Internet Journal of Food Safety, Vol.11, 2009, p. 81-87

Screening of Lactic Acid Bacteria for Antimicrobial Properties Against *Listeria monocytogenes* Isolated from Milk Products at Agra Region

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Abstract: and characterization of Lactic acid bacteria (LAB) strains with potential for the production of antimicrobial factors is essential to produce health promoting, microbiologically and chemically safe natural food products. Curd and cottage cheese are the traditional fermented food products, produced by natural fermentation using different utensils along with time honoured technology adapted to the local environment. For the present study curd and cottage cheese samples were collected seasonally from different parts of Agra city to isolate and characterize LAB and *L.monocytogenes*. LAB were evaluated for antimicrobial activity against *L.monocytogenes*. 400 LAB isolates (200 from each cottage cheese and curd) were examined for antimicrobial activity on *L.monocytogenes* using agar spot method. Out of the 400 LAB isolates 36 isolates displayed antimicrobial activity against *L.monocytogenes*. Of these 6 were identified as *Lactobacillus*, 4 as *Leuconostoc*, 1 as *Streptococcus* and 25 as *Lactococcus*. This study ascertained that curd and cottage cheese are rich sources of diverse LAB showing antimicrobial activity and the isolates have great potential to be used as natural preservatives for milk products.

Key words: Milk products, Lactic acid bacteria, antimicrobial activity, *L.monocytogenes*.

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Introduction

The genus Listeria represents a group of closely related, Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 mm in width and 1–1.5 mm in length. Taxonomically, it is divided into six species (i.e. *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri and Listeria grayi*), of which only *L.monocytogenes* and *L.ivanovii* are pathogenic (Robinson et al., 2000). *L.monocytogenes* is an opportunistic intracellular pathogen of both man and animals while *L.ivanovii* infects mainly ungulates, it rarely occurs in man (Low & Donachie, 1997).

L.monocytogenes shows characteristic tumbling motility and it is widely present in ready-to-eat-products, soil, water samples, and sewage, human and animal faeces and also in cattle, sheep, goat and poultry. Regular standard of FDA includes 'zero tolerance' for *L.monocytogenes* in all ready-to-eat products. *L.monocytogenes* is the only species in the genus *Listeria* that has been involved in known food-borne outbreaks of listeriosis, particularly in risk populations including neonates, immunocompromised hosts and pregnant women. During the early stages of infection, human listeriosis often displays non-specific flu-like symptoms (e.g. chills, fatigue, headache, and muscular and joint pain) and gastroenteritis. However, without appropriate antibiotic treatment, it can develop into septicaemia, meningitis, encephalitis, and may leed to abortion and, in some cases, death (Vazquez-Boland et al., 2001). Though listeriosis is relatively rare and sporadic, it is a severe disease with high fatality rate (20%-30%) (FAO/WHO, 2004). Food products most frequently associated

with listeriosis are soft cheeses, particularly those made from unpasteurized milk, and ready-to-eat meat-containing food products (Kaclikova *et al.*, 2001). Ability of *L.monocytogenes* to grow at temperatures ranging between -0.4^{0}C to 50^{0}C, its high tolerance for salt and its ability to initiate growth at relative low pH (5.0-5.7 at 4^{0}C and 4.3-5.2 at 30^{0}C) make the control of this pathogen in food very difficult. A novel approach to controlling *L.monocytogenes* in food is the use of antimicrobial compounds from LAB (De Vugst and Leroy 2007).

LAB are known for their probiotic properties and are considered as "food grade" organisms, used extensively in food microbiology and human nutrition (Isolauri *et al.*, 2001). Probiotics can be defined as mono or mixed culture of living microorganisms, which beneficially affect the host (human and animal) by improving the balance of the indigenous microflora, when consumed in an adequate amount as part of the food (FAO/WHO, 2001). LAB are gram-positive, facultative anaerobes. These are Catalase negative, non-spore-forming rods, cocci and cocco-bacilli generally present as indigenous microflora in raw milk and produce lactic acid as a major end product during fermentation of carbohydrates. LAB are widely distributed in different ecosystems such as several raw materials (milk, meat, flour), soil, water plants, silage, waste products and also in the intestinal tract of animals. Most representatives of LAB do not pose any health risk to man and are designated as "Generally Recognized as Safe" (GRSA). These have commonly been used as a starter culture and play an essential role in manufacturing of a wide variety of fermented food such as curd, cheese, yoghurts, dry sausages, beers and sourdoughs.

