hydrophilic substrata in a parallel – plate flow chamber , possibly through biosurfactant production [(16)], while in another study only a few lactobacilli were able to inhibit growth of E. faecalis(17).A ruminal isolate of L.fermentum produces an uncharacterized antimicrobial compound active against strains of Streptococcus bovis(18, 19). Viable LAB produces antibacterial material which inhibits growth of the pathogen (20, 21).The organic acids produced by some but not all strains of LAB such as benzoic acid, diacetyl, mevalonolactone, methylhydantoin and reuterin which maintain a competitive advantage (22).

In addition , several studies have revealed that certain LAB strains may also affect innate, humoral and cellular immune parameters as demonstrated by increased serum concentration of IgA , IgG and IgM(23), as well as killing of the cells by hydrogen peroxide and bacteriocin – like compound (24 , 25). The low pH makes organic acids lipossoluble, allowing them to break through the cell membrane and reach the cytoplasm of pathogens (26). Bacteriocins are proteins or complexed proteins biologically active with antimicrobial actionagainst other bacteria, principally closely related species (27).The cell wall of gram-positive bacteria allows passage of relatively large molecules, so that there is unlikely to be a requirement for bacteriocin receptors analogous to those in the outer membranes of gram negative cells. Anionic cell surface polymers like teichoic acid and lipoteichoic acid may be important in the initial interaction of cationic bacteriocins of Gram positive bacteria (28).The results of CFS of L. fermentumno. 5 agreed with findings of Mojgani et al. (2009) which reported that the increase in activity could be due to release of active monomers from bacteriocin complex (12) .Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive cells, this distinction being greatly influenced by several factors such as bacteriocin dose and degree of purification, physiological

state of the indicator cells and experimental conditions (5).

In conclusion,L. fermentum CFS showed significant activity against both Enterococcus isolates and the concentration 1000µg/ml has significantly higher effect than (250, 500, 750) µg/ml.

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