ISSN:2319-2607 IISUniv.J.Sc.Tech. Vol.2(1),17-23 (2013)

RESEARCH ARTICLE

Isolation and Identification of Lactic Acid Bacteria and their Antibacterial Activity Against Pathogenic Microbes from Dairy Products

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Abstract

Dairy Food products are complex ecosystems with beneficial and harmful bacteria. Lactic acid bacteria and their antimicrobial metabolites have potential as natural preservatives to control the growth of spoilage and pathogenic bacteria in these food products. Reports of co-existence of these probiotic and pathogenic bacteria in milk products are very limited. In the present study the presence and antagonistic impact of heterogeneous population of Lactic acid bacteria against foodborne pathogens (*Escherichia coli* and *Listeria monocytogenes*) were investigated in curd and cottage cheese samples during different seasons and from different areas of Agra city. Various genera of Lactic acid bacteria were differentiated from each other by RAPD-PCR analysis and species identification of the pathogenic as well as Lactic acid bacteria was done by 16S rRNA gene sequencing. The number of homofermentative Lactic acid bacteria exceeded the heterofermentative Lactic acid bacteria inhibiting *Listeria monocytogenes* and *Escherichia coli*. The antagonistic behaviour of lactic acid bacteria may either be due to their major metabolic end products or via the production of low-molecular weight compounds, the bacteriocins. This paper emphasises the application of Lactic acid bacteria for longer shelf-life of dairy products to extend their market reach and economically benefit to both the producers and the consumers.

Keywords

Antagonistic activity, Lactic acid bacteria, Pathogenic microbes

Introduction

In spite of modern advances in technology, preservation of food is still a debatable issue not only for developing countries but also for the industrialized world. The empirical use of microorganisms and their natural products for the bioprservation act as a good weapon for food spoilage and pathogenic bacteria *like Escherichea coli, Staphylococcus aureus, Enterobactor sakazakii, Salmonella typhimurium* and *Listeria monocytogenes* etc. Several Lactic acid bacteria (LAB) offer potential application in food treatment, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties. This can be used as a better alternative to satisfy the increasing consumers demand for safe, fresh, ready- to-eat minimally processed foods. LAB set up a competition for nutrients and drops the pH which causes the inhibition of above mentioned spoilage and other pathogenic bacteria (Pattnaik *et al,* 2005). They also produce an array of antimicrobial metabolites such as organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins (Magnusson and Schnurer, 2001). Bacteriocin produced by LAB offer several desirable properties that make them suitable for food preservation as they are generally recognized as safe substances are non toxic on eukaryotic cells and become inactivated by digestive proteases. LAB are usually pH and heat tolerant and have an antimicrobial spectrum against many food spoilage and pathogenic bacteria and they do not show cross resistance with antibiotics.

This clearly indicates the application of bacteriocin and their producer strain in food preservation (Thomas *et al,* 2000). This can offer several benefits including the increase the shelf life of foods and ameliorate the economic losses due to food spoilage. With these points in mind the present study addresses different aspects related to food preservation by screening of LAB inhibiting pathogenic bacteria (*Escherichea coli and Listeria monocytogenes*), and the co-dominance of LAB with aforementioned pathogens isolated from curd and cottage cheese from different region of Agra city. In order to achieve this goal, LAB and pathogenic bacteria were isolated and characterized by using phenotypic (cell morphology, Gram staining, physiological and biochemical tests) and genotypic methods (RAPD-PCR and 16S rRNA gene sequencing).

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Materials and Method

Samples

25 samples of each curd and cottage cheese were collected from different market areas of Agra city during summer, winter and monsoon season. All samples were collected aseptically in sterile plastic bags kept in an ice-box, and transported to the laboratory for further analyses.

