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COUNTER ACTIVITY OF PROBIOTICS WITH REFERENCE TO PATHOGENIC BACTERIA

S.SREEVANI AND J. PRAMODA KUMARI*

Department of Microbiology, S.V.University, Tirupati – 517502.

ABSTRACT

Most of the microbes play a vital role in health benefits, probiotics are one of them. In this study four potential bacterial probiotic isolates of *Lactobacillus* spp. were examined for their counter activity against pathogenic bacteria. Among the assays performed *viz.,* Bacteriocin, thermostability assay and pH sensitivity assay *L. plantarum*- MTCC2621 gives more yields but incase of Bile salt tolerance assay *L. rhamnosus*- MTCC-1408 gives more yield. The present study revealed that *L. rhamnosus*- MTCC-1408, *L. delbrueckii*-MTCC-911, *L. fermentum*-MTCC-903, *L. plantarum*- MTCC2621 inhibits *Bacillus subtilis*and *Staphylococcus aureus*more efficiently than *E. coli* and *Pseudomonas aeruginosa.*

KEY WORDS:Probiotics, *Lactobacillus* spp., Pathogenic bacteria, counter activity, Bacteriocin.

J. PRAMODA KUMARI
Department of Microbiology, S.V.University, Tirupati – 517502.

INTRODUCTION

The probiotics are defined as "living micro-organisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition"[1]**.** Most probiotic microorganisms belong to the lactic acid bacteria (LAB) group, such as Lactobacillus spp., and Enterococcus spp., or to the genus Bifidobacterium[2]. Different bacterial species belonging to the Lactobacillus genus are part of the human and animal commensal intestinal flora[3]. It's reported that, Lactobacilli isolated from dairy products have shown a long history of safe use[4]. They are used widely as starter cultures in the food industry, e.g. fermented milk or meat products, alcoholic beverages, sour dough and silage[5].Furthermore, cultures of various Lactobacillus strains have been developed for commercial use as probiotic bacteria[6].Lactic acid bacteria, particularly those belonging to beneficial and non-pathogenic genera (Lactobacillus, Lactococcus, Streptococcus, Pediococcus and Leuconostoc) are widely used in food industry. Among lactic acid bacteria; Lactobacilli are the most important group and are gaining increasing attention in food fermentation industry because of their potential biotechnological interest. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of an antimicrobial substance such as organic acids, hydrogen peroxide and bacteriocins[7]. Bacteriocins are small proteins with bactericidal or bacteriostatic activity[8]. The bacteriocin-producing Lactobacillus may be used as a protective culture to improve the microbial safety of foods[9]. The antagonistic effects of bacteriocins against food spoilage[10] which is usually achieved by inhibition of Pseudomonas, Staphylococcus aureus, Bacillus subtillis[11] and they have great potential as biopreservatives for food[12]. There is a growing consumer demand for processed dairy products containing no chemical preservatives, leading to indigenous studies in the field of screening bacteriocin as food preservatives. However, most of these indigenous research studies have always attempted to access the effectiveness of bacteriocin-producing lactic acid bacteria strains that were isolated from certain traditional dairy products. This present study was conducted to evaluate the antibacterial activity of bacteriocin producing Lactobacillus sp. isolated from traditional milk products.These Lactobacilli produced various antagonistic substances which can inhibit pathogenic and spoilage microorganisms. Among all these antagonistic substances, bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria. Lactobacilli exert a strong antagonistic activity of many microorganisms, including food spoilage organisms and pathogens[13].Bacteriocins are extracelluarlar released peptides or proteinaceous antimicrobial compounds, which exhibit a bactericidal effect against closely related bacteria[14]. Bacteriocins of lactic acid bacteria are considered as safe natural preservatives or bio preservatives as it is that are degraded by the proteases in gastro intestinal tract. Several types of bacteriocins from food associated lactic acid bacteria have been identified and characterized, of which the important ones are Nisin, Bacteriocin, Diplococcin, Acidophilin, Bulgaricin, Helveticins, Lactacins and Plantaricins[13] .The bactericidal activity of bacteriocins is attributable to destabilization of functions of the cytoplasmic membrane of the target cells, altering the permeability properties of the membrane. Lactobacillus species are primarily used as probiotics, but can also be used as starter cultures in various fermented foods[15.] B. subtilis is only known to cause disease in severely immune compromised patients, and can conversely be used as a probiotic in healthy individuals. It may contaminate food but rarely causes food poisoning. B. subtilis produces the proteolytic enzyme subtilisin. B. subtilis spores can survive the extreme heat during cooking. B. subtilis is responsible for causing ropiness sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread

