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Antagonistic Effects of Some Lactobacilli On Some Gram-Negative Bacteria

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ABSTRACT

Three lactobacilli strains were examined for the inhibitory activity against some gram-negative bacteria with a comparison of well diffusion and spot on lawn method. The inhibitory activity of lactobacilli under spot on lawn method showed significant clear zones. Although in spot on lawn method, lactobacilli have strongest antagonistic activity against *P. aeruginosa* ATCC 27853, in well diffusion method, it was the least sensitive tested bacteria. In spite of *E. coli* O157:H7 is inhibited secondly in well diffusion method (16mm), the value of the inhibition is lower than spot on lawn method (26mm). These results showed that spot on lawn method is better method than well diffusion method.

Key Words: *Antagonism, Gram-negative bacteria, Lactic acid bacteria, Spot on lawn method, Well diffusion method*

1. INTRODUCTION

Lactobacilli are fermentative and saccharoclastic microorganisms. Their production at least half of the end-product carbon is lactate. Major fermentation products from utilizable carbohydrates are mainly lactate, may give some acetate, ethanol, CO_2 [1], hydrogen peroxide, diacetyl [2] and bacteriocins [3] which have inhibitory effects towards other bacteria especially against pathogen bacteria like *E. coli* [4], *Pseudomonas aeruginosa* [2]. While inorganic metabolites like diacetyl, inhibit gram - negative bacteria by reducing the pH, bacteriocins are one of the organic metabolites which inhibit mostly gram-positive bacteria [2]. Bacteriocin-like substances may be defined as extracellulary released bacterial peptide or protein molecules that in low concentrations are able to kill some closely related bacteria by a mechanism against which the producer bacterium itself exhibit some specific immunity [4].

In this study we examine to determine the antagonistic effect of lactobacilli against some gram-negative bacteria by a comparison of spot on lawn and well diffusion assays which are commonly used methods for the measurement of antagonistic activity.

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2. MATERIALS AND METHODS

2.1. Bacterial Strains and Culture Media

The lactic acid bacteria strains used in this study are *Lactobacillus casei* 319 RSKK No: 706, *Lactobacillus plantarum* ATCC 80141 and *Lactobacillus helveticus* ATCC 15009. As indicator bacteria strains, *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* ATCC 25927, *Enterobacter cloaceae* ATCC 13047, *Escherichia coli* O157:H7, *Salmonella* EN 12824, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *Proteus mirabilis* ATCC 7002 were used.

L. casei and *L. plantarum*, were maintained anaerobically in de Mann Rogosa Sharpe (MRS) broth at 37ºC, *L. helveticus* at 42ºC for 24 hours and then transferred to MRS agar slants and stored at +4ºC. Pathogen indicator microorganisms were maintained on Brain Heart Infusion (BHI) agar, others on nutrient agar [8].

2.2. Well Diffusion Method

Well diffusion method of Kivanç [9] was followed with modifications. 16 h washed cells of indicator bacteria, had an inoculum's of 10^3 and 10^6 cells/mL, were added 800 µL in 10 mL nutrient agar-tween 80 mixtures (0.2 % Tween 80) and poured on plates. After solidification, 6 mm diameter wells were opened and covered with soft agar (0.75 % agar) then 30µL cell-free supernatant was filled [8]. After supernatant's diffusion, plates were incubated at 37°C for 24 h., anaerobically. Non-cultured nutrient agar-tween 80 mixtures were used as control. After incubation, a clear zone around the wells is an evidence for antimicrobial activity. All of these investigations repeated for 24, 48 and 72 h lactic acid bacteria's cell-free supernatant.

2.3. Spot on Lawn Method

Inhibitory activities of lactic acid bacteria on 16 h washed cells of indicator bacteria were determined by spot on lawn method, as described by Schillinger and Lucke [8]. The inhibition zone after 24 h and 37°C anaerobically incubation, is measured in millimeters.

3. RESULTS AND DISCUSSIONS

The inhibitory activity of lactobacilli against some gram – negative bacteria was compared with well diffusion and spot on lawn method (Table 1, 2). In both of methods, *L. helveticus* and *L. plantarum* strains exhibit significant inhibitory activity against indicator microorganisms mostly

Table 1. Antagonistic effect of lactobacilli against various gram -negative bacteria by well diffusion method.

