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Antagonistic activity of probiotic organism against *Vibrio cholerae* and *Cryptococcus neoformans*

Vidya, R. and Iyer, P. R.*

Women`s Christian College, College Road, Chennai-600006.
E-mail: brajuraj@yahoo.com

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ABSTRACT

The microbes are useful in many ways in the modern world. Probiotics one of them, which refers to, acid adherence bacteria in the intestinal cells, are able to survive at low pH and produce large amount of lactic acid. The present investigation deals with the antagonistic activity of *Lactobacillus acidophilus* organism against pathogens. The organism was isolated from the curd sample. Identification of bacteria was done by various biochemical testing. The present study revealed that *L. acidophilus* inhibits *Vibrio cholerae* more efficiently than *Streptococcus pneumoniae* and *Shigella dysentriae*. When *L. acidophilus and V. cholerae* were grown together, *L. acidophilus* dominated the growth and competitively inhibited the growth of *V. cholerae. L. acidophilus* was also found to inhibit *Cryptococcus neoformans.*

Keywords: Probiotics, acid adherence bacteria, lactic acid bacteria

INTRODUCTION

'Probiotics' are live microbial food and feed supplements that are supposed to benefit health by improving the balance of the intestinal microbial community. The term probiotics was first introduced by Parker (1974). According to him probiotics are "Organisms and substances which contribute to intestinal microbial ecological balance". Helpful bacteria, such as *L. acidophilus* and *B. bifidum*, have the ability to kill off other bacteria by secreting small quantities of antibiotic–like substances, including lactic-acid, acetic–acid, benzoic-acid, hydrogen peroxide, acidolin, lactocidin and acidophilin. Research has shown that the benefits of the friendly bacteria are achieved without the undesirable side effects of antibiotic therapy, including diarrhea, digestive problems and vaginal yeast infections (Fuller, 1989).

Lactobacilli of human intestinal origin have been shown to exhibit antagonistic activity against both Gram positive and Gram negative bacteria. Many strains belonging to the *L. acidophilus* groups have been reported to produce antimicrobial compounds, which show a great variety as to their inhibition spectrum. Antimicrobial substance produced are smaller peptides and do not contribute to the formation of resistance to pathogens (Alla *et al.*, 2003). Feed conservation rate is decreased due to the fact that many of the probiotic microorganisms produce enzymes like amylase, protease and lipase, whether in gut or in environment. The pathogens are kept at bay by probiotic microorganisms by the process called competitive inhibition (Akshat *et al.*, 2004; Attai *et al.*, 1987).

MATERIALS AND METHODS

Microscopic examination

A loop full of curd was placed on clean glass slide. Cover slip was placed on it. The slide was observed under the microscope.

Serial dilution

The locally procured curd sample was homogenized and then it was serially diluted to all seven test tubes till 10^{-9}

Plating: spread plate method

Rogosa Medium was used for the isolation and enumeration of Lactobacilli in the oral and intestinal microbial flora, meat, milk and other food stuffs. 0.1 mL of the serially diluted sample was spread plated on Rogosa Medium plates. The plates were incubated in Anareobic Gas Pack Jar for 48 h at 37 °C (De Man *et al.*, 1960).

Identification of *L. acidphilus* (Harringan and Margaret, 1993)

A control sample of the *L. acidophilus* was used. This culture was procured from the microbiology lab of the local Aavin dairy.

Gram staining

Gram staining was performed with the colonies obtained from the Rogosa Plate after incubation using Crystal Violet and Safranine stains.

***Corresponding author**

Catalase test

Catalase test was performed by transforming the bacterial culture to the slide containing hydrogen peroxide using glass rod. The slide was then examined for the formation of bubbles within few minutes.

Motility test

Motility test was performed using hanging drop method.

Detection of carbon dioxide production from glucose

Gibson's semi-solid medium was prepared and pH was adjusted to 6.5 and was distributed to test tubes to a depth of 5 to 6 cm and sterilized by steaming. Incubation was made with inoculation needle. The tubes were incubated in anaerobic jar for 48 h at 37 °C.

Detection of ammonia production from arginine

Arginine MRS broth was prepared and distributed in tubes and sterilized at 15 lb for 15 min. The culture was inoculated and incubated in anaerobic jar for 7 days at 25 °C. Nessler's reagent was added to detect the ammonia production.

Fermentation tests

MRS fermentation containing 0.004% chlorophenol red was prepared and distributed in tubes and sterilized at 15 lb for 15 min. 10% of test substrate (Mannitol) was prepared, sterilized (filtration) and was aseptically added to the sterilized MRS broth to give a final concentration of 2% .Culture was inoculated and incubated in Anaerobic Jar for 3 days.

