IN VITRO ANTIMICROBIAL ACTIVITY SHOWED BY *LACTOBACILLUS RHAMNOSUS* GG AGAINST PATHOGENS OF CLINICAL AND PUBLIC HEALTH SIGNIFICANCE[#]

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ABSTRACT

Probiotic supplementation is now being a focused alternative to control several diseases worldwide. Antimicrobial activity of *Lactobacillus casei rhamnosus* GG (Lb) spent culture supernatant (SCS) was tested against pathogens of clinical and public health significance viz. *Salmonella typhimurium* MTCC 098, Shiga-toxin producing *E. coli* (STEC), *Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeroginosa, Rhodococcus equi, Listeria monoctogenes* and *Bacillus stereothermophilus* using agar well diffusion assay. The pattern of antibacterial activity of Lb SCS was also monitored. A varying zone of inhibition ranging from 13 mm to 20.5 mm were obtained. It was observed that the antibacterial activity appeared only after 24 hours of growth and increased till 72 hours. Our results suggest that the *Lactobacillus rhamnosus GG* produces substances having inhibitory effect on pathogens.

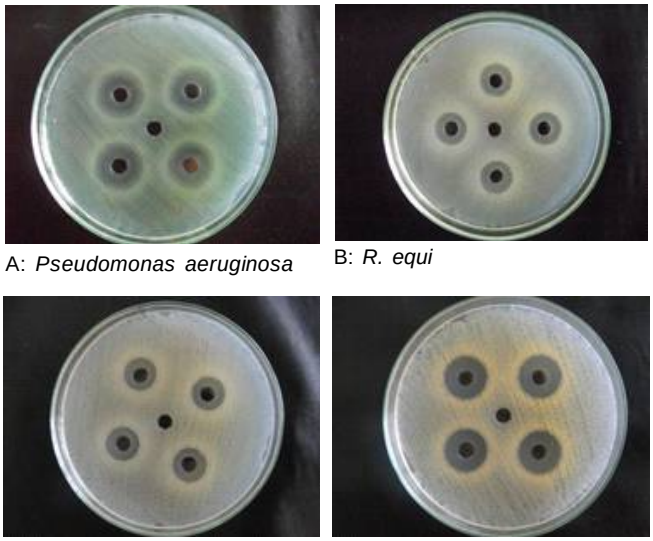
Key words: Antibiotic resistance, Lactobacillus rhamnosus, antimicrobial activity, pathogens

Introduction

Extensively used antibiotics have already led to the emergence of antibiotic resistance in both pathogenic as well as commensals in human health, animal husbandry and agriculture. Besides this, antibiotics and pathogens also disturb the normal intestinal microbial flora. Probiotics are a potential alternative to revolutionize the current scenario. Microorganisms which when administered in adequate amounts, can confer a health benefit on the host, are referred to as probiotic strains (FAO/WHO, 2001). The most often implicated probiotic organisms are LAB, having antagonistic property to disease-causing bacteria. LABs have also been reportedly used in the prevention of various types of diarrhoea (traveller's diarrhoea, rotavirus diarrhoea, infectious diarrhoea), recurrent *Clostridium difficile* disease and reduction in the incidence of common pathogenic infections (Biller *et al.,* 1995). *Lactobacillus rhamnosus* GG (Sherwood and Gorbach, 1996) is one such strain for which many health benefits have been postulated, including the defeat of intestinal pathogens.

The ability of exclusion and reduction demonstrated for several enteropathogens has been attributed to the production of organic acid, hydrogen peroxide, bacteriocins and bacterial peptides (Close, 2000; Breashers and Durre, 1999), which suppress the multiplication of pathogenic and putrefying bacteria. However, the antimicrobial effect of the organic acids on target microorganism consists of lowering the pH at intervals inhibiting their metabolism (Caplice and Fitzgerald, 1999), even more complex mechanisms are involved in the bacteriocin action.

This study was undertaken to understand the *in vitro* antagonistic effect of *Lactobacillus rhamnosus* GG to inhibit the pathogens of clinical and public health significance including several gram positive bacteria and gram negative bacteria such as *Salmonella*, *E. coli, Bacillus cereus, Listeria monocytogenes MTCC, Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeroginosa, Rhodococcus equi*; which are normal contaminants in synthetic creams, eggs, high salt foods such as hams, dairy foods, milk and cheese, rice dishes and milk items and causal agents of gastroenteritis, bacillary dysentery, invasive colitis or dysentery like syndrome and the spoilage causing bacteria, *Bacillus stereothermophilus,*.



A: *Pseudomonas aeruginosa* B: *R. equi*

C: *B. stearothermophilus* D: *Staphylococcus aureus*

Materials and Methods

Bacterial cultures

Culture collection

The reference strains of *Salmonella typhimurium*

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(pathogenic) (MTCC 0098), *Lactobacillus rhamnosus* (MTCC 1408) and *Listeria monocytogenes* 4b (pathogenic) (MTCC 1143) strains were procured from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India in freeze-dried form. All the strains were cultivated thrice before use in experiments and maintained by monthly subculturing as per the instructions of IMTECH. The isolated pathogens used were Shiga toxin producing *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* (isolated from clinical and food samples and characterised in the division). The cultures of *Rhodococcus equi* and *Bacillus stearothermophilus* were revived from glycerol stored cultures, provided kindly by Dr. S.K Kotwal, Division of Veterinary Public Health and Epidemiology, SKUAST- Jammu.