Fermentation of foods with LAB can exert several beneficial effects:

- LAB set up a competition for nutrients and drop the pH which causes the inhibition of spoilage and other pathogenic bacteria.
- They reduce the toxicity by degradation of noxious compounds of plant origin such as cyanogens.
- Probiotic effect of LAB improves the host intestinal microbial balance.
- Fermentation produces a variety of antimicrobial compounds, which act as a natural competitive substance to inhibit other microorganisms sharing the same niche; these include H_2O_2, ethanol, CO_2, acetic acid and bacteriocins.

LAB can be divided into two groups 'homofermentative and heterofermentative', based upon the products produced from the fermentation of glucose. Depending on the fermentation of glucose and cell morphology LAB are classified into different genus including *Lactobacillus, Lactococcus, Pediococcus, Leuconostoc and Streptococcus*.

The purpose of this study was to isolate and characterize LAB from fermented milk products, curd and cottage cheese collected form unorganized sectors of Agra region and to study their antimicrobial activity against *L.monocytogenes* also isolated from the same samples.

Material and Methods

Standard strains: Standard strain of *L.monocytogenes* (MTCC-1143) was procured from MTCC Chandigarh. All the isolates were confirmed through biochemical tests by comparing with the results of the standard strain.

Collection of samples: Samples of cottage cheese and curd were collected from in and around the different market areas of Agra City including Ram Bagh (East), Sultan Pura (West), Dayalbagh (North), and Sikandra (South) for a period of 12 months and examined for the presence of *L.monocytogenes* and Lactic acid bacteria (Fig. 1).

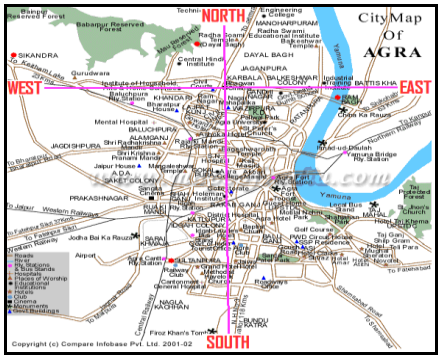
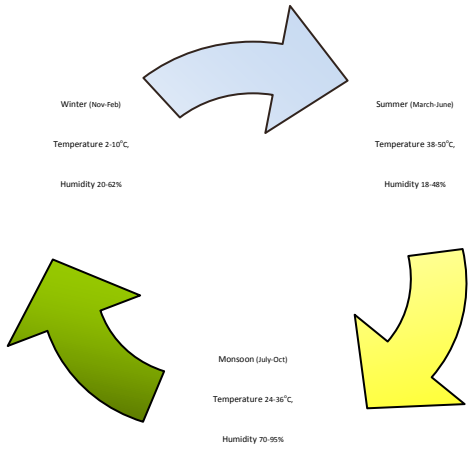


Fig. 1: Selected sampling area of Agra city.

These samples were collected aseptically, transferred to sterile plastic bags and directly transported to the laboratory under cold conditions. They were stored at 4^0C and analyzed within 24 hrs.

Climatic conditions of the sampling Area:



Bacterial strains and culture conditions: A portion (10g or 10ml) from the centre of each sample was extracted aseptically and homogenized with 90 ml sterile enrichment broth. UVM-2 broth was used for the isolation of *Listeria* from curd and cottage cheese using the method of McClain and Lee, 1988. The inoculum from enriched UVM-2 was streaked on Dominguez-Rodriguez Isolation Agar (DRIA) (Dominguez-Rodriguez *et al.,* 1984) and incubated at 37^0C for 24-48 hrs. morphologically typical colonies (greenish-yellow glistening, iridescent and pointed colonies surrounded by diffuse black zone) at least 4/plate were confirmed by Gram's staining, Catalase reaction, tumbling motility at 20-25^0C, Methyl red test, Voges-Proskauer test, Nitrate reduction, fermentation of sugars (Ramnose, α-methyl d-mannoside and Xylose) (Table 1).

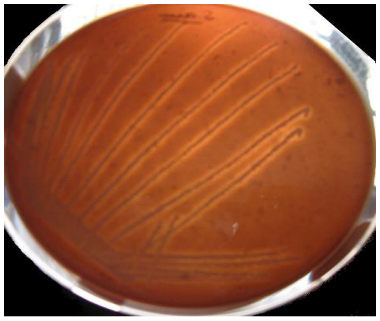


Fig. 2: Haemolysis on 5% sheep blood agar.

