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A Comparison of Two Methods Used for Measuring Antagonistic Activity of Lactic Acid Bacteria

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Abstract: In this research, we have aimed to determine antagonistic effects of various lactic acid bacteria against Gram (+) and Gram (-) bacteria with a comparison of disc diffusion and spot-on-lawn method. In spot-on-lawn method, *P. aeruginosa* ATCC 27853 was the most sensitive of the tested bacteria, followed by *E. coli* ATCC 25927 and *P. aeruginosa* ATCC 10145. On the other hand, in disc diffusion assay *B. subtilis* ATCC 6633, *E. coli* O157:H7 and *P. aeruginosa* ATCC 10145 were the most sensitive indicator microorganisms. The spot-on-lawn method was suitable for assessing the antagonistic effect of lactic acid bacteria. In general, inoculum density had little effect on inhibition.

Key words: Antagonistic activity, disc diffusion assay, spot on lawn method

Introduction

In the food industry, lactic acid bacteria are widely used as starter cultures (Hammes, 1986; Skytta and Mattila-Sandholm, 1991; Harris *et al.*, 1989). Lactic acid bacteria have an important role in the inhibition of food-borne pathogenic and spoilage microorganisms with antimicrobial metabolites, including lactic acid, acetic acid, and other organic acids, hydrogen peroxide, bacteriocins and bacteriocin-like substances (Juven *et al.*, 1991, 1992). Lactic acid and acetic acids cause a reduction in pH (Çetin, 1983), hydrogen peroxide is a non-stable thermodynamic compound and destroys bacterial enzymatic activity (Collins and Aramaki, 1980). Bacteriocins produced by lactic acid bacteria are biologically active proteins, bactericidal, plasmid mediated and reactive with specific binding sites on sensitive bacteria. In many publications, inhibition of Gram (+) bacteria by lactic acid bacteria were reported, but investigations about Gram (-) bacteria are very few (Daeschel and Klaenhammer, 1985; Klaenhammer, 1988; Neve *et al.*, 1984; Bhunia *et al.*, 1987).

Lactic acid bacteria have antagonistic effects on food borne pathogenic and spoilage microorganisms (Schillinger and Lucke, 1989), e.g. inhibition of *B. subtilis* which contaminates bread and causes spoilage (Vogel *et al.*, 1999). Survival of *E. coli* O157:H7 in dairy products is a potential health hazard because of the link with dairy cattle and raw milk (Saad *et al.*, 2001). Earlier studies had shown that, some *Lactobacillus* strains had an inhibitory activity on *E. coli* (Rodriguez *et al.*, 1989). *Pseudomonas aeruginosa* is an opportunistic pathogen and a spoilage organism. It causes especially inner-ear and urinary system infections. Psychrophilic *Pseudomonas* species, spoil foods by their lipolytic and proteolytic activities (Unluturk and Turantas, 1998). Hydrogen peroxide produced by *Lactobacillus* species

inhibits *Pseudomonas* species (Daeschel, 1989). The antibacterial effect of neutralized supernatant fluid of a *L. casei* strain inhibits *S. aureus*, *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Salmonella typhimurium* and *P. aeruginosa* (Vignolo *et al.*, 1993).

There are many techniques for detecting antimicrobial activity. For example, critical dilution assays (Parente *et al.*, 1995), flip-streak method (Lewus and Montville, 1991; Spelhaugh and Harlander, 1989), well diffusion assay (Stecchini *et al.*, 1992; Sarkar and Banerjee, 1996; Kivanç, 1990), disc diffusion assay (Pulusani *et al.*, 1979; Fleming *et al.*, 1975), spot-on-lawn method (Schillinger and Lucke 1989; Çon *et al.*, 2001) etc. Some of them are based on dilution of antimicrobial agent in broth but most of the techniques based on the diffusion through solid or semi-solid culture media to inhibit the growth of sensitive organisms.

The purposes of this study were to compare the disc diffusion assay and spot-on-lawn method to determine the most reliable method for detection of antimicrobial activity and to show the effect of antimicrobial agents' inhibitory activity against some Gram (+) and Gram (-) bacteria.

Materials and Methods

Bacterial strains and culture media: The strains used in this study are listed in Table 1. *L. casei*, *L. plantarum* were cultured at 37°C and *L. helveticus* at 42°C anaerobically for 24 h. in de Mann Rogosa Sharpe (MRS) broth and then transferred to MRS agar slants incubated at 37°C and 42°C for 24 h and stored in the refrigerator. Pathogen indicator microorganisms were maintained on Brain Heart Infusion (BHI) soft-agar, other bacterial cultures were maintained on nutrient agar for 15 days at +4°C (Kivanç, 1990).

