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Bifidobacteria bifidum and *Bifidobacteria infantis* Effects on *Salmonella enteritidis*

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During the last decades, the prevalence of foodborne diseases due to contaminated food as well as demand for natural and healthy foods has increased. Using probiotics for this purpose and for inhibiting growth of food pathogens is an interesting topic. The aim of this study was to investigate the antibacterial effects of *Bifidobacterium bifidum* and *Bifidobacterium infantis* against *salmonella enterica* serotype Enteritidis by three different methods namely spot on lawn assay, agar well diffusion assay and agar disk diffusion assay. Supernatant and sediment of the two probiotic bacteria culture was tested in three different assays (spot-on-lawn, well diffusion and disk diffusion) against salmonella. Results showed that in all three assays, sediment and supernatant of *Bifidobacterium infantis* culture had a greater inhibition effect on salmonella than *Bifidobacterium bifidum* but the difference was not significant from statistical analyses point of view. In this study in all three methods, the supernatant was significantly more effective than sediment in inhibiting the pathogen. This inhibition could be related to metabolites such as Acids, Diacetyle , Hydrogen proxide, Bacteriocins, produced by probiotics.

Keywords: *Probiotic, Bifidobacterium, Salmonella,* antagonism.

Bifidobacterium spices are one of the most abundant microbes in natural micro flora of colon. About 25% of adult stool bacteria and 80% of infant stool bacteria are Bifidobacterium[19]. This bacterium is gram positive, rod shaped, immobile, non-spore forming, catalase negative and the major product of their metabolism is acetic and lactic acid[6].

Bifidobacterium spices play an important role in human health by prevention of intestinal infections, decreasing cholesterol, stimulating immune system therefore decreasing cancer risks[6, 12,13]. Some of the spices in this genus are categorized as probiotics.

Probiotics are a big group of bacteria consisting of lactic acid bacteria (like Lactobacillus, Bifidobacterium, some Streptococci, Pediococcus and Lactococci) and none lactic acid bacteria like Propionibacterium, Bacillus and some yeasts like saccharomyces[6].

Many in vivo and in vitro experiments have shown the antagonistic effect of probiotics against many pathogens. Probiotics inhibit the growth of many microorganisms by producing lactic and acetic acid, bacteriocins, hydrogen peroxide, diacetyl, acetaldehyde and ammonia [1,4,18]). In these researches some really valuable characteristics like resistance to intestinal pathogens, prevention and curing of bacterial and viral diarrhea have been related to probiotics[3, 8, 17, 20]. Inhibition of salmonella spices by probiotics is a proof of their beneficial effect[11, 13, 14, 15].

Objectives

The aim of this study was to investigate the antibacterial effects of *Bifidobacterium bifidum*

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and *Bifidobacterium infantis* against salmonella enterica serotype Enteritidis by three different method namely spot on lawn assay, agar well diffusion assay and agar disk diffusion assay .

MATERIALS AND METHODS

Preparing the probiotic and pathogen culture

Lyophilized Bifidobacteria strains (*Bifidobacterium bifidum* Bbis015 and *Bifidobacterium infantis* Bins012) were obtained from Zist Takhmir Company and were anaerobically (with anaerocult A Merck company) activated in MRS broth for 3-5 days. Then the cultures were frozen in micro tubes containing 30% glycerol as cryoprotectant and held in -80 °C freezer. Before experimental tests, cultures were propagated overnight in broth media.

The pathogen used for antagonistic test was *Salmonella enterica* serotype Enteritidis ATCC 13311 which was obtained at lyophilized form and activated in TBS broth culture then the cultures were frozen in micro tubes containing 30% glycerol and held at -80 °C freezer. Before experimental tests, culture were propagated overnight in broth media .

Preparation of Cell-Free Supernatants

Strains *Bifidobacterium bifidum* Bbis015 and *Bifidobacterium infantis* Bins012 to be tested for antimicrobial activity were incubated in MRS broth for 48 h at 37°C. Bacterial cells were removed by centrifuging the culture at 3500 g for 25 min at 4°C. The supernatants were membrane filtered (0.22µm) and stored at 4°C in sterile conditions. The sediments also at 4°C in sterile conditions.

Antimicrobial assay

The assay was performed with three different methods

a) Spot on lawn assay
b) Agar well diffusion assay (Cup plate assay)
c) Agar disk diffusion assay

A) Spot on lawn testing was carried out on MRS agar (Merck1.10660.0500) and soft. Muller-Hinton Broth (QUELAB QB-65-8547 100G) layers. MRS agar (Merck1.10660.0500) as first layer was poured in sterile plates then plates were inoculated with approximately ($1.5*10^8$ CFU/ml) equal to 0.5 McFarland turbidity of Salmonella enterica serotype Enteritidis ATCC 13311 inoculum as pathogen bacteria by a sterile swab. 2 microliter Spots of supernatant and sediments were put on this layer (3 replicates, a positive and a negative control) and then plates were incubated for a short while(15 minutes at 37ÚC. Second layer consisting of soft. Muller-Hinton Broth (QUELAB QB-65-8547 100G) (0.7% agar and 2% glycerol) was poured and plates were incubated for 3-5 days in anaerobe conditions at 37°C. The clear zone around spots then was recorded. Gentamicin was used as positive control and deionized water as negative control.

