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ANTAGONISTIC PROPERTIES OF DIFFERENT BACTERIA ISOLATED FROM SALADS

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Abstract : Salads viz. carrot, coriander and cucumber collected from various local markets including producers around Jaipur city showed presence of a number of gram positive as well as gram negative bacteria; however, the population of the later was more. The isolates, based on biochemical tests, tentatively belonged to *Lactobacillus, E. coli, Enterobacter, Pseudomonas, Bacillus* and *Streptococcus*. Out of 110 isolates, some of these showed inhibitory effects on the human pathogens especially on *E. coli, Salmonella typhi* and *Staphylococcus aureus*. The synthesis of antibacterial compound (bacteriocin) from one of the isolate MRS-4 showed highest production at pH of 6.5 and temperature of 37°C.

Key words: Bacteriocin, *E.coli*, Food borne pathogens, *Lactobacilli*, Salads

INTRODUCTION :

Salad is a mixture of fresh vegetables and fruits; eaten raw or partially cooked that promotes good health but, at the same time they harbour a wide range of microbial contaminants. Salads are good source of antioxidants and phytonutrients. They are low in calories and are rich in complex carbohydrates, vitamins and minerals. One health benefit of consuming salad is an increase in fiber intake. Salads provide the body with a lot of fiber which result in lower calorie intake and cholesterol level.

Pathogens on edible plants present a significant potential source of human illness. A significant portion of enteric pathogens can persist on the surface and proliferate. These pathogens can increase the occurrence of food-borne diseases. Fresh vegetables and fruits become contaminated with microorganisms during production, harvest, packaging, and distribution (Bartz and Wei, 2003).

Several outbreaks of gastroenteritis have been linked to the consumption of contaminated fresh vegetable. One such type of incidence occurred in Japan in 1996 in which 11,000 people were affected and about 6,000 pathogenic cultures were confirmed resulted in the death of three children by infection of *Escherichia coli*. The most common bacterial enteropathogens associated with fruits and vegetables are *Salmonella sp., E. coli etc.* (Thunberg *et al.*, 2002; Beuchat, 2002).

The ill-health because of consumption of contaminated fruit juices at several places in India and elsewhere are also reported (Bhaskar *et al.*, 2004; Chumber *et al.*, 2007; Ghosh *et al.*, 2007). Such juices have shown to be potential sources of bacterial pathogens notably *E. coli* 0157:H7, species of *Salmonella, Shigella,* and *Staphylococcus aureus* (Buchmann *et al.*, 1999). In India, the presence of coliforms and *staphylococci* in kinnow and mandarin juices in Patiala city could be reported (Ganguli *et al.*, 2004). Similarly, coliforms were observed in fresh fruit and vegetable juices sold by the street vendors of Nagpur city (Titarmare *et al.*, 2009).

Lactic acid bacteria play an important role in the preservation, microbiological stability and production of aroma compound in these products. The preservative effect is mainly due to the acidic conditions that these bacteria create in food during their development but, they are capable of producing and excreting inhibitory substances other than lactic and acetic acid. These include hydrogen peroxide, ethanol, diacetyl, carbon dioxide, bacteriocin or antibiotic-like substances (De Vuyst and Vandamme, 1994a). Most of the bacteriocins from lactic acid bacteria have been isolated from species of the genus *Lactobacillus* because of the diversity of its species and habitats (Klaenhammer, 1988; De Vuyst and Vandamme, 1994b).

Several strains of lactic acid bacteria isolated from commercial salads were active against coliforms, *Enterococci, Aeromonas hydrophila, Listeria monocytogenes, Salmonella typhimurium* and *Staphylococcus aureus*, when tested in solid agar plates, in contaminated salads and juice of vegetable salads.

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The highest activity against many types of pathogens was demonstrated by *Lactobacillus casei* (Vescovo *et al.*, 1996). Application of bacteriocin producing lactic acid bacteria such as *Lactobacillus casei* strains had a remarkable inhibitory effect on the growth of indigenous micro flora and pathogens inoculated in mixed salad vegetables (Vescovo *et al*., 1996). Lactic acid bacteria inhibit the growth of food-borne pathogens by producing bacteriocin, generating H_2O_2 and producing organic acid and therefore lowering the pH (Breidt and Fleming, 1999; Bennik *et al.*, 1999).