The pathogenicity of *L.monocytogenes* was tested by haemolysis on 5% Sheep Blood Agar and growth of CHROMagar characterized by specific sky blue colonies surrounded by white halo (Fig. 2 and Fig. 3)

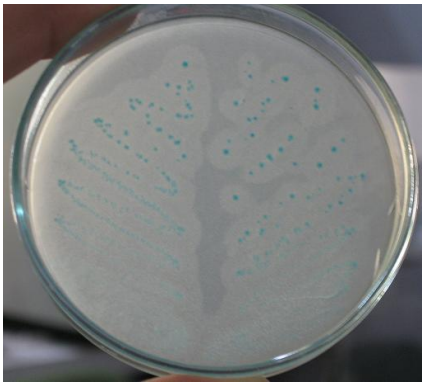


Fig. 3: Pathogenicity test on CHROMagar.

Table 1: Biochemical characterization of *L.monocytogenes*

Reaction		***L.monocytogenes***
Catalase		+
Oxidase		-
Indole Production		-
Nitrate Reduction		-
Methyl Red		+
Voges-Proskauer		+
Haemolysis		+
Acid From Sugar	Ramnose	+
	α-methyl d-mannoside	+
	Xylose	+

For the isolation of LAB curd samples were enriched in MRS (de Mann Rogosa Sharp) (De Man *et al.,* 1960) broth and cottage cheese samples were enriched in RSS (Ringer's Salt Solution) and incubated for 24 hrs at 37^0C. Serial dilutions were made from the enriched sample in

the same diluent and one ml from tube was Sharp) agar and incubated at 37^0C for 24 hrs. Plates showing growth in between 30-300 colonies were selected and 8 well isolated identical colonies (white cream coloured colonies, 2-3 mm in diameter, with entire margins) were picked up and transferred to MRS broth. They were propagated twice and streaked on MRS agar to check the purity of the isolates and then stored in MRS broth overlaid with 15% glycerol at -80^0C. Working cultures were also kept in MRS broth at 4^0C and sub-cultured at the interval of 4 weeks.

Before biochemical examination each strain was sub-cultured twice overnight in MRS broth for the isolation of LAB. All strains were initially tested by Gram's staining, Catalase reaction and spore formation. Gram positive, Catalase-negative, cocci or rod shaped and non spore forming isolates were considered as LAB. Further grouping into different genera was made by gas production from glucose by the hot loop test (William and Janice, 1976).

Screening of LAB: 10µl overnight cultures of LAB were spotted on the surface of Muller Hinton Agar plates, pre-inoculated with 100µl overnight culture of Indicator strain and incubated at 37^0C and examined after 24 hrs for the zone of inhibition.

Result and discussion

The present research concentrates on screening of LAB for antimicrobial activity that inhibit pathogenic *L.monocytogenes* from milk products (curd and cottage cheese), collected seasonally from different unorganized sectors of Agra region. Table-2 depicts the sampling data which consists of various numbers of samples analyzed and confirmed as *L.monocytogenes.*

A total of 200 milk product isolates comprising 100 from curd and 100 from cottage cheese were analyzed for the identification of *L.monocytogenes* throughout the sampling period (2007-2008), collected from different sites including Ram Bagh (east) Sikandra (west) (Dayalbagh (north) and Sultan Pura (south),) of Agra city in three different seasons viz. winter (November 2007-February 2008) Summer (March-June 2008) and monsoon (July to September 2008) to observe the seasonal occurrence of *L.monocytogenes.*

inoculated on plates of MRS (de Mann Rogosa

Table-*2. L. monocytogenes* detected in test samples during various seasons.

Seasons	Number of isolates		Number of isolates positive for *L.monocytogenes*	
	Curd	Cottage cheese	Curd	Cottage cheese
Summer	32	32	6	2
Winter	36	36	5	0
Monsoon	32	32	7	10

Curd samples: Out of a total of 32 isolates collected in each summer and monsoon season**,** 6 and 7 isolates respectively were confirmed as *L.monocytogenes,* while out of the 36 isolates collected in the winter season**,** 5 isolates were confirmed as *L.monocytogenes.*

Cottage cheese samples: Out of a total of 32 isolates collected in each summer and monsoon season**,** 2 and 10 isolates respectively were confirmed as *L.monocytogenes,* while out of the 36 isolates collected in winter season**,** none of the isolates was confirmed to be *L.monocytogenes.*

On analysis of the data of curd samples, collected from the Southern part (Sultan Pura) and cottage cheese samples collected from the Western part of Agra (Sikandra), 55% and 50% respectively were found to be highly contaminated with *L.monocytogenes.* Occurrence of *L.monocytogenes* in curd and cottage cheese samples was higher in the monsoon season, followed by the summer and the winter season respectively. Of the total samples analysed 18% of curd and 12% of cottage cheese were contaminated with *L.monocytogenes.* Of total 30 isolates, 26 isolates confirmed as pathogenic *L.monocytogenes* of the bases of haemolysis on sheep blood agar and growth on CHROMagar. These confirmed isolates were then subjected to antimicrobial activity through LAB.