Antibacterial assay

Bacterial cultures of *V. cholerae*, *S. pneumoniae* and *S. dysentriae* were obtained from the Microbiology labs of a Private Laboratory, Chennai. *Aspergillus niger* and *C. neoformans* was procured from IMTECH, Chandigarh, INDIA.

Well assay

Muller Hinton agar plates were prepared surface of the MHA plates was inoculated with the swab containing 24 h culture (*V. cholerae*). Wells were punched with the gel puncher 20 mL of supernatant of culture (*L. acidophilus*) were added to the wells punched in the centre of the plates. Plates were incubated for 24 h at 37 °C. *S. pneumoniae* and *S. dysentriae* were also incubated on the MHA plates, separately and wells were punched as mentioned above.

Well assay using solvents

A 24 h culture of *L. acidophilus* from MRS broth were taken (3 mL) in eppendorf and centrifuged at 10,000 rpm for 15 min. Supernatant were collected separately and to equal amounts of solvents like butanol, chloroform, propanol, methanol, acetone, isoamylalcohol, diethyl ether, petroleum ether and hexane was added in separate eppendorf. Solvents were allowed to evaporate and the sample (30 µL) was added to the well punched in the plates. Plates were incubated for 24 h at 37 °C.

Colony inhibition

Hundred microlitre of *V. cholerae* culture was added to first tube containing nutrient broth. 100 mL of sample culture was added to second tube containing nutrient broth.

Hundred microlitre of *V. cholerae* was added to the third tube containing nutrient broth. To the same tube 100 mL of sample culture was added. All the three tubes were kept for overnight incubation. Each tube was serially diluted to 10^{-7} tube. *V. cholerae* was serially diluted and spread plated on TCBS agar.

Serial dilution performed with sample culture was spread plated in MRS agar plate.

The mixture (*V. cholerae* with sample) which was serially diluted was placed both on MRS agar and TCBS agar plates. The plates were incubated at 37 °C for 24 h.

Mycological methods

Preparation of culture filtrate

Twenty four hours culture (sample) was centrifuged at 15,000 rpm for 10 min. Sterile cell–free supernatant was suspended in 10 mM acetic acid.

Fungal inocula

Inocula containing spores or conidia were prepared by growing the molds on PDA slants for 7 days. Spores or conidia were collected after vigorously shaking the slants with sterile peptone water. 24 h yeast cell inocula were prepared from washed cultures grown in PDA broth at 30 °C.

Antifungal activity assays

Agar- diffusion assay

MRS agar plates containing *A. niger* conidia and *C. neoformans* were prepared. Wells, with a diameter of 5 mm, were then cut in the agar using sterile gel puncher. 30 µL of samples were added to each well and allowed to diffuse into the agar during a 5 h preincubation period at room temperature, followed by aerobic incubation at 30 °C (Muriana and Klaenhammer, 1991; Magnusson and Schnürer, 2002).

Overlay method

Overlay method was performed using MRS agar plates on which *L. acidophilus* were inoculated as two (2) cm–long lines. Inoculated plates were incubated at 30 °C for 48 h in Anaerobic jar. The plates were then overlaid with 10 mL of Potato Dextrose Soft agar containing yeast cells or fungal spores (conidia). The plates were then incubated aerobically at 30 °C for 48 h. The plates were examined for clear zones of inhibition around the bacterial streaks (Jacobsen *et al.*, 1999; Sjögren *et al.*, 2003).

RESULTS

L. acidophilus was isolated on Rogosa media from curd sample. The organism is identified as *L. acidophilus* based on the Bergey's Manual of Bacteriology and the control organism results (8th Edition) (Table 1).

Antibacterial assay

Well assay

The inhibitory activity against *V. cholerae*, *S. pneumoniae* and *S. dysentriae* was seen in (Figure 1, 2 and 3 and Table 2) zone of clearance obtained with each organism (Piard and Desmazeaud, 1992; Ogunbanwo *et al.*, 2003).

Well assay using solvents

The inhibitory activity against *V. cholerae, S. pneumoniae* and *S. dysentriae* using solvents such as acetone, butanol, chloroform, propanol, methanol, isoamyl alcohol, diethyl ether, petroleum ether and hexane was observed (Figure 4, 5 and 6 and Table 3) as zone of inhibition obtained with each organism.

Colony inhibition

The colony inhibitory effect of Lactobacillus on *V. cholerae* on TCBS agar plate is shown in (Figure 7 and 8 and Table 4) shows total number of colonies obtained in 10^{-4} and 10^{-6} dilution (Klaenhammer, 1983; Kastmoglu and Akgun, 2004; Alkaline *et al.*, 2004).