dough[16].P. aeruginosa flourishes in hospital environments, and is a particular problem in this environment since it is the second most common infection in hospitalized patients (nosocomial infections). This pathogenesis may in part be due to the proteins secreted by P. aeruginosa. The bacterium possesses a wide range of secretion systems, which export numerous proteins relevant to the pathogenesis of clinical strains.[17] E. coli normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or with the individuals handling the child. In the bowel, it adheres to the mucus of the large intestine. It is the primary facultative anaerobe of the human gastrointestinal tract[18].S. aureus also produce an enterotoxin that is the causative agent of S. aureus gastroenteritis. The gastroenteritis is self-limiting, with the person recovering in 8 to 24 hours. Symptoms include nausea, vomiting, diarrhea, and major abdominal pain[19].

MATERIALS AND METHODS

(i) Culture Collection

The sample i.e. lyophilized Lactobacillus rhamnosus -MTCC 1408, Lactobacillus delbrueckii- MTCC-911, Lactobacillus fermentum- MTCC-903 and Lactobacillus plantarum- MTCC-2621was purchased from IMTECH, Chandigarh.

(ii) Screening for Bacteriocin Producers

The enriched broth was screened for antimicrobial activity by swab paper disc method[20]. In this method, supernatant of bacterial isolates, about 20µl was suspended in the sterile paper disc against the indicator organisms. The indicator organisms, viz, Bacillus subtilis, Staphylococcus aureus , Pseudomonas aeruginosa, E.coli were procured from Microbial Type Culture Collection . All the plates were incubated at 37°C for 24 hours. Colonies showing zone of inhibition were considered as potent bacteriocin producers. The bacterial strains that were selected as potent bacteriocin producers were subjected to morphological characterization.

(iii)Bacteriocin assay

The isolates from the food sample were tested for their ability to produce bacteriocins. The isolates maintained in MRS agar were propagated in MRS broth and incubated at 37°C for 48 hours. Cells were separated by centrifugation at 5000 rpm for 10 minutes. The pH of the cell free supernatant was adjusted to 5.0 with 2N NaOH. Cell free supernatant was passed through 0.22µm membrane filter and evaluated for antimicrobial activity by agar well diffusion method. The antagonistic effects of the culture supernatants of bacteriocin producing Lactobacillus were tested on various indicator organisms on Nutrient agar. MRS agar was used for lactic strains. All cultures were grown aerobically at 37°C for 48 hours. Inhibition zones around the wells were measured[20].

(iv)Agar well diffusion method

The inhibitory effects of Lactobacillus strains on indicator organisms were carried by agar well diffusion method [18]. Petri dishes with nutrient agar that were previously inoculated with 0.1ml of 24 hours old nutrient broth culture of individual test bacteria were poured. Once solidified, petri dishes were stored for 2 hours at 4°C. Four wells of 5mm diameter were made and filled with 10µl of culture supernatant. The inoculated plates were kept at 4°C for 2 hours and then incubated at 37°C for 24 hours. Inhibition zones around the wellswere measured.

(v) Bile Salt Tolerance

Isolated Lactobacillus sp. were inoculated into MRS medium of varying pH, i.e. pH 2, 3, 4 and 5; as well as broth with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2.0%), and incubated at 37°C for 48h. Then 0.1ml inoculums was transferred to MRS agar by pour plate method and incubated at 37°C for 48h. The growth of LAB on MRS agar plate was used to designate isolates as acid or bile salt tolerant[20].

(vi)Sensitivity to pH and Thermo stability

To test sensitivity to pH, the supernatant was adjusted to pH between 2 to 12 with HCl or

NaOH and incubated. To test heat stability, the supernatant fluid was heated in boiling water for 10 min, at 56ºC for 15 min, or autoclaved at 121ºC for 15 min. In all cases, the activity remaining after treatment was measured by spotting procedure. This experiment repeated and the solutions were kept at 4 and -20ºC for 4 weeks, then antibacterial activity was measured[20].

RESULTS

1. Isolation and Screening of Lacto Bacillus species

Lactobacillus species, like L.rhamnosus**-** MTCC-1408**,** L.delbrueckii**-**MTCC-911**,** L.fermentum**-**MTCC-903**,** L.plantarum**-** MTCC2621 were characterized and identified in Figure 1.

Isolated colonies of various Lactobacillus Spp.