Microbiological Analysis

Samples (10g/10ml) of each milk product were homogenised with 90 ml of sterile MRS (DeMan Rogosa Sharpe) broth for LAB, Lactose broth for *E. coli* and DRIA (Dominguez-Rodriguez Isolation Agar) for *L. monocytogenes* and incubated for 24 hrs at 37°C. Serial dilutions were made from the incubated samples of LAB and 1 ml of each dilution was spread on MRS plate (M641, HiMedia, India). Eight Isolated colonies based on colony morphology were selected randomly from the dilutions showing 30-300 colonies on MRS plates. Purity of the isolates was checked by streaking and sub-culturing again on fresh agar plates of the isolation specific media, followed by microscopic examination. Pure isolates of LAB were preserved at 20 °C in MRS broth with 15% (v/v) glycerol. All the pathogenic isolates (100 from curd and 100 from cottage cheese for each) were confirmed morphologically and biochemically (Singh and Prakash, 2008) by referring standard strains of *E. coli* (MTCC-723) and *L. monocytogenes* (MTCC-1143) as a control, procured from MTCC Chandigarh, India.

Phenotypic Characterization

Isolates of LAB were Gram-stained and tested for catalase production, and spore formation following the methods of Schillinger and Lücke (1987), and Dykes *et al,* (1994). Differentiation between homofermentative and heterofermentative groups was done by the hot loop test (William and Janice, 1976). Sugar fermentation patterns of LAB isolates were determined by fermentation of 1% carbohydrate solution including galactose, arabinose, mellibiose, maltose, raffinose, xylose, mannose, melizitose, trehalose, salicine, glucose, lactose and sucrose (Tserovska *et al,* 2002).

Screening of LAB: 10µl overnight cultures of LAB were spotted on the surface of Muller Hinton Agar plates, pre-inoculated with 100 µl overnight culture of indicator strain and incubated at 37^{0}C and examined after 24 hrs for the zone of inhibition. All the isolates showing inhibitory activity against both the pathogens were further characterized genotypically (Schillinger and Lucke, 1989; Toba *et al,* 1991).

Genotypic Characterization

DNA Extraction

Total genomic DNA of LAB isolates and pathogenic isolates were extracted from 100µl of overnight cultures grown in respective broths of the isolates at 37 °C, according to the methods of Tsai and Olson (1991).

RAPD-PCR Analysis

Amplification was performed in a thermal cycler (Applied Biosystem, 2720) using a single primer P1 of arbitrary nucleotide sequence (5′ ACGCGCCCT 3′) (Angelis *et al,* 2001). 50µl of master mix was prepared containing 1 X PCR buffer, 0.2mM dNTP mix, 2mM MgCl2, 1µM primer, 1.25U Taq DNA Polymerase and 5 µl of extracted DNA. The PCR mixture was subjected to thermal cycler (initial incubation 94^{0}C for 5 min, 34 cycles of 1 min at 94^{0}C for the denaturation, 1.5 min at 55^{0}C for annealing and 2.5 min at 72^{0}C for extension).

16S rRNA Gene Analysis

The 16S rDNA nucleotide sequence (~ 1400bp) was determined for two representative *Lactococci*, one *Lactobacillus* and one *Pediococcus* isolate and one representative of each *E. coli* and *L. monocytogenes*. The 16S rDNA gene was amplified by PCR using universal primers set 27F (5 AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACTTGTTACGACTT-3) (Frank *et al,* 2008; Acedo-Felix and Perez-Martinez 2003). DNA was amplified in 50µl volumes containing 1X of 10 X PCR buffer, 0.2mM dNTP mix, 2mM $MgCl_2$, 5pM of each primer, 1.25U Taq DNA Polymerase, 5µl template DNA and MiliQ water. DNA was amplified in 34 cycles (denaturation, 95^{0}C for 30 s, annealing, 55^{0}C for 30 s and 30 s at 72^{0}C for extension).