As the salads have a very high consumer preference and eaten raw or partially cooked due to their various important properties; the present study was undertaken to determine the antagonistic properties of some of the bacteria isolated from salads towards human pathogens.

MATERIALS AND METHODS:

Collection of salad samples

Different salads such as carrot, coriander and cucumber were purchased from various local markets (Vaishali Nagar, Jhotwara, Sodala, Achrol and Chandwazi) of Jaipur city and placed in sterile container. The samples were transferred to the laboratory and analyzed within two hours from procurement. Total fifteen samples were collected and processed for the isolation of bacterial colonies.

Isolation, purification and characterization of bacterial isolates

Isolation of different bacterial strains was determined after imposing following main and sub-treatments. Main treatments were (i) washing with ordinary tap water and (ii) washing with warm tap water (40 C). The sub treatments were performed by (i) peeling of the respected samples and (ii) without peeling. 20g of each salad sample was washed with 200 ml ordinary tap water for two minutes. Then rinsed salad sample was divided into two parts equally; one part of salad sample (10g) was used without peeling and the other part was peeled off and transferred in sterilized 250 ml conical with 100 ml of sterile distilled water separately. The sample was finally placed on an orbital shaker at 100 rpm for 10 minutes. The isolation of the colonies was then performed using tenfold serial dilution and then spreaded on Nutrient Agar, MacConkey Agar and MRS Agar media plates and purified by several streaking at 37 C. Similar procedure was used for the isolation with the sample washed with warm tap water (40 C) (Rajvanshi, 2010). The purified isolates of respective medium were characterized on the basis of morphological analysis and biochemical tests.

Determination of production of lactic acid

A loopful of the purified bacterial isolate from MRS Agar plate was aseptically transferred to 20 ml MRS broth (pH 6.5) and incubated for 24 h at 37 C. After the completion of growth, the broth was centrifuge at 10000 rpm for 20 min at 4 C. The supernatant was collected in sterilized eppendroff tubes and **added drop-wise to Uffelman's reagent, prepared by adding two drops of 1N ferric chloride to 10 ml of 1% phenol solution. The color of solution turns from bluish violet to yellow, indicating the presence of lactic acid** (Walker and Stiles, 2008). The production of lactic acid was also confirmed by paper chromatography using the solvent system acetone, water, chloroform, ethanol and ammonia in the ratio 60:2:6:10:22. The spot developed was visualized by spraying with a solution of bromophenol blue (0.2%) and methyl red (0.2%) in 70% methanol and RF value was calculated (Lee, Heo, 1998).

Antagonistic interaction of isolates against different human pathogens

Three pathogenic cultures *Staphylococcus aureus* MTCC 3160, *Salmonella typhi* MTCC 733 and *Escherichia coli* 901 were used as test organisms for the evaluation of antagonistic properties of the bacteriocin produced from the MRS medium grown cells. The inhibitory activity was determined by using the supernatant of the broth after adjusting its pH to 7.0 by means of 1M NaOH to exclude antimicrobial effect of organic acid. The method adapted for the evaluation of antagonistic property was agar well diffusion assay (Vescovo *et al.*, 1993). The assay involved seeding of Mueller Hinton agar plates with test organisms and introducing 100 µl of supernatant into the well of a diameter of 6 mm. The plates were incubated at 4 C for 1 h for diffusion of the cell free extract and then incubated at 37°C for 24 h for the development of zone of inhibition.

Development of seed inoculum

The seed inoculum for growth profiling was prepared in MRS broth by inoculating with a loopful of cells and incubated at 37°C for 24 h without agitation. The turbidity of broth was adjusted with sterile MRS and nutrient medium to a turbidity of 0.5 Mac Farland standards, which resulted in a suspension containing approximately 1.5×10^8 bacteria/ml.