Table 1: Zone of inhibition of different organisms using Lb SCS

Organism	Zone of Inhibition (mm)
STEC	15.5
S. aureus	19
P. aeruginosa	20.5
P. mirabilis	15
R. equi	13
B. stearothermophilus	14
L. monocytogenes	14.5
S. typhimurium	14

Diameter of zone of inhibition expressed as mean (mm) of four replicates

Table 2: Antibacterial activity of Lb SCS against *S. typhimurium* during growth of *L. rhamnosus* in MRS broth

Growth time in hours	0 hour	24 hours	48 hours	72 hours	96 hours
Zone of inhibition (mm)	0	9	13	14	14

Diameter of zone of inhibition expressed as mean (mm) of three repititions

Table 3: The pH of spent culture supernatants (SCS) of *L. rhamnosus* and *S. boulardii*

	0 hours	24 hours	48 hours	72 hours	96 hours
Lactobacillus rhamnosus	6.5	4.21	4.15	3.96	3.96

Values indicate pH meter readings, measured time to time

Antibacterial activity of the *Lactobacillus rhamnosus* spent culture supernatant (Lb SCS) against microorganisms

The antagonistic activity of *Lactobacillus rhamnosus* spent culture supernatant (Lb SCS) was studied using well diffusion assay of Schillinger and Lucke (1989) with a slight modification. *L. rhamnosus* was grown in MRS at 37°C for 72 hours, followed by centrifugation (10,000 rpm) at 4°C for 10 min to remove the cells. The supernatant thus obtained was passed through a millipore filter (pore size 0.40 µ) and was then used for well diffusion assay. Bacteria were grown in nutrient broth until it achieved an optical density corresponding to 0.5 McFarland (1.5 X 10^8 cfu/ml, usually in 3 hr.) and were spread over petri plates (90 mm) containing solidified nutrient agar (20 ml) using a sterile cotton swab. Wells (6 mm diameter) were cut into these plates using a sterile cork borer and 60 ìl of supernatant was added to the wells. The plates, with unsealed wells, were kept at 4°C for 1hr followed by incubation for 24 h at 37^0 C and examined for clear zone of inhibition around the wells. The inhibition zones were measured. PBS was inoculated in the control well. Each test was performed in triplicate.

Pattern of Lb-SCS antibacterial activity

The antibacterial activity against *Salmonella typhimurium* was examined at 24 hour intervals (24, 48, 72 and 96 hrs of growth) during the growth of *L. rhamnosus* in MRS broth, using the agar well diffusion assay as discussed earlier and pH was also measured at these corresponding intervals (Coconnier *et al.,* 1997).

Results and Discussion

Antibacterial activity of the *Lactobacillus rhamnosus* spent culture supernatant (Lb SCS) against other microorganisms of public health significance

The inhibition recorded was higher for Gram negative bacteria as compared to Gram positive. The maximum inhibition zone was observed against *P. aeruginosa* (20.5 mm) followed by *Staph. aureus* (19 mm). The minimum inhibition was observed in *R. equi* (13 mm) (Table 1). Similar findings were reported by Tambekar *et al.* (2009) who reported inhibiton (zone of inhibition-18 to 24 mm) of *E. coli*, *Enterobacter aerogenes*, *Kl. pneumoniae*, *P. vulgaris* and *S. typhi* by *L. rhamnosus* and *L. plantarum* isolated from cow, goat and buffalo milk. Lactobacillus rhamnosus GG spent culture supernatant was reported to be antimicrobial against *Salmonella* enterica serovar *typhimurium* (*S. typhimurium*) and other intestinal pathogens (Silva *et al*., 1987; Hudault *et al.*, 1997; Lehto and Salminen, 1997). Prasad and Ghodekar (1990) reported that *Lactobacillus* isolated from dahi showed antimicrobial activity against *Staphylococcus auerus, Bacillus cereus, E. coli* and *Salmonella* spp. Similiar results were documented by Sarkar and Sharmistha (1996).

Pattern of *Lactobacillus rhamnosus* spent culture supernatant antibacterial activity

The antibacterial activity against *Salmonella typhimurium* was tested at 24 hour intervals and it was observed that the antibacterial activity appeared after 24 hours of growth and increased till 72 hours, afterwards there was no increase in activity. There was a reduction in the pH of Lb SCS from 6.5 to 4.21, 4.15, 3.96 at 24 hr, 48 hr and 72 hr, respectively. However, no further decline in the pH was seen at 96 hrs of growth as shown in Table 2 and Table 3. The role of lactic acid in the antimicrobial activity of *L. rhamnosus* GG is controversial with hypotheses being formulated that ranges from no role at all (Silva *et al.,* 1987;

Fayol-Messaoudi *et al.,* 2005) to lactic acid being the major player (Makras *et al.,* 2005).

On the other hand, some studies on the antibacterial components of various *Lactobacillus* strains regard them to be proteinaceous in nature viz, bacteriocins (Atanassova *et al.,* 2003; Ocana *et al*., 1999). It was also reported that the *Lactobacillus* strains vary from each other regarding antimicrobial activity; some strains are non-protective (La Ragione *et al.*, 2004*,* Lema *et al.*, 2001), while many others are protective to infectious agents (Silva *et al.,* 1999; Shu *et al.,* 2000). However, this aspect needs to be explored for the *Lactobacillus* strain under study.

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