Agarose Gel Electrophoresis

Amplified products of RAPD-PCR and 16S rDNA were subjected to electrophoresis on 1.5% and 1% agarose gels respectively containing 0.5µg of ethidium bromide per ml in 0.5XTris-borate-EDTA buffer at 7V/cm. A DNA ladder (10 bp and 1000bp DNA ladder Bangalore genei) was. The gel was visualized and photographed over the UV transilluminator (Zenith gel documentation system) and analyzed by gel doc software named UN-SCAN-IT gel 6.1.

Sequence Analysis

Amplified samples were sent to the Institute of Molecular Medicine, Delhi, for sequencing. The sequences obtained were aligned with the already established sequences available online through the National Centre for

Biotechnology Information (NCBI) database using the Basic Local alignment Tool (BLAST 2.0 search program).

Results and Discussion

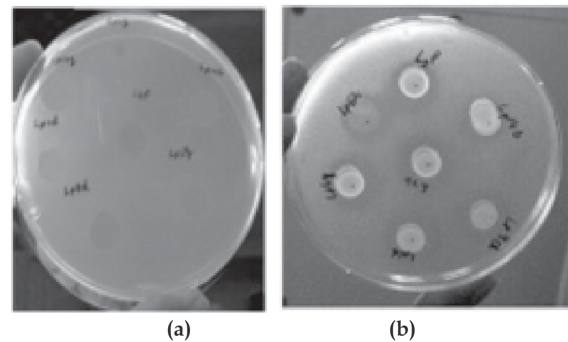
On the basis of Gram's staining and biochemical characterization (MR, VP, Indole, Nitrate, citrate utilization, H_2S production, fermentation of various sugars, haemolysis, and growth on chromogenic medium) 5 isolates were confirmed as pathogenic *E. coli* (1 from curd and 4 from cottage cheese), and 18 isolates as pathogenic *L. monocytogenes* (12 from curd and 6 from cottage cheese).

In comparison to *E. coli* occurrence of *L. monocytogenes* was found to be higher in the milk products. Gray and Killinger, (1966) reported that multiplication of the *L. monocytogenes* was inhibited or decreased at a pH lower than 5.6. The pH of the curd is 6.0-6.3 and that of cottage cheese is 5.6-6.0. So according to pH, fresh curd is more susceptible to contamination of *L. monocytogenes* than cottage cheese and with age these milk products becomes more susceptible to rapid growth of *L. monocytogenes* already present in it. The initiation of this microbe requires a pH of 5.0-5.7 at 4^0C and a pH of 4.3-5.2 at 30^0C Farber and Peterkin (1991). This indicates that even under refrigeration *L. monocytogenes* can start growing in these milk products whose pH range is ideal for its growth. *E. coli* is inactivated faster than *L. monocytogenes* in curd and cottage cheese during refrigerated storage (Velani and Gilbert, 1990). Hence, milk products free from *E. coli* may not be necessarily free of *L. monocytogenes*.

The results of our findings are in agreement with the aforesaid facts as the incidence of pathogenic *L. monocytogenes* was found to be higher in curd samples as compared to the cheese samples. Study done by Shaw, (1993) also supported our findings that pH of the curd before salting does not decrease below 5.0 and therefore the risk of survival and growth of *L. monocytogenes* increases. The heat treatment step during its preparation would also inhibit these microbes.

These milk products are prepared by traditional methods in the unorganized sectors whose activity is not regulated under legal provisions. Uyttndaele and Donachie, (1999) reported that the ready to eat products that do not undergo any substantial heat treatment and are refrigerated before consumption are of particular concern with respect to *Listeria* and other microbial infections.