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Table 1: Reference of bacterial strains

Lactic Acid Bacteria	Reference Company
<i>Lactobacillus casei</i> 319 RSKK No: 706	Institute Superiordi Santa Roma
<i>Lactobacillus plantarum</i> ATCC 80141	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Lactobacillus helveticus</i> ATCC 15009	Karadeniz Technical University, Faculty of Medical, Microbiology Department
Indicator Bacteria Strains	
<i>Pseudomonas aeruginosa</i> ATCC 10145	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Escherichia coli</i> ATCC 25927	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Enterococcus faecalis</i> ATCC 29212	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Bacillus subtilis</i> ATCC 6633	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Enterobacter cloacae</i> ATCC 13047	Karadeniz Technical University, Faculty of Medical, Microbiology Department
<i>Escherichia coli</i> O157:H7 DraftBS150–16654	Ankara Control Lab.
<i>Staphylococcus aureus</i> ATCC 25923	Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology
<i>Escherichia coli</i> ATCC 25922	Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology
<i>Pseudomonas aeruginosa</i> ATCC 27853	Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology
<i>Proteus mirabilis</i> ATCC 7002	Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology

Preparation of cell-free filtrate: *L. casei* and *L. plantarum* were cultured at 37°C and *L. helveticus* at 42°C for 16-h. in MRS broth. Resulting 16-h cultures of lactic acid bacteria were inoculated 1% by volume into 150 ml MRS broth and incubated anaerobically at 37°C for 24, 48 and 72 h. The lactic acid bacteria cultures were centrifuged at 15000 rpm for 15 min and supernatants were collected in conical flasks and the pH was adjusted to 4,5. The cell-free supernatant was sterilized by membrane filtration (0,22µm pore size: Sartorius; (Kivanç, 1990).

Preparation of washed cell: Washed cells were prepared as described by Kivanç (1990), with some modifications. 16-h cultures of indicator bacteria were centrifuged at 15000 rpm for 15 min and cells were collected and supernatants discarded. Cells, washed twice with sterile physiological saline solution (PSS), were adjusted to 10³ and 10⁶ cells/ml.

Disc diffusion method: The disc diffusion method of Bhunia *et al.* (1988) was followed with modifications. Washed cells of indicator bacteria, at concentrations of 10³ and 10⁶ cells/ml, were added at 560 µl into 7 ml BHI soft agar (0,75% agar) and mixed. Then this inoculum was transferred on to MRS agar plates. 30µl cell-free supernatant from 24, 48 and 72 h lactic acid bacterial cultures were added to paper discs (disc diameter

6mm) and placed on the MRS agar surface and incubated at 37°C for 24 h., in anaerobe chamber. Sterile BHI broth was absorbed to a disc and placed on MRS agar as above, as a control. After incubation, a clear zone around a disc was evidence of antimicrobial activity.

Spot-on-lawn method: In spot-on-lawn method, 24, 48 and 72 h cultures of lactic acid bacteria cultures were spotted on to the surface of MRS agar plates and incubated at 37°C for 24 h. anaerobically. Suspensions of 16 h washed cells of indicator bacteria, were added to (8% by volume) 7 ml. BHI soft-agar and this mixture transferred on to MRS agar plates which contained overnight lactic acid bacteria cultures. Sterile MRS broth was spotted as a control. The inhibition zone after 24 h and 37°C anaerobic incubation was measured in millimeters (Schillinger and Lucke, 1989).

Results

Three *Lactobacillus* strains were tested against 10 indicator microorganisms. The results of the spot on lawn and disc diffusion method to determine the antimicrobial activity of the *Lactobacillus* strains on indicator bacteria are listed in Table 2 and 3.

As can be seen in Table 2, under the disc diffusion method, *L. plantarum* and *L. helveticus* exhibited inhibitory activity while under the spot on lawn method,

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Table 2: Antagonistic effect of lactobacilli against indicator microorganisms by disc diffusion method

Culture of Bacteria	<i>L. helveticus</i>						<i>L. plantarum</i>						<i>L. casei</i>					
	24h		48h		72h		24h		48h		72h		24h		48h		72h	
	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶
<i>B. subtilis</i> ATCC 6633	11*	8	21.5	21	12	10	12	11.5	21.5	20.5	12	11.5	9	11	11	9	12	10.5
<i>S. aureus</i> ATCC 25923	11	10.5	7.5	9	10	10	11	9	8.5	9	9.5	10	11.5	9.5	7.5	9	12	10
<i>E. faecalis</i> ATCC 29212	11	10.5	12	10	11.5	10	14	13.5	13	10	13	12.5	9	8.5	10.5	10.5	10	9.5
<i>E. cloaceae</i> ATCC 13047	10.5	9.5	8	7.75	9.5	10	10	8.5	8	7	10	9	9.5	9	8	7.5	10	10
<i>E. coli</i> O157:H7	14	11	16	14.5	15	14.5	13.5	11	15	14.5	12	10.5	10.5	10	11.5	11	12.5	11.5
<i>E. coli</i> ATCC 25927	12	11	13	12.5	12.5	11.5	12	11.5	14	13	13	12.5	9.5	9	10	9	11	10
<i>E. coli</i> ATCC 25922	11	9	8	9	11	8.5	11	10	9	9	9	9	10	10	10	10	10	8.5
<i>P. aeruginosa</i> ATCC 10145	11	10	20	18	10	9	12	10.5	20.5	20	11	9.5	8	7.5	12	9	10.5	9.5
<i>P. aeruginosa</i> ATCC 27853	11	9	6.25	10	7.5	7.5	10	8	6.25	10	8	7.5	11	9	6.25	10	8.5	7.5
<i>P. mirabilis</i> ATCC 7002	11.5	11.5	8	8	9.5	9	10	10	8	8	10	8.5	10	10	10	7.75	11	8