On the basis of Gram's staining and hot loop test the isolates were genus classified as follows, 6 *Lactobacillus,* 4 *Leuconostoc,* 1 *Streptococcus,* and 25 *Lactococcus* (Table 4). The antimicrobial

activity of 400 LAB isolates from fermented milk products (200 from each curd and cottage cheese) were analyzed. Despite the high number of LAB strains isolated only 36 isolates (6 from each curd and 30 from cottage cheese) showed inhibition activity against *Listeria monocytogenes* (Table 3, Fig 4).

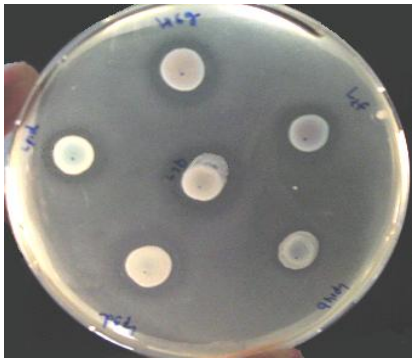


Fig. 4 Inhibition Zone of LAB against *L.monocytogenes*

Table-3 LAB samples showing inhibitory activity against *L.monocytogenes* in various seasons.

Season	**Number of isolates**		**Number of isolates showing inhibition against *L.monocytogenes***	
	Curd	**Cottage cheese**	**Curd**	**Cottage cheese**
Summer	64	64	3	12
Winter	72	72	3	8
Monsoon	64	64	0	10

Out of 36 isolates 9 isolates were confirmed as heterofermentative (4 from curd and 5 from cottage cheese) and 27 as homofermentative (2 from curd and 25 from cottage cheese). Most natural LAB isolates inhibiting *L.monocytogenes* belonged to the genus *Lactococcus* (69.44%) (Table 4).

Table-4 Classification of LAB isolates depending on fermentation of Glucose and Cell morphology.

Heterofermentative				**Homofermentative**			
Bacillus	**Coccus**		**Ovoid cocci**	**Bacillus**	**Coccus**		**Ovoid cocci**
Lactobacillus	**Lactococcus**	**Streptococcus**	**Leuconostoc**	**Lactobacillus**	**Lactococcus**	**Streptococcus**	**Leuconostoc**
5	None	None	4	1	25	1	None

The results show that the occurrence of *L.monocytogenes* was higher in monsoon season while the number of LAB samples that inhibited *L.monocytogenes* was lesser in this season, on the other hand the occurrence of *L.monocytogenes* was more in summer and It has been reported that environmental factors including stressful conditions are the key parameters that influence the magnitude of the production of antibacterial substances by bacteria as a means to overcome competitive strains living in the same environment (Pattnaik *et al.*, 2005). 55.55% of natural isolates inhibiting *L.monocytogenes* show an inhibition zone within the range of 7-10 mm, 27.77% show an inhibition zone within the range of 10-13 mm and only 16.66% show an inhibition zone greater than 13 mm (Table 5).

These isolated strains can positively boost their use as starter cultures for traditional fermented winter and the number of LAB samples showing inhibition was lesser in this season. This showed that LAB are capable of inhibiting pathogenic microorganisms in the food environment and display crucial antimicrobial properties with respect to food preservation and safety. foods, with a view to improving the quality and microbiological safety of these fermented milk products. They can also be used more specifically to selectively inhibit certain high-risk bacteria like *L.monocytogenes* in food. Thus LAB can act as a barrier and can inhibit food spoilage and pathogenic bacteria in milk products. Furthermore, due to synergistic properties, these can also help to reduce the addition of chemical preservative in food and can alternatively satisfy the consumer's demands for safe, fresh-tasting, ready-to-eat and minimally processed foods.

Table 5: Diameter of inhibition zone (mm) produced by LAB isolates against *L. monocytogenes* isolated from curd and cottage cheese.

LAB belonging to genus →	**Lactobacillus**		**Leuconostoc**	**Streptococcus**	**Lactococcus**
Type → / **Diameter of zone ↓**	**Heterofermentative**	**Homofermentative**	**Heterofermentative**	**Homofermentative**	**Homofermentative**
1	**5**	**-**	**2**	**-**	**13**
2	**-**	**1**	**-**	**1**	**8**
3	**-**	**-**	**2**	**-**	**4**

1 – Diameter of zone >7 and <10, **2** – Diameter of zone >10 and <13, **3** – Diameter of zone >13.

Acknowledgement:
The authors are greatful to "Frederic Eudes from CHROMAGAR, Paris, France"for generously providing the CHROMagar for the identification of Pathogenic *Listeria monocytogenes*.

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