DISCUSSION

The result obtained for antimicrobial activity using well assay inferred *Lactobacillus acidophilus* inhibits *V. cholerae, S. dysentriae* and *S. pneumoniae*. It could be seen from the Table 1 that of the three bacteria used for antibacterial activity of *L. acidophilus*, *V. cholerae* was found to be the most sensitive. Similar results have been reported for *Lactobacillus* sp. (Juven *et al.*, 1992). Lactobacillus sp. of organism has also been reported to be active against the vaginal pathogens. The results also prove the natural medicine correct where buttermilk is used for diarrhea (Piard and Desmazeaud, 1992).

Table 1: Biochemical characteristics of *Lactobacillus acidophilus*

Sl. No	Test	*L. acidophilus*
1.	Grams Reaction	Positive
2.	Catalase test	Negative
3.	Motility test	Non.motile
4.	Gas from Glucose	Negative
5.	Arginine test	Negative
6.	Mannitol	Negative
7.	Lactose	Positive
8.	Glucose	Positive

Table 2: Overlay method to check antimicrobial activity

Organisms	Zone of clearance
Vibrio cholerae	10mm
Streptococcus pneumoniae	3 mm
Shigella dysentriae	8 mm

Figure 1: Zone of inhibition on *Vibrio cholerae*

Figure 2: Zone of inhibition on *Streptococcus pneumoniae*

Figure 3: Zone of inhibition *on Shigela dysentriae*

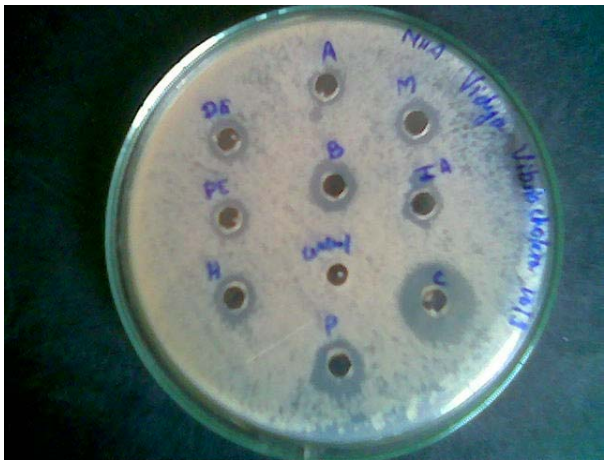

Figure 4: Zone of inhibition on *Vibrio cholerae*

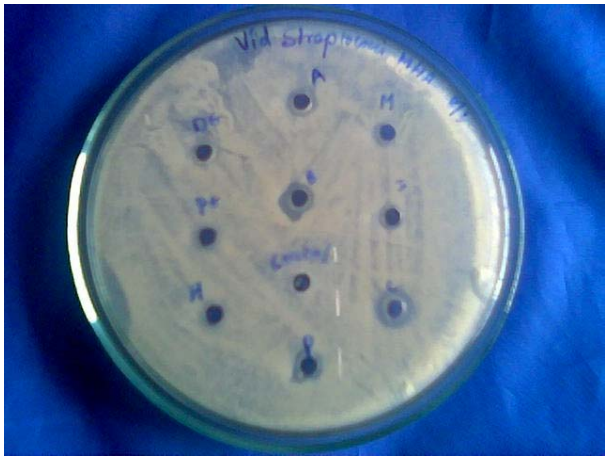

Figure 5: Zone of inhibition on *Streptococcus pneumoniae*

Figure 6: Zone of inhibition *on Shigela dysentriae*

Table 3: Well Assay using solvent extracts to check antimicrobial activity

Organisms	Solvents	Zone of clearance
Vibrio cholerae	Acetone	10 mm
	Butanol	10 mm
	Propanol	9 mm
	Methanol	9 mm
	Iso Amyl Alcohol	10 mm
	Chloroform	12 mm
	Hexane	9 mm
	Petroleum Ether	8 mm
	Diethyl Ether	8 mm
Shigela dysentriae	Acetone	2 mm
	Butanol	10 mm
	Propanol	5 mm
	Methanol	-
	Iso Amyl Alcohol	2 mm
	Chloroform	-
	Hexane	-
	Petroleum Ether	-
	Diethyl Ether	1 mm
Streptococcus pneumoniae	Acetone	1 mm
	Butanol	2 mm
	Chloroform	8 mm
	Propanol	1mm
	Methanol	1mm
	Iso Amyl Alcohol	1 mm
	Hexane	-
	Petroleum Ether	-
	Diethyl Ether	-

