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Antagonistic Effects of Lactobacilli on Gram-Negative Bacteria

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Abstract: Three lactobacilli strains were examined for the inhibitory activity against some gram-negative bacteria with a well diffusion method. Lactobacilli have strongest antagonistic activity against *P. aeruginosa* in well diffusion method; it was the least sensitive tested bacteria. In spite of *E. coli* 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 a better method than the well diffusion method.

Keywords: Antagonism, Gram-negative bacteria, Lactic acid bacteria, Well diffusion method.

1. Introduction

Antagonistic effects of some microorganisms against others *in vivo* and *in vitro* have been reported by many investigators. On many raw foods, the bacterial microbiota is often composed of mixed species. The activities of one of bacterial species may be influenced by the growth activities of others (1). Bacterial antagonism could arise from the combined effects of several mechanisms during their growth in the media. For example, one group of microorganisms may remove a growth factor required for the growth of another, synthesize a substance inhibitory to another or produce an adverse pH or Oxidation Reduction Potential (Eh) (2). Lactobacilli are fermentative and saccharolytic 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 [3], hydrogen peroxide, di-acetyl [4] and bacteriocins [5] which have inhibitory effects towards other bacteria especially against pathogen bacteria like *E. coli* [6], *Pseudomonas aeruginosa* [4]. While inorganic metabolites like di-acetyl inhibit gram-negative bacteria by reducing the pH, bacteriocins are one of the organic metabolites which inhibit mostly gram-positive bacteria [4]. Bacteriocin-like substances may be defined as an extracellularly 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 [6]. 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.

2. Materials and Methods

2.1 Bacterial Strains and Culture Media

The lactic acid bacteria strains used in this study are *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus fermentum*. As indicator bacteria strains, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella*, and *Proteus mirabilis* were used. *L. casei* and *L. plantarum* was maintained anaerobically in de Man, Rogosa and Sharpe (MRS) broth at 37^0C, *L. fermentum* at 42^0C for 24 hours and then transferred to MRS agar slants and stored at $+4^0$C. Pathogen indicator microorganisms were maintained on Brain Heart Infusion (BHI) agar, others on nutrient agar [10].

2.2 Well Diffusion Method

Well diffusion method of Kivanç [11] was followed with modifications. 16 h washed cells of indicator bacteria, had inoculums of 103 and 106

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cells/mL, were added 800 CL in 10mL nutrient agar-tween 80 mixtures (0.2% Tween 80) and poured on plates. After solidification, 6mm diameter wells were opened and covered with soft agar (0.75% agar) then 30 CL cell-free supernatant was filled [10]. After supernatant's diffusion, plates were incubated at 37^0C for 24 h, anaerobically. Non-cultured nutrient agar-tween 80 mixtures were used as a 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 lawn method

Inhibitory activities of lactic acid bacteria on 16 hours washed cells of indicator bacteria were determined by spot lawn method, as described by Schillinger and Lucke. The inhibition zone after 24 hours and 37^0C, anaerobically incubation, is measured in millimeters.

3. Result and Discussion

The inhibitory activities of lactobacilli against some gram-negative bacteria were compared with well diffusion and spot on lawn method (Table 1, 2). In both of methods, *L. fermentum* and *L. plantarum* strains exhibit significant inhibitory activity against indicator microorganisms mostly in our study, in a spot on lawn method, *P. aeruginosa* was determined as the most sensitive tested bacteria followed by *P. mirabilis* and *P. aeruginosa* with 30-33mm inhibition zones (Table 2). On the other hand, in well diffusion assay, *P. aeruginosa* was the most inhibited indicator microorganisms with 23mm inhibition zones (Table 1). Although *E. coli* is inhibited secondly in well diffusion method (16mm), the value of the inhibition is lower than spot on lawn method (26mm) (Table 2).

The most resistant indicator microorganisms were in a spot on lawn method, *E. cloacae*, and in well diffusion assay, *P. aeruginosa* (Table 1 and 2). In spite of *Salmonella* was one of the most resistant strains in well diffusion method, it was inhibited 28mm in spot on lawn method. *P. aeruginosa* was sensitive in spot on lawn method while it was resisted in well diffusion method. These results might be due to the cells present in spot on lawn method. According to Schillinger and Lucke [10], spot on lawn method is more effective method than a well diffusion method for measuring antimicrobial activity. Similar results were found by Con and Gokalp [11]. 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 the 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, di-acetyl, bacteriocin etc. In the 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.

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

Strains of Bacteria	*L. fermentum*						*L. plantarum*						*L. casei*					
	24 h		48 h		72 h		24 h		48 h		72 h		24 h		48 h		72 h	
	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	13	12	14	13	12	11	11	9.5	12	11	10	8	8.5	6	9.5	11	8	9
E. cloacae	11	11.5	11	11	12	10.5	9.5	10	10	9	10	10.5	11	12	9.5	12	11.5	9.5
E. coli	15	13	16	14	14	12.5	12	16	14	12	17	10	15	11.5	14	13	15.5	13
E. coli	11	10	13	12	10	10	13	12	14	13	12	11.5	5	7	16	12	11.5	9.5
E. coli	11	10	12	10	10.5	11	11.5	11	12	8.5	12.5	10	9.5	9.5	11	10.5	10	12.5
P. aeruginosa	19	18	21	19	16	14	22	20	23	21	15	14	7	6	11	9	10.5	12
P. aeruginosa	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	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. fermentum*						*L. plantarum*						*L. casei*					
	24 h		48 h		72 h		24 h		48 h		72 h		24 h		48 h		72 h	
	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	16	15	24.5	22	25.5	23.5	16	16.5	27	25	28	26	18	18	24	26	26.5	27
E. cloacae	23	13	16	15	21	21	16	15.5	18	17	18.5	18	16	17	20	21	24	11
E. coli	16	19	23	23	25	26	15.5	16.5	26	24	24	21	18.5	18	25	24	25.5	25
E. coli	21	19	27	24.5	26	28	17.5	17	28	27	27	22	20	19	27	25.5	27.5	24
E. coli	13	14.5	20	23	17	19	17	19	17	16	27	25	14	12	21.5	17	27.5	25
P. aeruginosa	20	15.5	23	21	28	19	17	15	28	25	26	27	19	22	26	22.5	30	30
P. aeruginosa	19	20	33	31	25.5	25	16.5	14	26	23	25	26	20	21	26	25	28	30

*Measured in millimeters

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