Table 3: Antagonistic effect of lactobacilli against indicator microorganisms by spot-on-lawn method

Culture of Bacteria	<i>L. helveticus</i>						<i>L. plantarum</i>						<i>L. casei</i>					
	24h		48h		72h		24h		48h		72h		24h		48h		72h	
	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶	10 ³	10 ⁶
<i>B. subtilis</i> ATCC 6633	18	18	34	27	29	25	20	19	23	22	25	23.5	20.5	19	21.5	23	23	18.5
<i>S. aureus</i> ATCC 25923	17	17	28	27	23.5	24	17	13	28	22	20.5	21	18	18	21	21	26	24
<i>E. faecalis</i> ATCC 29212	15	15	22.5	19	19.5	22	14.5	13.5	22	20	20	19	15	17	20	18.5	19.5	20
<i>E. cloaceae</i> ATCC 13047	23	13	16	15	21	21	16	15.5	18	17	18.5	18	16	17	20	21	24	11
<i>E. coli</i> O157:H7	16	19	23	23	25	15.5	16.5	26	24	24	21	18.5	18	25	24	25.5	25	
<i>E. coli</i> ATCC 25927	21	19	27	24.5	26	28	17.5	17	28	27	27	22	20	19	27	25.5	27.5	24
<i>E. coli</i> ATCC 25922	13	14.5	20	23	17	19	17	19	17	16	27	25	14	12	21.5	17	27.5	25
<i>P. aeruginosa</i> ATCC 10145	20	15.5	23	21	28	19	17	15	28	25	26	27	19	22	26	22.5	30	30
<i>P. aeruginosa</i> ATCC 27853	19	20	33	31	25.5	25	16.5	14	26	23	25	26	20	21	26	25	28	30
<i>P. mirabilis</i> ATCC 7002	16	17	26	27	23.5	25.5	14.5	14.5	26	25	23	26	18	16	21	20	30	31

L. helveticus and *L. casei* strains exhibit significant inhibitory activity against indicator microorganisms mostly (Table 3).

In the disc diffusion method *B. subtilis* ATCC 6633, *E. coli* O157:H7 and *P. aeruginosa* ATCC 10145 were the most inhibited indicator microorganisms. In the spot-on-lawn method, *P. aeruginosa* ATCC 27853 was the most sensitive of the tested bacteria followed by *E. coli* ATCC 25927 and *P. aeruginosa* ATCC

10145. Our results obtained from the disc diffusion assay showed that *P. aeruginosa* ATCC 27853 was the most resistant strain to *L. helveticus* and *L. casei*, however in the spot-on-lawn method this organism was the most sensitive indicator bacteria. Similarly, *L. helveticus* possessed inhibitory activity against *E. coli* ATCC 25927 in the spot-on-lawn method, but it was less active in the disc diffusion assay.

As in Table 2 and 3, it is that Gram (-) bacteria were mostly inhibited. *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25927, *P. aeruginosa* ATCC 10145, *E. coli* O157:H7 were the most sensitive Gram (-) bacteria in our research, especially in the spot-on-lawn method.

Discussion

We determined the antagonistic effects of various lactic acid bacteria against Gram (+) and Gram (-) bacteria in a comparison of the disc diffusion and the spot-on-lawn methods.

In the disc diffusion assay *B. subtilis* ATCC 6633, in the spot-on-lawn method, *P. aeruginosa* ATCC 27853 were the most sensitive bacteria. Kivanç (1990) indicated that *P. aeruginosa* spoiled foods at low temperatures as a result of its lipolytic and proteolytic activity, and was sensitive to *L. casei* and *L. plantarum*. These results were confirmed also by Çon and Gökalp (2000), Schillinger and Lucke (1989) and Kaya (1992). Some antimicrobial metabolites, like diacetyl, inhibited Gram (-) bacteria more than Gram (+) bacteria (Narasimhan *et al.*, 1988).

As a result, the inhibitory activity on tested bacteria by the spot-on-lawn method is seen as better, but it could be because of all metabolites; lactic acid, acetic acid, diacetyl, bacteriocin etc., are present and being produced during the assay period (Çon and Gökalp, 2000). But in the disc diffusion method, supernatants of lactic acid bacteria cultures were used, and anaerobic conditions were imposed to decrease H₂O₂ inhibitory activity, and the pH was adjusted to 4,5. So, the inhibition zone that was seen around discs may be dependent solely on bacteriocin activity.

In addition, in antimicrobial activity research the spot-on-lawn method is a practical and suitable technique. However in bacteriocin investigations, the spot-on-lawn method should be controlled with the disc diffusion method. The antimicrobial compounds, produced by lactic cultures, have a great potential for controlling the growth of food spoilage and pathogenic microorganisms.

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