B) Agar well diffusion assay was carried out on Muller-Hinton agar. Muller-Hinton agar (Merck1.05437.0500) was poured in sterile plates and plate's surfaces were inoculated with pathogen. Wells were cut on plate by sterile pipet (with an approximate distance of 19 mm so that zones did not collide). Wells were filled by supernatant or sediment and incubated 3-5 days at 37°C with closed lid and anaerobe conditions. The clear zone around spots then was recorded[7,10].

c) Agar disk diffusion assay(Cup plate assay) was carried out on Muller-Hinton agar by Kirby-Bauer disk diffusion susceptibility test protocol. MullerHinton agar was poured in sterile plates and plate's surfaces were inoculated with approximately ($1.5*10^8$ CFU/ml) equal to 0.5 McFarland turbidity of Salmonella enterica serotype Enteritidis ATCC 13311 inoculom as pathogen bacteria by a sterile swab. The inoculum optical density (OD) had been adjusted between 0.08-0.13 in 620 nm in spectrophotometer. Standard blank disk with 6.4 mm diameter were put on plate (with an approximate distance of 19 mm so that zones did not collide[7,10].

RESULTS

Adjusting pathogen culture optical density

Overnight culture of Salmonella in TBS broth were diluted by fresh culture media until their OD was set to 0.08-0.13 in 625 nm in spectrophotometer. Total cells were counted by Muller – Hinton agar plates cultured with this diluted pathogen . This test was done to evaluate

the approximate1.5*10^8 CFU/ml of pathogen which is inhibited by probiotic bacteria.

Assaying inhibitory effect of both Bifidobacteria

Results of studying the effect of Bifidobacteria supernatants on growth of *Salmonella Enteritidis* are presented in Figure 1.

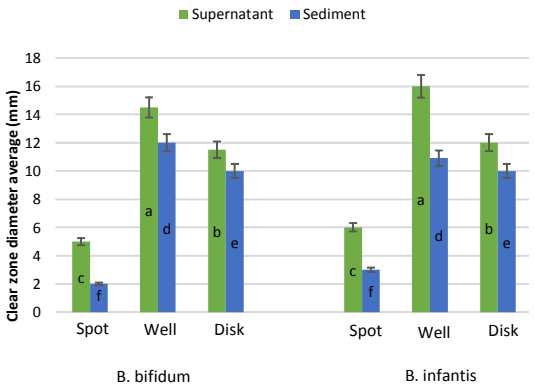

Fig. 1. Inhibitory effect of Bifidobacterium bifidum and Bifidobacterium infantis (supernatant and culture sediment) against Salmonella in 3 different assays

As it's shown both strains had inhibitory effect and a clear zone was formed around the spot, well or disk with the inhibition zone ranging from 8.4 to 16 mm (with considering Disk diameter 6.4 mm in disc diffusion assay and well diameter 6.0 mm in cup plate assay). These results complied with[2, 9,16].

DISCUSSION

Salmonella is a very important bacterium in food borne pathogens. This pathogen exists in food stuffs and play a main role in food microbiology[21] , Bifidobacteria are one of the most important groups of microorganisms to mankind being involved in prevention of intestinal infections, decreasing cholesterol, stimulating immune system therefore decreasing cancer risks (6, 12 and 13). With every day passing a new aspects of probiotics is discovered and a new use is defined for them.one of these new aspect is the antagonism between Bifidobacteria and pathogens and it is related to the various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins produced by these microorganisms (1, 4 and 18).

During this study it was concluded that *Bifidobacterium bifidum* and *Bifidobacterium infantis* both had inhibitory effect against *Salmonella Enteritidis*, the infantis strain was slightly more effective but the difference was not statically significant. Makras et al. (2006) stated that *Bifidobacterium bifidum* had inhibitory effect against *Salmonella Enteritidis* and the reason is acid production and lowered pH which seems true since bacteriocins of Gram positive bacteria like Bifidobacteria is less effective against Gram negative bacteria such as Salmonella spices[16].

The microbial quality of poultry paste as raw material, cooked and raw meats study show that microbial contamination especially Salmonella contamination in these food stuffs, and necessity for preventing ways of contamination[22].

Gibson and Wang (1994) investigated the regulatory effect of Bifidobacteria in intestine and decided that Bifidobacteria are of the most numerically important bacteria in intestine and maintain their host's health by some biological activities. One of these actions is inhibiting pathogens by producing acidic compounds like lactate and acetate. They also discovered that 8 strains of Bifidobacteria were able to produce antimicrobials with a large range of inhibitory and inhibit pathogens like Salmonella, Listeria, campylobacter, Shigella and vibrio spices[9].

Researches about inhibitory effect of *Bifidobacterium infantis* were rare. Antimicrobial Activity of *Lactobacillus gasseri* as Probiotic Bacteria Against *Salmonella Enterica* Sero type Entertidis had been reported at 2015 by Moulood Barzavar *et al.*,23.

Investigating the antibacterial effectiveness of *Lactobacillus plantarum* on *Salmonella Entrica* serotype entritidis had been reported at 2015 by Moulood Barzavar et al [24]

The result from comparison of assays was in contrast with the results obtained by cadirci and citak (2005) who investigated antagonism of LAB against Gram negative bacteria with two methods namely Spot on lawn assay and well diffusion assay and concluded that spot method was best for evaluation of LAB inhibitory effect[5].

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