Strains of Bacteria	*L. helveticus*						*L. plantarum*						*L. casei*					
	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
Salmonella EN 12824	13*	12	14	13	12	11	11	9,5	12	11	10	8	8,5	6	9,5	11	8	9
E. cloaceae ATCC 13047	11	11,5	11	11	12	10,5	9,5	10	10	9	10	10,5	11	12	9,5	12	11,5	8,5
E. coli O157:H7	15	13	16	14	14	12,5	12	16	14	12	17	10	15	11,5	14	13	15,5	13
E. coli ATCC 25927	11	10	13	12	10	9	13	12	14	13	12	11,5	5	7	16	12	11,5	9,5
E. coli ATCC 25922	11	10	12	11	10,5	11	11,5	11	12	8,5	12,5	10	9,5	9,5	11	10,5	10	12,5
P. aeruginosa ATCC 10145	19	18	21	19	16	14	22	20	23	21	15	14	7	6	11	9	10,5	12
P. aeruginosa ATCC 27853	10	9	10	9	9	9	9,5	11,5	11	9,5	10	10	11	10	12,5	12,5	12	9
P. mirabilis ATCC 7002	13,5	10,5	12	12	12	8,5	13	11,5	13	11	10	8	12	10,5	10	10	10,5	10

*Measured in millimeters

Table 2. Antagonistic effect of lactobacilli against various gram -negative bacteria by spot on lawn method.

Strains of Bacteria	*L. helveticus*						*L. plantarum*						*L. casei*					
	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
Salmonella EN 12824	16	15	24,5	22	25,5	23,5	16	16,5	27	25	28	26	18	18	24	26	26,5	27
E. cloaceae ATCC 13047	23	13	16	15	21	21	16	15,5	18	17	18,5	18	16	17	20	21	24	11
E. coli O157:H7	16	19	23	23	25	26	15,5	16,5	26	24	24	21	18,5	18	25	24	25,5	25
E. coli ATCC 25927	21	19	27	24,5	26	28	17,5	17	28	27	27	22	20	19	27	25,5	27,5	24
E. coli ATCC 25922	13	14,5	20	23	17	19	17	19	17	16	27	25	14	12	21,5	17	27,5	25
P. aeruginosa ATCC 10145	20	15,5	23	21	28	19	17	15	28	25	26	27	19	22	26	22,5	30	30
P. aeruginosa ATCC 27853	19	20	33	31	25,5	25	16,5	14	26	23	25	26	20	21	26	25	28	30

In our study, in spot on lawn method, *P. aeruginosa* ATCC 27853 was determined as the most sensitive tested bacteria followed by *P. mirabilis* ATCC 7002 and *P. aeruginosa* ATCC 10145 with 30-33 mm inhibition zones (Table 2). On the other hand, in well diffusion assay, *P. aeruginosa* ATCC 10145 was the most inhibited indicator microorganisms with 23 mm inhibition zones (Table 1). Although *E. coli* O157:H7 is inhibited secondly in well diffusion method (16mm), the value of the inhibition is lower than spot on lawn method (26mm) (Table1-2).

The most resistant indicator microorganisms were in spot on lawn method, *E. cloaceae* ATCC 13047, and in well diffusion assay, *P. aeruginosa* ATCC 27853 (Table 1 and 2). In spite of *Salmonella* EN 12824 was one of the most resistant strains in well diffusion method, it was inhibited 28 mm in spot on lawn method. *P. aeruginosa* ATCC 27853 was sensitive in spot on lawn method while it was resistant in well diffusion method. These results might be due to cells presence in spot on lawn method.

According to Schillinger and Lucke [8], spot on lawn method is more effective method than well diffusion method for measuring antimicrobial activity. Similar results were found by Con and Gokalp [9]. They showed that *L. plantarum* inhibited *C. perfringens*, *C. botulinum* and *B. cereus* with spot on lawn method but, there was no inhibition zone with well diffusion method.

As a result, the inhibitory activity of lactobacilli on tested bacteria under spot on lawn test could be due to all metabolites; lactic acid, acetic acid, diacetyl, bacteriocin etc. In well diffusion method, supernatant of lactic acid bacteria were used, anaerobic conditions were prepared to decrease H_2O_2 inhibitory activity and pH was adjusted to 4.5. So, the inhibition zone which had been seen around wells could be a result of bacteriocin.

We conclude that spot on lawn method has several advantages towards well diffusion method by means of the efficiency of the inhibition and the facility of the application of the method.

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