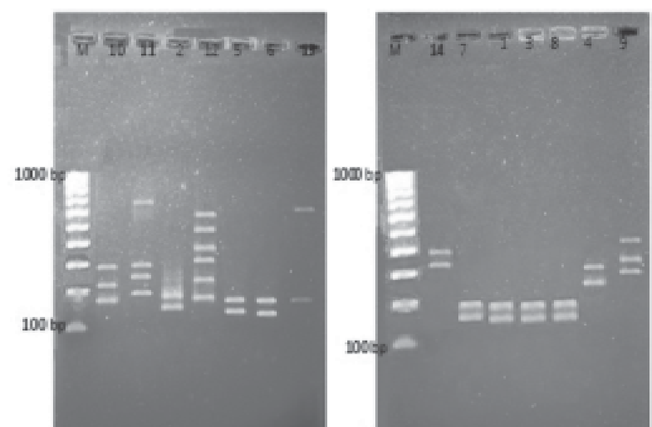
Of the 400 isolates only 30 isolates were able to inhibit at least one of the selected pathogenic bacteria as detected by agar spot test method (Fig.1). Of the 30 isolates, 14 Isolates were capable of inhibiting both the indicator microbes (*E. coli* and *L. monocytogenes*) and were from the following genera- 3 from *Lactococci* (from cottage cheese), 7 from *Pediococci* (2 from curd and 5 from cottage cheese), 1 from *homofermentative lactobacilli* (from cottage cheese), 1



(a) (b)

Fig.1. Inhibition Zone of Lab against (a) *E. coli* (b) *L. monocytogenes*

from *heterofermentative Lactobacillus* (from cottage cheese) and 2 from *Leuconostoc* (1 from curd and 1 from cottage cheese). These genera were confirmed on the basis of biochemical tests (Gram staining, gas from glucose and sugar fermentation test) (Table 1 and 2) and molecular analysis (RAPD-PCR) (Fig.2). After grouping all the LAB isolates into various genera all the isolates were numbered



M-100 bp DNA Ladder, Number 1 to 14 - serial number of the isolates grouped into various genera

Fig.2. RAPD profile of the 14 LAB isolates inhibiting the two selected path pathogenic indicator bacteria

serially from 1 to 14. One isolate of each *E. coli,* and *L. monocytogenes* were selected randomly after their biochemical confirmation and 4 isolates of LAB (1,3,5,12) were selected based on their ability to inhibit all the pathogens, for further confirmation up to species level by the amplification of 16S rDNA coding ~ 1400 bp 16S rRNA gene sequence using universal primer set 27F/1492R. Amplified products were submitted to the Institute of Molecular Medicine, New Delhi for sequencing. Obtained sequences were aligned through National centre for biotechnology Information (NCBI) database by using the Basic Local Alignment Tool (BLAST, 2.0 search program) to determine their approximate phylogenetic affiliations.

Sequence alignment with BLAST database shows the best match of the two pathogenic bacteria as *Escherichia coli* F11 gcontig_1112495919726 and *Listeria monocytogenes* FSL N1-017 cont5.59, and LAB isolates as LAB isolates 1, 3, 5 and 12 show the best hit with *Lactococcus lactis subsp. lactis* KF147, *Lactococcus lactis subsp. lactis* KF147, *Pediococcus acidilactici* DSM 20284 contig00019, *and Lactobacillus fermentum* ATCC 14931 contig00160 (Fig.3).