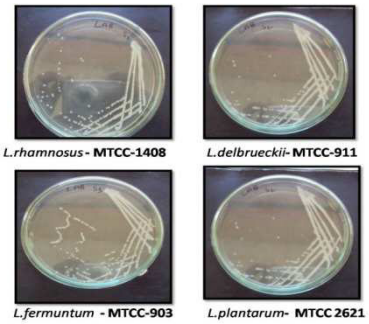


Figure 1

Pure cultures of Lactobacillus spp., were maintained on MRS agar medium which is specific medium, lyophilized cultures were retrieved followed by streak plate technique

2. Antimicrobial activity

Antimicrobial activity was evaluated against different food borne pathogens viz., Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and E.coli., Lactobacillus sps.viz., L.rhamnosus MTCC-1408, L.delberueckii MTCC-911, L.plantarum MTCC-2621 and L.fermentum MTCC-903 were maintained in Lactobacillus selective media (MRS medium) and well defined colonies were observed and shown in Figure 2.

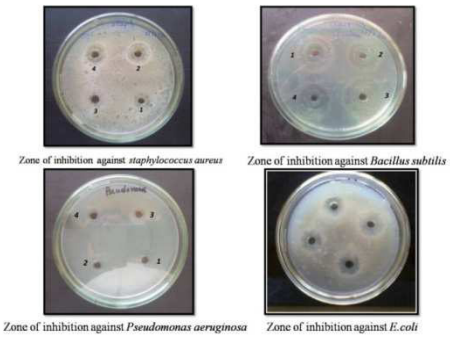


Figure 2

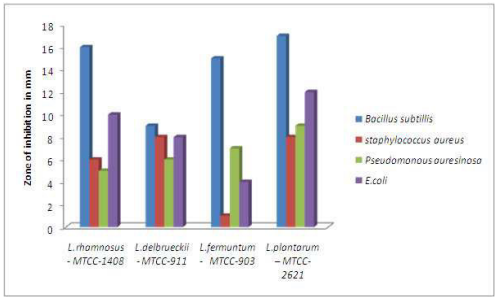
Zone of inhibition by Lactobacillus spp., against various pathogens, among various probiotics organisms used Lactobacillus plantarum MTCC -2621 shows maximum zone of inhibition against Bacillus subtilis

3. *Bacteriocin assay*

Table 1
Bacteriocin assay of probiotics against to pathogens

TESTING ORGANISM	INHIBITION ZONE OF DIAMETER(MM)			
	L.rhamnosus - MTCC-1408	L.delbrueckii - MTCC-911	L.fermentum - MTCC-903	L.plantarum – MTCC-2621
Bacillus subtilis	13 mm	9 mm	15 mm	17 mm
Pseudomonas aeurosinosa	5 mm	6 mm	7 mm	9 mm
Staphylococcus aureus	6 mm	7 mm	2 mm	8 mm
E.coi	10mm	8mm	4mm	12mm

Graph 1
Measurement of Zone of Inhibition



Bar graph represents the zone of inhibition (mm) by various Lactobacillus spp. against pathogenic bacteria; maximum zone of inhibition was shown byL. Plantarum against all the pathogens used in present study.

4. *Thermal Stability Test*

Different temperatures (4ºC, 60ºC, 80ºC, 100ºC and 121ºC) were used for bacteriocin production, at 37ºC maximal anti microbial activity against Bacillus subtilis(Figure.3,Table 2,Graph 2) and Staphylococcus aureus (Figure 4, Table 3,Graph 3) observed.

Detection of Anti microbial activity by Thermal Stability Test against Bacillus subtilis

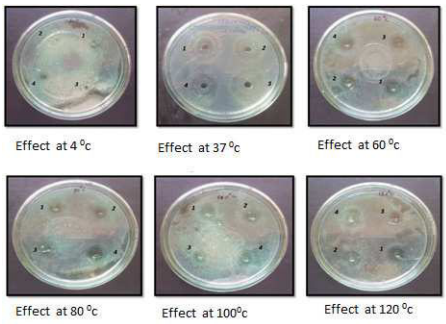
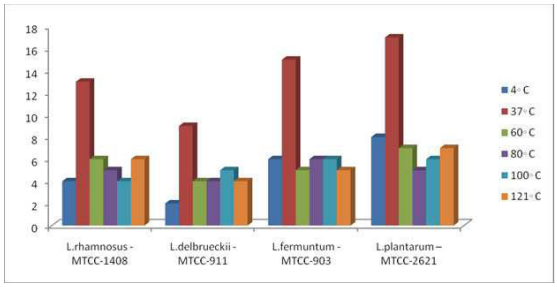