Growth profiling of lactic acid bacteria

The growth of lactobacilli screened on MRS medium was investigated by inoculating 1% (v/v) of seed inoculum in sterile MRS broth with the initial pH of 6.5 and incubated for 18 h, 24 h, 48 h and 72 h at 37°C in an anaerobic condition.

RESULTS AND DISCUSSION

Based on our preliminary investigation about the abundance of different bacterial isolates and the antimicrobial activity of some of the bacteria isolated from salads, showed that maximum population belong to the genus *Bacilli* (79%) followed by *Cocci* (21%) as shown in Figure 1. The presence of *Lactobacilli* was reported from carrot while *Leucomostoc* was sereened from cucumber and coriander as given in Table 1. Similar type of work has been performed by Uhlman et al., 1992 and Vaughan *et al.*, 1994. They isolated antimicrobial substances producing vegetable associated lactic acid bacteria.

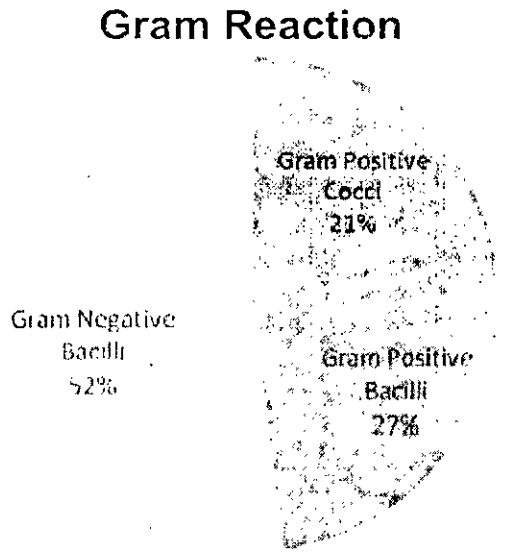


Figure 1: Abundance of different bacteria isolated from salads.

Table 1: Identification of isolates on the basis of biochemical tests.

Name of bacteria	No. of bacteria (cfu× 10⁷/ ml after washing)		
	Carrot	Coriander	Cucumber
E coli	04	05	04
Enterobacter	03	04	03
Staphylococcus	02	06	04
Streptococcus	03	02	02
Pseudomonas	05	04	04
Klebsiella	02	03	01
Citrobacter	01	01	02
Bacillus	08	13	06
Lactobacillus	02	-	-
Lactococcus	-	01	01
Not identified	06	05	03

Studies on antagonistic properties of different isolates: Out of 110 strains, thirteen isolates were evaluated for their antagonistic properties using *E. coli* MTCC 901 as pathogen and the results of zone of inhibition obtained are represented in Table 2. The observation of the table revealed that all the isolates inhibited *E. coli* confirming the presence of antibacterial compounds in the broth with maximum inhibition by *Pseudomonas*. It was also noted from the table that all the MRS grown cells also inhibited the pathogen with maximum inhibition by MRS-4. These isolates are lactic acid producing bacteria while Co10k is a non lactic acid producing bacteria. The present study confirms the findings of Klaenhammer, 1988; De Vuyst and Vandamm, 1994a,b; Vescovo *et al.*, 1996; Breidt and Fleming, 1999; Bennik *et al.*, 1999. They isolated bacteriocin producing lactic acid bacteria from juices fruits and vegetable salads. Similarly, epiphytic species of *Pseudomonas* have been identified and commercialized for the control of postharvest decays caused by fungi and bacteria in fruits (Janisiewicz, *et al.*, 2002).

Table 2: Antagonistic isolates against *E. coli* with inhibition zone.