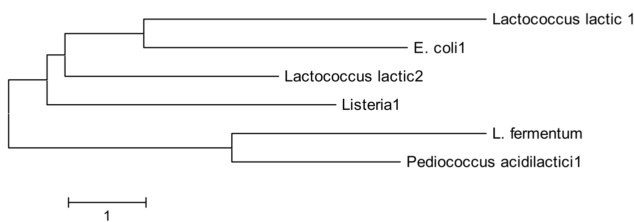


Fig.3. Phylogenetic tree of the identified pathogenic and lactic acid bacteria

Lactic acid bacteria may prove to be good natural preservatives to prevent the deterioration of these milk products from spoilage and pathogenic organisms. Our results show that selected dairy products provide a good source for LAB exhibiting antimicrobial activity against *E. coli* and *L. monocytogenes*. The highest number of LAB isolates inhibiting the selected pathogenic bacteria was obtained from cottage cheese samples followed by the curd samples (Table 1). This may be attributed to the factors like the pH of milk products and also their nutritive value. The pH of cottage cheese is more acidic (5.6-6.0) than curd (6.0-6.3). The LAB require more acidic conditions for their growth. The curd procured from the market is relatively more fresh then the cottage cheese. Thus the cottage cheese is able to harbor more LAB during this storage period. We observed a high number of isolates which could inhibit *L. monocytogenes* (15/30). While only 1/30 isolates were able to inhibit *E. coli* (Table 3 and 4), *L. monocytogenes* being gram positive while *E. coli* is gram-negative. Initial screening of our study is in agreement of Erdogrul and Ervilir, (2006) reported that gram-positive indicator bacteria are more sensitive to anti microbial substance of LAB strains than the gram-negative indicator bacteria.

The results obtained in the present study interestingly correlate in most of the samples, that samples having more LAB do not have the pathogenic bacteria or if present their number are very less. As the sample numbers 1, 2, 6, 9 and 16 of cottage cheese and sample numbers 2 of curd yielded LAB that inhibit all the selected pathogenic microbes none of the selected pathogenic bacteria were found in those sample. Similarly LAB were isolated from the samples number 10, 18 and 21 of cottage cheese and samples number 14 of curd which could inhibit either one or two of the selected pathogenic bacteria but no pathogenic bacteria were isolated from these samples. This shows that the LAB bacteria themselves inhibit the pathogenic microbes in the natural environment. Bezkorovainy, (2001) also reported that the inhibition against the pathogenic bacteria may be due to the production of more acidic and lactic acid that lowered the pH of the medium. Interestingly in some of the samples where we get LAB inhibiting the selected pathogenic bacteria we also found the pathogenic microbes (sample number 14, 15 and 17 in cottage cheese and 7 in curd). This may be due to the low number of the probiotic bacteria present. The environmental conditions and the sample handling and processing techniques may be responsible for the contamination of these samples. The pathogenic bacteria were also obtained from the samples where we did not find any probiotic LAB bacteria (sample number

Table 1. Classification of the LAB isolates showing inhibitory activity against *E. coli* and *L. monocytogenes* isolated from curd and cottage cheese

Type of LAB on the bases of hot loop test →	Homofermentative				Heterofermentative		Total Number of isolates
Genus	*Lactococcus*	*Streptococcus*	*Lactobacillus*	*Pediococcus*	*Lactobacillus*	*Leuconostoc*	
Cell morphology	Cocci single and in pair	Cocci in chain	Rods	Cocci in pair and tetrad	Rods	Cocco-bacillus or in chain	
Gas production from Glucose	-	-	±	-	±	+	
Number of isolates inhibiting *E. coli*, and *L. monocytogenes* from curd	-	-	-	2	1		3
Number of isolates inhibiting *E. coli*, and *L. monocytogenes* From cottage cheese	3	-	1	5	1	1	11

Table 2. Sugar fermentation pattern of LAB inhibiting all the pathogenic indicator bacteria

Genus	S.No. of isolate	Galaetose	Arabinose	Mallibose	Maltose	Raffinose	Xylose	Mannose	Melezitose	Trilalose	Salicine	Glucose	Lactose	Sucrose
Lactococcus	1	+	-	-	-	+	+	+	+	+	+	+	+	+
Lactococcus	2	-	-	+	-	+	+	+	-	+	+	-	+	+
Lactococcus	3	+	-	-	-	+	+	+	+	+	+	+	+	+
Homofermentaive Lactobacillus	4	+	-	-	-	+	+	+	+	+	+	+	+	+
Pediococcus	5	+	-	-	-	+	+	+	+	-	+	+	+	+
Pediococcus	6	+	-	-	-	+	+	+	+	-	+	+	+	+
Pediococcus	7	+	-	-	-		+	+	+	+	+	+	+	+
Pediococcus	8	+	-	-	-	+	+	+	+	+	+	+	+	+
Pediococcus	9	+	-	-	-	+	-	+	+	+	+	-	+	-
Pediococcus	10	+	-	+	-	+	+	+	+	-	+	+	+	-
Pediococcus	11	-	-	-	-	+	-	-	-	+	+	+	+	+
Heterofermentative Lactobacillus	12	+	-	-	-	+	+	+	+	+	-	+	+	+
Hererofermentative Lactobacillus	13	+	-	-	-	+	+	+	+	+	+	+	+	+
Leuconostoc	14	+	-	-	-	+	+	+	+	+	+	+	+	+