Figure 3
Thermal stability test of Lactobacillus spp., against Bacillus subtilis shows maximum inhibition by L. plantarum at 37^0C and minimum inhibition by L. delbrueckii at 4^0C

Table 2
Detection of Antimicrobial activity by Thermal Stability Test against Bacillus subtilis

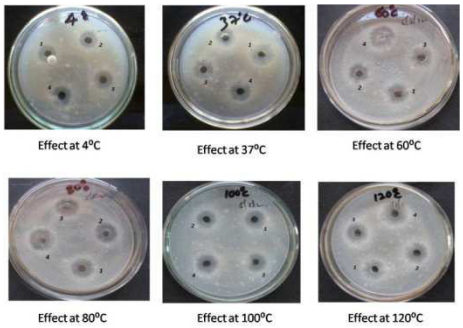
	INHIBITION ZONE IN DIAMETER(MM)					
SPECIES	4° C	37° C	60° C	80° C	100° C	121° C
L.rhamnosus - MTCC-1408	4	13	6	5	4	6
L.delbrueckii - MTCC-911	2	9	4	4	5	4
L.fermentum -MTCC-903	6	15	5	6	6	5
L.plantarum – MTCC-2621	8	17	7	5	6	7

Graph 2
Thermal Stability Test against Bacillus subtillis



Column chart represents Thermal stability test of Lactobacillus spp., against Bacillus subtilis shows maximum inhibition by L. plantarum at 37^{0}C and minimum inhibition by L. delbrueckii at 4^{0}C

Figure 4
Detection of Thermal stability test against Staphylococcus aureus

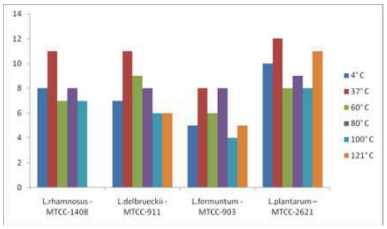


Thermal stability test of Lactobacillus spp., against Staphylococcus aureus shows maximum inhibition by L. plantarum at 37^{0}C and minimum inhibition by L. fermentum at 100^{0}C

Table 3
Detection of Antimicrobial activity by Thermal Stability Test against Staphylococcus aureus

SPECIES	4° C	37° C	60° C	80° C	100° C	121° C
L.rhamnosus - MTCC-1408	8mm	11mm	7mm	8mm	7mm	9mm
L.delbrueckii- MTCC-911	7mm	11mm	9mm	8mm	6mm	6mm
L.fermentum -MTCC-903	5mm	8mm	6mm	8mm	4mm	5mm
L.plantarum – MTCC-2621	10mm	12mm	8mm	9mm	8mm	11mm

Graph 3
Thermal Stability Test against Staphylococcus aureus



Bar chart represents thermal stability of probiotic organisms against Staphylococcus aureus at various temperatures. Maximum activity was elevated by L. plantarum at all encountered temperatures whereas remaining organisms shown fluctuations

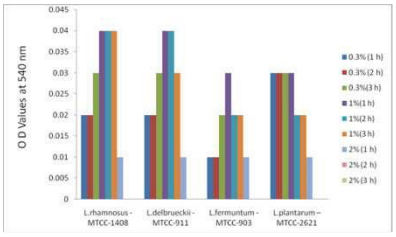
5. Bile salt tolerance test

Table 4
Growth of organisms at different bile salt concentrations with time interval variance

ISOLATES	0.3% (1 h)	0.3% (2 h)	0.3% (3 h)	1% (1 h)	1% (2 h)	1% (3 h)	2% (1 h)	2% (2h)	2% (3h)
L.rhamnosus -MTCC-1408	0.02	0.02	0.03	0.04	0.04	0.04	0.01	0	0
L.delbrueckii - MTCC-911	0.02	0.02	0.03	0.04	0.04	0.03	0.01	0	0
L.fermentum - MTCC-903	0.01	0.01	0.02	0.03	0.02	0.02	0.01	0	0
L.plantarum – MTCC-2621	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0	0

Graph 4
Bile Salt Tolerance Test Graphical

Representation



Bar chart represents growth of organisms at different bile salt concentrations with time interval variance, which shows maximum yield at 1% concentration for 1 – 2 hours incubation time by L.rhamnosus and L.delbrueckii

6. pH sensitivity test

Table 5
Growth of organisms at different pH levels

ISOLATE	pH-3	pH-4	pH-5	pH-6	pH-7	pH-8	pH-9
L.rhamnosus - MTCC-1408	0.15	0.18	0.23	0.72	0.92	0.69	0.11
L.delbrueckii - MTCC-911	0.11	0.15	0.31	0.81	0.88	0.54	0.12
L.fermentum - MTCC-903	0.7	0.14	0.09	0.48	1.53	0.38	0.12
L.plantarum – MTCC-2621	0.18	0.19	0.16	0.76	0.98	0.28	0.18