Salads	Isolate No.	Diameter of inhibition zone (mm)	Genus of isolates
Carrot	Ca1c	12 ± 2.828	*Bacillus*
	Ca2c	10 ± 1.414	*Bacillus*
	Ca4c	12 ± 3.423	*Streptococcus*
	MRS1	10 ± 2.828	*Lactobacillus*
	MRS2	8.5 ± 0.707	*Lactobacillus*
Coriander	Co8g	10 ± 1.414	*Bacillus*
	Co10k	15 ± 3.423	*Pseudomonas*
	MRS3	10 ± 2.828	*Lactococcus*
Cucumber	Cu11c	12 ± 1.414	*Bacillus*
	Cu12c	10 ± 1.414	*Streptococcus*
	Cu12e	12 ± 2.828	*Bacillus*
	Cu13h	12 ± 3.423	*Streptococcus*
	MRS 4	12 ± 1.414	*Lactococcus*

*Values are mean ± SD and significant at ($p < 0.05$)

Based on the observation from table 2, we have selected only three isolates (MRS-1, MRS-4 and Co10k) for further studies. Figure 2 shows the antagonistic properties of these isolates against three

Table 3: Effect of treatment on *E. coli* 901 as monitored by absorbance at 600 nm.

Time (h)	Supernatant (ml)	*E.coli* Control	*E. coli* + MRS-1	*E. coli* + MRS-4	*E. coli* + Co 10k
24	0.5		0.26±0.020	0.24±0.042	0.21±0.028
	1.0	0.32±0.010	0.23±0.010	0.21±0.048	0.18±0.042
	2.0		0.18±0.020	0.16±0.057	0.14±0.057
48	0.5		0.25±0.028	0.21±0.057	0.19±0.048
	1.0	0.41±0.010	0.19±0.014	0.16±0.052	0.14±0.028
	2.0		0.13±0.016	0.11±0.047	0.10±0.057

*Values are mean + SD and significant at ($p < 0.05$).

human pathogenic organisms as mentioned in the material and methods section. We have also carried out the different treatments on *E. coli* using the supernatants of three antibacterial synthesizing isolates as shown below.

Treatment 1) 9 ml NA broth + 1 ml *E. coli* seed inoculums (as control)

2) 8.5 ml NA + 1 ml seed + 0.5 ml supernatant

3) 8.0 ml NA + 1 ml seed + 1.0 ml supernatant

4) 7.0 ml NA + 1 ml seed + 2.0 ml supernatant

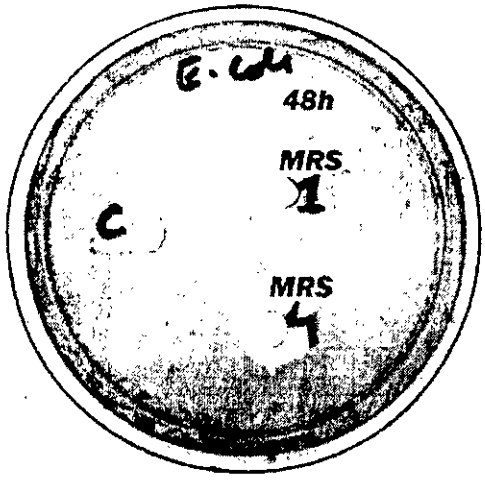
The result of this experiment as observed in the decrease in cell O. D. is represented in Table 3.

It was found that there was a drastic decline in absorbance indicating the cell lyses of *E. coli* which was due to the antibacterial activity of bioactive compound synthesized by lactic acid as well as non lactic acid bacterial isolates screened from salads. To confirm the decrease in cell optical density of *E. coli*, we have performed the viable cell count as given in Table 4 and found the reduction in cell number. Similar type of work have been performed by Yazid *et al.*, 1999, they checked antibacterial activity[4] of four lactic acid producing *Bifidobacterium* strains against food borne pathogens by study cell optical density and viable count of *E.coli* and other used pathogens.

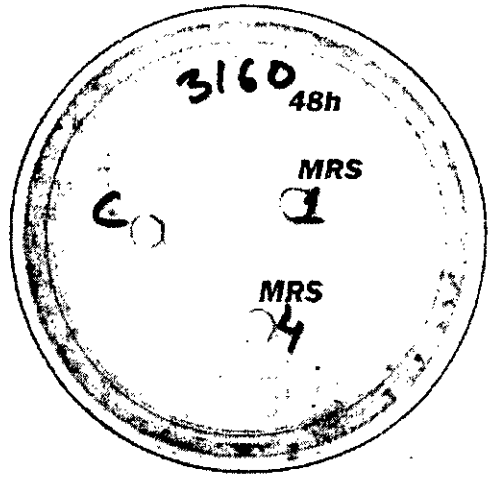
Table 4: Viable cell count (CFU×10^6/ml broth) of treated *E. coli* broth.

