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Isolation and identification of antagonistic *Lactobacillus* spp. isolated from dairy products against selected pathogens

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Abstract
Lactic acid bacteria occur naturally as indigenous micro flora. In fermented foods, starter culture of lactic acid bacteria is added for fermentation which results in desired changes in the food and dairy products. Isolation and identification of *Lactobacillus* spp. in various food products reveals the indigenous microflora of that region. Isolation of such regional strains helps in identification the best isolates which can be utilized for further study. Five hundred forty eight lactic acid bacteria were isolated from food (Dosa batter) and dairy samples (Raw milk, Curd and Paneer). Out of which 242 isolates were identified as *Lactobacillus* spp. and others included *Bacillus* spp., *Streptococcus* spp., and *Lactococcus* spp. Isolates of *Lactobacillus* spp. were identified to species level on the basis of cultural, morphological and biochemical characteristics - *Lactobacillus casei subsp. casei, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus bulgaricus* and *Lactobacillus casei subsp. rhamnosus.*

Keywords: *Lactobacillus* spp., MRS Agar, Raw milk, Curd, Paneer, Dosa batter.

1. Introduction
Lactic acid bacteria are widely distributed in nature as indigenous micro flora. Lactic acid bacteria play multifunctional role in the production of fermented foods like curd, yoghurt, idli and dosa batter, alcoholic beverages, vegetables, fish and meat products. Lactic Acid Bacteria (LAB) have been utilized for thousands of years in fermentation of foods due to their ability to produce changes in the taste, flavour and texture as well as inhibit pathogenic and spoilage microorganisms. Since they are prevalent in raw and fermented foods, it is assumed that most representatives of this group do not pose any health risk to man, and are designed as GRAS (Generally Recognized as Safe) organisms.
LAB have the ability to produce a number of antimicrobial substances, such as organic acids (lactic acid, acetic acid), free fatty acids, ammonia, diacetyl, H_2O_2 and bacteriocin which have the capacity to inhibit growth of variety of food-borne spoilage and pathogenic organism (Jack *et al.*, 1995; Vandenbergh 1993) [8, 23]. These bacteria producing different antimicrobials can inhibit pathogenic and spoilage causing microorganisms, extending the shelf life and enhancing the safety of food products.
Species of *Lactococcus, Lactobacillus, Streptomyces, Staphylococcus, Bacillus, Pediococcus, Leuconostoc, Enterococcus*, and *Carnobacterium* have been reported as Bacteriocins producing Lactic acid bacteria.
Isolation and identification of *Lactobacillus* spp. in various food products reveals the indigenous microflora of that region. Isolation of such regional strains helps in identification the best isolates which can be utilized for further study.
With the widespread increased interest in biological preservation of food stuffs, we focused on the antagonistic activity of bacteriocin produced by *Lactobacillus* spp. that may be utilized in inhibiting the growth of pathogenic and spoilage causing bacteria in order to keep food products healthy and also with increased shelf life.

Material and methods
Collection of Samples
Samples of food and dairy products like Milk, Curd, Paneer and Dosa Batter were collected from dairies and shops of different localities of Allahabad. Samples were collected in sterilized bottles. After the collection of samples, bottles were transported to laboratory and maintained at 4 ^{0}C until further use.

Isolation of lactic Acid Bacteria

Samples were serially diluted by ten folds in 1% peptone water blank. 0.1ml of the final dilution of each sample was then spread on preformed MRS Agar plates and was inoculated on triplicate plates of MRS Petriplates and then incubated at room temperature 37 ^{0}C for 24 - 48 hours under anaerobic condition in candle jar extinction method. One plate of each medium was kept as media control.

Identification of isolated lactic acid bacteria

The isolates were identified on the basis of cultural, morphological and biochemical characteristics as given in the Bergey's Manual of Systematic Bacteriology vol. 2 (Holt *et al.,* 1984) [7].

Cultural Characteristics

Appearances of colony on surface of MRS agar were studied.

Morphological Characteristics:

Isolates were Gram stained and observed under microscope.

Biochemical Characteristics

The isolates were subjected to following biochemical tests: motility, catalase, oxidase, Indole Production, methyl Red reaction, voges proskauer reaction, citrate utilization, growth at different temperature and carbohydrate fermentation tests.

Statistical Analysis

The data recorded during the course of investigation was subjected to three-way analysis of variance (ANOVA) and the conclusion was drawn accordingly (Fisher and Yates, 1968) [24].

Result and Discussion

A total of 548 bacterial strains were isolated from the sample of Raw Milk (68), Curd (265), Paneer (87) and Dosa batter (128).

The percentage incidence of the *Lactobacillus* spp. was reported to be in Raw Milk 37 (54.41%), Curd 98 (36.98%), Paneer 42 (48.27%), and Dosa batter 65 (50.78%) isolated from the samples. (Table 1. and Fig.1)

Similar results were reported by other researchers where the incidence of *Lactobacillus* spp. was found as low as 30-38% as reported (Abd El Gawad *et al*, 2010) [1] and a higher % as high as of 44-50% was reported (Aziz *et al,* 2009) [3].

Similarly, a study reports isolation of 67 lactic acid bacteria from curd, dosa and idli samples (Puttalingamma *et al*., 2006) [15]. In another study, isolation of 23 (41.82%) *Lactobacillus* spp., 10 (18.18%) *Streptococcus thermophilus* and 22 (40%) *Bifidobacterium* spp. (22) was reported from yoghurt, yoghurt milk and pharmaceutical products. (D'Aimmo *et al.,* 2007) [26]

Another investigation on other sources for isolation of lactic acid bacteria has been successfully reported. Isolation of six *Lactobacillus* spp., one bacteriocin producing strain *Lactobacillus fermentum* UN01 and 35 lactic acid bacteria was isolated from other products like Mango pulp, Chick intestine and different other sources origins respectively (Ravi *et al*., 2011, and Udhayashree *et al*., 2012) [16, 22].

