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Comparison of Different Methods for Detection of Antimicrobial Activity of *Lactobacillus* spp.

AIJAZ HUSSAIN SOOMRO, TARIQ MASUD, SHEHLA SAMMI AND HABIB AHMED RATHORE

Department of Dairy Technology, Sindh Agriculture University Tandojam, Pakistan (AHS), Department of Food Technology, University of Arid Agriculture, Rawalpindi, Pakistan (TM, SS) and Department of Food Technology, University College of Agriculture Rawalakot, AJK (HAR)

Abstract.- The aim of this study was to determine antagonistic effect of *Lactobacillus* spp. against Gram positive and Gram negative bacteria with a comparison of deferred antagonism, spot-on-lawn and paper disc methods. For this purpose *Lactobacillus acidophilus* strains isolated from fermented milk product yoghurt were used. Only one strain *Lactobacillus acidophilus* J1 showed an inhibitory effect against *Escherichia coli* in all the methods used. The paper disc method showed pronounced effect as compared with other methods.

Key words: Antibacterial effect, *Lactobacillus* spp.

INTRODUCTION

Lactic acid bacteria (LAB) are traditionally used as natural or selected starters in the manufacture of yoghurt, cheese and other fermented products. During fermentation LAB are known to cause acidification, produce flavor compounds production, and protect food from spoilage and pathogenic microorganisms by producing organic acids, hydrogen-peroxide, diacetyl, and bacteriocins (Messens and De Vuyst, 2002).

Bacteriocins are protein or peptides, which do not harm the producer strain but have lethal antibacterial activity against food spoilers and/or food borne pathogens. In different studies inhibition of Gram +ve organisms by LAB is reported but investigations about Gram –ve bacteria are very few. It is reported that some *Lactobacillus* strains had an inhibitory activity against *E. coli* (Rodriguez *et al*., 1989).

Most of the bacteriocins from lactic acid bacteria have been isolated from species of the genus *Lactobacillus,* especially from *L. acidophilus.* It is a nonpathogenic Gram-positive bacterium and occurs as the predominant lactobacilli in the human intestine, display interesting probiotic properties (Klein *et al*., 1998) and is widely used for the production of fermented dairy products such as acidophilus yoghurt and sweet acidophilus milk (Tamime and Marshall, 1997).

There are many techniques for detecting antimicrobial activity such as, flip-streak method (Spelhaugh and Harlander, 1989), well diffusion assay (Stecchini *et al*., 1992), paper disc assay (Ohmomo *et al.,* 2000) and spot-on-lawn method (Schillinger and Lucke 1989). Some of them are based on dilution of antimicrobial agent in broth but most of the techniques are based on the diffusion through solid or semi-solid culture media to inhibit the growth of sensitive organisms.

The purposes of this study was to compare the deferred antagonism, spot on lawn and paper disc methods in order to determine the most reliable method for detection of antimicrobial activity against some Gram +ve and Gram -ve bacteria.

MATERIALS AND METHODS

Isolation and identification of Lactobacillus acidophilus

The streak plate method was used to isolate the *L. acidophilus* on solid selective media. For this purpose a loopful of each sample was streaked on MRS agar (Oxoid, England) plates and incubated at 37°C for 24 h. After incubation the culture was observed for growth, single and isolated colonies were picked and sub cultured on MRS agar media and incubated at 37°C for 24 h to obtain a pure

*Correspondence author: Email; aijaz68@hotmail.com
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culture of the isolates. Simultaneously the smears were prepared and stained with Gram's stain and examined under microscope for staining characteristics and morphology of the isolates.

The identification was done according to the morphological, cultural and biochemical characteristics described by Collins and Lyne (1980).

Bacterial strains and culture media

Among the lactic acid bacteria isolated from indigenous fermented milk product yoghurt 14 strains of *L. acidophilus* were maintained in MRS broth with 20% glycerol and stored at -20^{o}C. Before use the strains were propagated twice in their respective broth at 37^{o}C under anaerobic conditions. Among the indicator strains, all the LAB were isolated in this laboratory, whereas pathogens were provided by Department of Pathology, Army Medical College, Rawalpindi. The cultures were maintained at 4^{o}C in Nutrient agar slants and were transferred to fresh broth 24 hours before start of the experiment.

Detection of antimicrobial activity

For the determination of antagonistic activity, the deferred antagonism, spot-on-lawn method as described by Kim *et al.* (2000) and paper disc method (Ohmomo *et al*., 2000) were used against different indicator strains.

Deferred antagonism method

In deferred antagonism method the strains of *L. acidophilus* were grown in MRS broth. Ten µl of overnight broth cultures were spotted onto the surface of MRS agar plates and incubated for 2 h at 37^{o}C to allow colonies to develop. Hundred µl of indicator strains mixed with 3.5 ml of soft (0.75%) MRS agar was poured over the plates. After incubation at the optimum growth temperature of the indicator strains the plates were checked for the formation of inhibition zones around the producer colonies.

Spot-on-lawn method

The *L. acidophilus* strains were grown in MRS broth at 37^{o}C for 16 h. The supernatants were collected by centrifugation at 13000xg for 20 min at 4^{o}C, the pH of the supernatants were adjusted to 5.5 with 1M NaOH and filter sterilized by using 0.45 µm porosity filters (Sartorius, Germany). Hundred µl of indicator strains grown in broth were mixed with 3.5 ml of soft MRS agar and were overlaid over the MRS agar plates. The plates were incubated at 37^{o}C for 2 h. Thirty µl of the cell-free supernatant of these strains were spotted onto the overlaid surface. The plates were incubated at 37^{o}C for 18 h and were subsequently examined for inhibition zones.