(+) = showing fermentation of sugar (-) = no fermentation

Table 3. Seasonal occurrence of pathogenic *E. coli, L. monocytogenes* and the LAB inhibiting them isolated from cottage cheese samples

Seasons	Summer								Winter									Monsoon							
Samples	1D	2-S	3-K	4-SI	5-D	6-S	7-K	8-SI	9-D	10-S	11-K	12-K	13-SI	22-D	23-S	24-K	25-SI	14-SI	15-S	16 D	17-K	18-S	19-D	20-SI	21-K
Probiotic bacteria inhibiting pathogenic *E. coli*																									
Occurrence of pathogenic *E. coli* in the samples																									
Probiotic bacteria inhibiting pathogenic *L. monocytogenes*																									
Occurrence of pathogenic *L. monocytogenes* in samples																									

Inhibiting *E. coli* and *L. monocytogenes*.

Inhibiting only one microbe from *E. coli* and *L. monocytogenes*.

Showing occurrence of pathogenic *E. coli* and *L. monocytogenes* in the sample.

Table 4. Seasonal occurence of pathogenic *E. coli, L. monocytogenes* and the LAB inhibiting them isolated from curd samples

Seasons	Summer								Winter									Monsoon								
Samples	5-S	6-D	7-K	8-SI	14-K	15-S	16-S	17-D	1-D	2-S	3-K	4-SI	9-D	10-S	11-K	12-K	13-SI	18-SI	19-S	20-D	21-K	22-D	23-S	24-K	25-SI
Probiotic bacteria inhibiting pathogenic *E. coli*																									
Occurrence of pathogenic *E. coli* in the samples																									
Probiotic bacteria inhibiting pathogenic *L. monocytogenes*																									
Occurrence of pathogenic *L. monocytogenes* in the samples																									

Inhibiting *E. coli* and *L. monocytogenes*

Inhibiting only one microbe from *E. coli* and *L. monocytogenes*

Showing occurrence of pathogenic *E. coli* and *L. monocytogenes* from the sample

4, 5, 8 and 12 in cottage cheese and 5, 8, 19 and 20 in curd) (Table 3 and 4).

A major benefit of some LAB is their ability to produce inhibitory compounds like Organic acid, hydrogen peroxide and bacteriocins at 4^{0}C while not multiplying themselves (Brashears *et al*, 1998). Bacteriocins produced by LAB have considerable potential for food preservation, as well as for human therapy because of their advantage over known antibiotics. Ingestion of these compounds does not alter the digestive tract ecology and causes no risk related to the use of common antibiotics. Present study suggested that the bacteriocin extracted from *Pediococcus acidilactici* can be used as abroad sprectrum bacteriocin while bacteriocin from *Lactobacillus fermentum* and *Lactococcus lactis subsps, lactis* can be used more specifically for selective inhibition.

The exact identification of this inhibitory compound using sophisticated analytical techniques in order to purify the molecules responsible for inhibiting the pathogenic indicator bacteria would lead to further important research findings. Further study is needed to unravel the importance of bacteriocin in this process as well as in the genetic engineering of the already identified probiotics and those newly discovered to make them more efficacious against pathogenic bacteria.

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