Time (h)	Supernatant (ml)	*E. coli* Control	*E. coli* + MRS-1	*E. coli* + MRS-4	*E. coli* + Co 10k
24	0.5 ml		182±8.265	164±5.637	136±8.465
	1.0 ml	304±8.485	114±6.845	104±8.385	84±5.867
	2.0 ml		72±5.657	56±5.462	32±2.426
48	0.5 ml		48±7.271	40±5.245	24±5.457
	1.0 ml	415±7.071	28±8.645	24±5.657	16±2.628
	2.0 ml		12±2.828	06±1.414	03±2.121

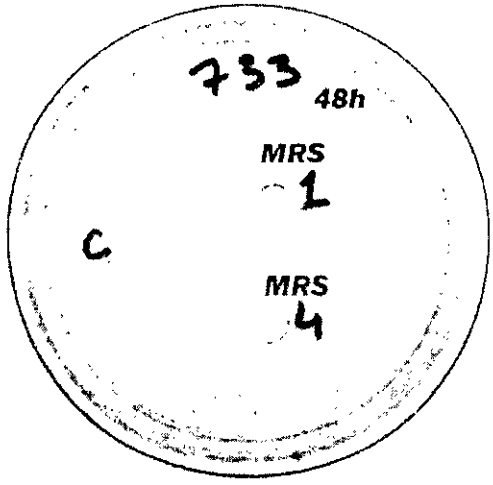
*Values are mean + SD and significant at ($p < 0.05$).



MRS1 and MRS4 against *E. coli* 901

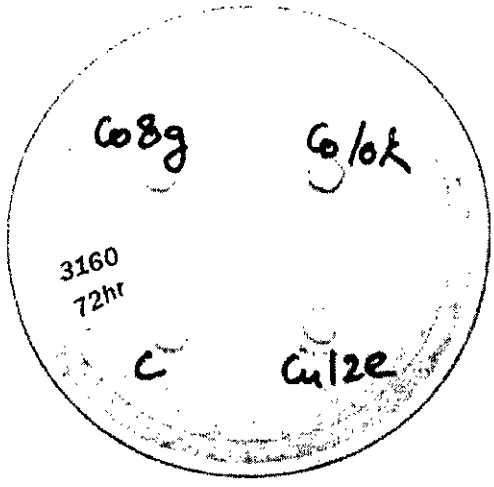


MRS1 and MRS4 against *S. aureus* 3160

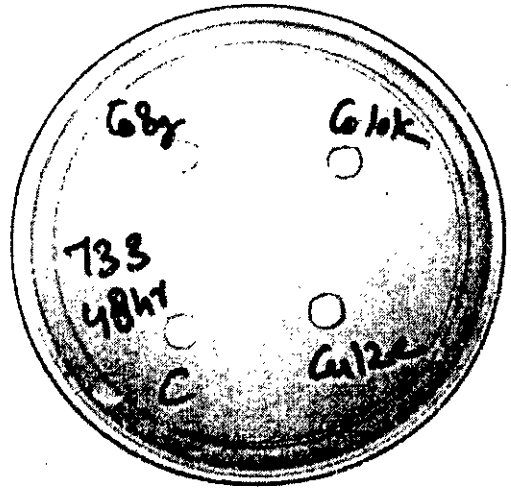


MRS1 and MRS4 against *S. typhi* 733

Co10k against *E. coli* 901



Co10k against *S. aureus* 3160



Co10k against *S. typhi* 733

Figure 2: Antagonistic activity of supernatant against all human pathogenic bacteria.