Paper disc method

For the determination of antimicrobial activity sterile filter paper discs measuring 6 mm diameter, thin type with an adsorbed aliquot of 20 µl of cell-free supernatant were placed on MRS and Nutrient agar plates containing target strain. After incubation for 37°C the inhibitory activity was evaluated, which was based on the formation of a clear zone around the paper disc.

RESULTS AND DISCUSSION

The phenotypic identification revealed that all the 14 isolates were Gram positive rods occurred singly, in pairs and short chains, catalase negative, non-motile, non spore former. On MRS agar gray white rough colonies were observed. The isolates produced acidic reaction in glucose, lactose, fructose, sucrose and maltose whereas did not ferment mannitol.

In order to determine the antimicrobial activity three methods deferred, spot on lawn and paper disc assay were used against different indicator organisms but only one isolate *L. acidophilus* J1 showed positive results. However, variation was observed among the examined methods in the zone of inhibition produced by the *L. acidophilus* J1 against indicator strain *E. coli* (Fig. 1).

The deferred method is common and simple method but the inhibition observed may also be due to certain other factors such as production of organic acids. These results are supported by Kim *et al.* (2000), that the antagonism deferred method was more suitable when compared with the streak and

well methods due to its reproducibility, rapidity and simplicity.

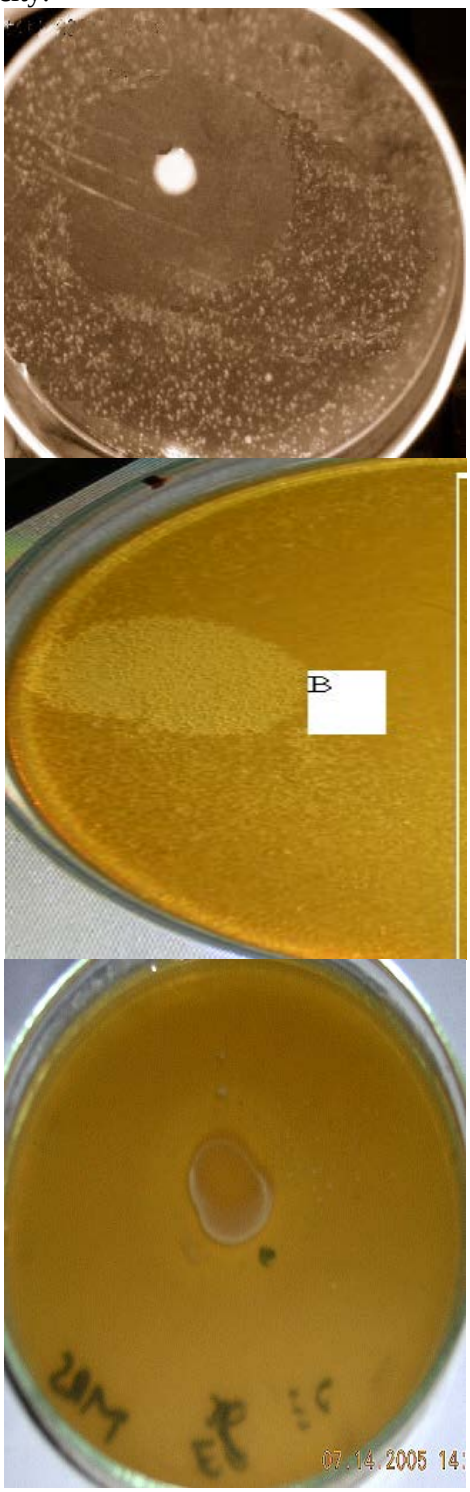

Fig. 1. Zone of inhibition observed by *L. acidophilus* J1 against *E. coli* in deferred (A), Spot-on-lawn (B), and paper disc (C) methods.

Whereas in spot-on-lawn method culture filtrate was used by eliminating the effects of organic acid and hydrogen peroxide and this showed inhibitory effect which is due to production of antimicrobial compound bacteriocin against the indicator strain *E. coli*. It was also observed that in spot on lawn method the zone of inhibition was higher as compared to the deferred method against test organism *E. coli*. These results confirmed the findings of Kim *et al.* (2000), where as the Lewus *et al.* (1991) reported that only a few strains showed positive results in the spot-on-lawn method. Moreover, Lewus *et al.* (1991) were of the view that spot-on-lawn method was more reproducible, rapid and easy to score than other methods. Similar observations were made by Spelhaug and Harlander (1989) that in a study of three bacteriocin producing strains against a large panel of food borne pathogens they found that spot method was superior to other methods. However, McLean and McGroarty (1996) were of the view that about 60% of the antimicrobial activity of culture filtrates of LAB was removed when the filtrates were neutralized to pH 6.5 with NaOH. Whereas Varadaraj *et al.* (1993) observed moderate inhibition of some food borne pathogens and other bacterial species by neutralized culture filtrates of LAB.

In paper disc method the inhibition zone was about 10 mm against *E. coli* and this showed that the paper disc method was more appropriate as compared to the other methods. As a result the inhibitory effect produced by producer strain against *E. coli* by paper disc method was well observed. These results are further supported by Ohmomo *et al.* (2000), Rattanachaikunsopon and Phumkhachorn (2000) and Yurdugul and Bozoglu (2002).

However, in antimicrobial activity research the spot-on-lawn method is a practical and suitable technique but in the present investigation it was observed that paper disc method is more appropriate and was easy to use. However, in bacteriocin investigations, the spot-on-lawn method should be used with the paper disc method. The antimicrobial compound produced by lactic cultures, have shown great potential in controlling the growth of food

spoilage and pathogenic microorganisms.

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