Graph 5
pH sensitivity test Graphical representation

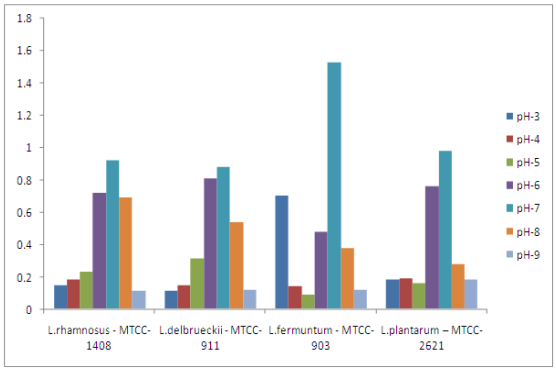


Chart indicates the role of pH in growth of organisms, L. fermentum shows maximum growth at neutral pH besides that, remaining organisms shows less growth or decreases steeply with respect to the pH employed

DISCUSSION

Cultures of Lactobacillus spp., L.rhamnosus MTCC-1408, L.delberueckii MTCC-911, L.plantarum MTCC-2621 and L.fermentum MTCC-903 were maintained in Lactobacillus selective media (MRS medium), well defined colonies were presented in Figure 1. Those colonies were screened for bacteriocin assay. In screening antimicrobial nature of bacteriocin was studied using four organisms viz., Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and E.coli. Screening shows that all the organisms were susceptible to the antimicrobial nature of bacteriocin, of four particularly Staphylococcus aureus and Bacillus subtilis were strongly inhibited. The results were represented in Figure 2. The zone of inhibition (mm) by various Lactobacillus spp. against pathogenic bacteria shows that maximum zone of inhibition was observed by L. plantarum against all the pathogens used in present study (Table 1 and Graph 1). Thermal stability test of Lactobacillus spp., against Bacillus subtilis shows maximum inhibition was noticed by L. plantarum at 37^0C and minimum inhibition by L. delbrueckii at 4^0C (Figure 3, Table 2 and Graph 2). Thermal stability of probiotic organisms against Staphylococcus aureus at various temperatures were presented in Figure4, Table 3 and Graph 3. Maximum activity was elevated by L. plantarum at all encountered temperatures whereas remaining organisms shown fluctuations. Growth of organisms at different bile salt concentrations with time interval variance, shows maximum yield at 1% concentration for 1 – 2 hours incubation time by L.rhamnosus and L.delbrueckii (Table 4 and Graph 4). The role of pH in growth of organisms was also presented in Table 5 and Graph 5. L. fermentum shows maximum growth at neutral pH besides that, remaining organisms shows less growth or decreases steeply with respect to the pH employed. Out of these four probiotic organisms two of them were identified as an excellent probiotics on the basis of their acid and bile tolerance, antibacterial activity, antibacterial potential of bacteriocin, high temperature tolerance of bacteriocin. The best of probiotics were L. rhamnosus(MTCC-1408), L.plantarum(MTCC-2621) which is having commercial probiotic preparations and standard probiotic bacterial strains

CONCLUSION

The present study revealed that probiotics Lactobacillus rhamnosus -MTCC 1408, Lactobacillus delbrueckii- MTCC-911,

Lactobacillus fermentum- MTCC-903 and Lactobacillus plantarum- MTCC-2621 probiotic bacterial strains were bile salt tolerant at 2% bile salt concentration, antibacterial against enteric pathogens, antibiotics resistant to most of the antibiotics and their bacteriocin stable at temperature 37^0C, 120^0C and pH 3 to 8. From comparative study of probiotics it was concluded that plantarum-MTCC-2621 probiotics and their bacteriocins showed strong antibacterial potential as compared to the Lactobacillus rhamnosus -MTCC 1408, Lactobacillus delbrueckii- MTCC-911, Lactobacillus fermentum- MTCC-903 can be used for oral therapy and as prophylactic to prevent the enteric infections such as food poisoning, and gastro intestinal infection etc. Study suggested that these isolated prominent probiotics can be used in milk or milk products supplement to provide restoration and maintenance of normal microbial flora of intestine and prevention of side effect of antibiotics. The study will affirm their use in the development of new pharmaceutical preparations and functional foods that contain milk probiotics for the betterment of the health of the public.

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