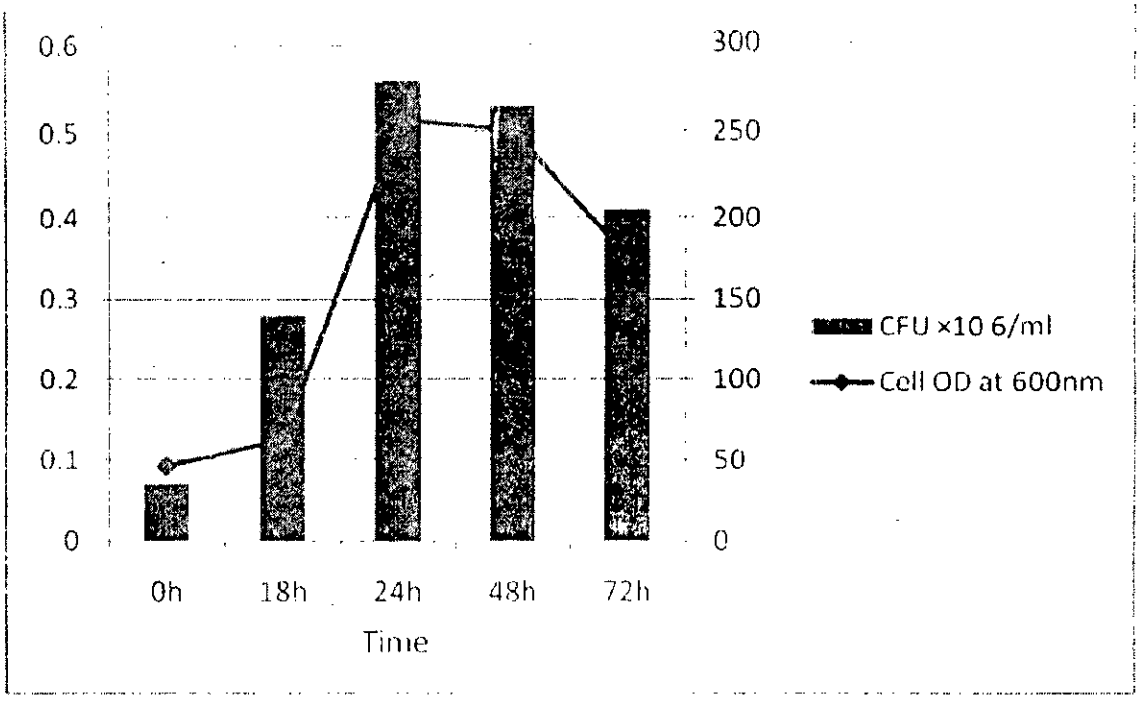


Figure 3: Growth profiling of MRS-4 in terms of O.D. as well as CFU.

Growth kinetics and production of bacteriocin from MRS-4:

The growth profiling of MRS-4 (*Lactococcus*) was investigated in the liquid broth at optimal conditions. Different flasks were used for different time intervals to analyze the increase in cell optical density as well as colony forming unit. The result of increase in cell density with respect to time is shown in Figure 3.

It indicates that maximum cell biomass was achieved during 24 h of incubation and remained more or less constant till 48 h of incubation as shown by the number of colonies observed on the agar plate. As far as the production of bioactive compound is concerned it starts from 18 h of incubation and remains constantly produced throughout the stationary phase. The zone of inhibition as recorded against pathogens is represented in Table 5. Similar observations were reported by earlier investigators while studying growth profiling of Lactic acid bacteria (Leroy and De Vuyst, 2001; Todorov and Dicks, 2005) by studying growth profiling of Lactic acid bacteria.

Table 5: Production of bacteriocin from MRS-4 at different time intervals, represented by zone of inhibition (mm).

Time (h)	*S. aureus* 3160	*E. coli* 901	*S. typhi* 733
18	10±1.414	9.5±0.707	9.5±0.707
24	14±1.414	12±1.414	12±1.414
48	14±2.828	12±2.828	12±2.828
72	14±1.414	12±1.414	12.5±0.707

*Values are mean ± SD and significant at ($p < 0.05$)

CONCLUSION

Based on our preliminary work, it can be concluded that the metabolite produced by MRS-4 could be used as biocontrol agent for inhibiting the human pathogenic organisms. The bacteriocin produced from this isolate has potential application as a bio preservative in the food industry.

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