Table 2 shows that of the solvents used, chloroform seems to be the best solvent in the extraction of antibacterial compound to prevent the growth of *V. cholerae* and *S. pneumoniae* whereas butanol was found to extract antibacterial compound efficiently to inhibit *S. dysentriae.* A control was tried where only solvent was

Table 4: Colony inhibition

Sl. No	Organism	Agar plate	Serial dilution	Number of colonies
1.	*Vibrio cholerae*	TCBS	10^{-4}	520
			10^{-6}	110
2.	*Lactobacillus acidophilus*	MRS	10^{-4}	570
			10^{-6}	130
3.	*Lactobacillus acidophilus* +	TCBS	10^{-4}	200
	Vibrio cholerae		10^{-6}	37
4	*Lactobacillus acidophilus* +	MRS	10^{-4}	Numerous to count
	Vibrio cholerae		10^{-6}	Numerous to count

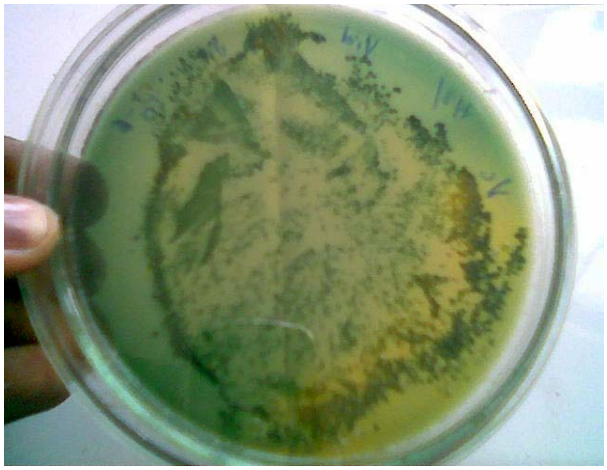

Figure 7: *Vibrio cholerae* on TCBS

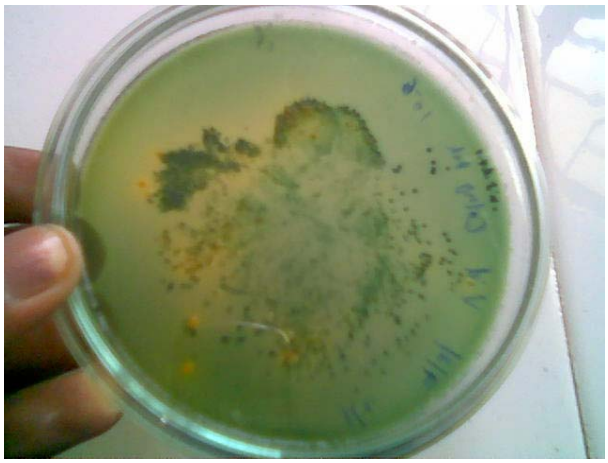

Figure 8: Mixed culture on TCBS

added to the wells to see if solvent caused the inhibition but solvent alone without the antimicrobial compound had no effect on the organisms (Muriana and Klaenhammer, 1991; Juven *et al*., 1992; Peant and Lapointe, 2004).

The use of solvent caused better extraction of the antimicrobial in comparison to water extracts.

The growth of *V. cholerae* was much reduced in the presence of Lactobacillus. When the growth of *V. cholerae* and *L. acidophilus* in nutrient broth is considered, both had more or less similar growth. When the growth together is seen, *L. acidophilus* dominated the growth .This result clearly shows that competitively inhibits the growth of *V. cholerae*. The presence of Lactobacillus was sufficient for the inhibition of growth of *V. cholerae* (Malinen and Bigret, 1998; Miller *et al.*, 2001; Corcoran *et al.*, 2004).

These results indicate the ability of the antimicrobial produced by *L. acidophilus* against the pathogen but its ability to grow with normal flora is also clearly observed (Reddy *et al.*, 1984).

L. acidophilus also showed antifungal activity. It inhibited the growth of *C. neoformans*. But no inhibition was observed against *A. niger*. This could be because the antifungal compound produced by the *L. acidophilus* could be specific in inhibiting *C. neoformans* but not *A. niger* (Jacobsen *et al.*, 1999; Magnusson and Schnürer, 2002; Kastmoglu and Akgun 2004).

CONCLUSIONS

The curd sample was used to isolate the *L. acidophilus* organism. The organism was tested for antibacterial activity against Gram positive and Gram negative organism (*S. pneumoniae, V. cholerae and S. dysentriae*) using well assay and also using solvents to extract the antibacterial compounds. Inhibition of *V. cholerae* was done by *L. acidophilus* by the colony inhibition method. The organism was tested for antifungal activity against *A. niger* and *C. neoformans* by overlay method and agar diffusion method. The work will be useful to understand the importance of probiotics against pathogenic organisms.

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