The statistical analysis of the data obtained revealed significant difference between bacteria as well as samples.

Isolation of 10 Lactobacilli, Out of which 5 (50%), 3 (30%) and 2 (20%) were reported from curd, idli and dosa samples respectively. Bhattacharya and Das (2010) [4]

Similarly, another study reported isolation *Lactobacillus acidophilus*, *Pediococcus* spp. *Bifidobacterium* spp. and *Lactococcus* spp. from the various type of samples like Paneer, Cheese, yoghurt, milk sausage, pickle, beer, fermented olives, chicken and mutton and environment waste materials (Malini Maria and Janakiraman (2012) [10].

Another study reports isolation 244 bacterial isolates which were identified as strains of *Lactobacillus spp.* 152 (62.30%), 27 (11.07%) genus *Lactococcus* spp and 65 (26.63%) *Streptococcus* spp. was isolated from Dairy and Pharmaceutical products (Gad *et al*., 2014) [27].

The high incidence of lactic acid bacteria in raw milk can be attributed to contamination from the animal, especially the exteriors of Udder and the adjacent area, bacteria found in manure, soil, and water may enter (Garbutt, 1997) [28].

Table 1: Incidence of bacteria in food and dairy samples

S. No	Sample	Bacterial isolates (avg. value)	Incidence of lactic acid bacteria (%)			
			Lactobacillus isolates (avg. value)	*Bacillus* isolates (avg. value)	*Streptococcus* isolates (avg. value)	*Lactococcus* isolates (avg. value)
1.	Raw Milk	68	37 (54.41%)	13 (19.12%)	8 (11.76%)	10 (14.71%)
2.	Curd	265	98 (36.98%)	74 (27.92%)	46 (17.36%)	47 (17.74%)
3.	Paneer	87	42 (48.27%)	27 (31.03%)	9 (10.35%)	9 (10.35%)
4.	Dosa Batter	128	65 (50.78%)	41 (32.03%)	13 (10.16%)	9 (7.03%)
		548	242	155	76	75

(Parantheses indicates percentage incidence)

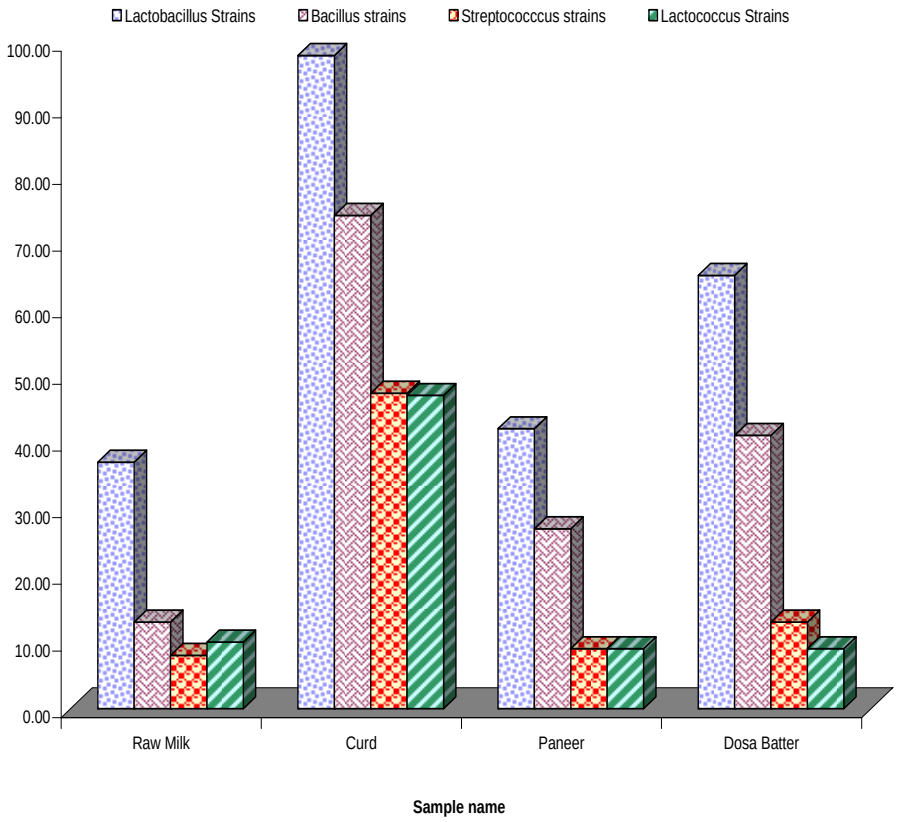

Fig 1: Incidence of total lactic acid bacteria in food and dairy samples

Identification of *Lactobacillus* spp.

Lactobacillus colonies were identified on the basis of typical characteristics namely white, small colonies with entire margin were transferred and maintained on MRS. Isolates were then subjeed to classification on basis of morphological and biochemical characteristics.

All isolates of *Lactobacillus* spp. gave positive reaction to gram staining when observed under the light compound microscope. The bacterial cells were mostly long rods and sometimes they were coccoid/ cocco bacilli. Isolates were non motile without flagella, Indole negative, citrate and catalase negative, produced acid but no gas from fermentation of glucose. All *Lactobacillus* spp. isolates were found to ferment glucose, lactose, except few which are able to ferment mannitol. The strains of *Lactobacillus* spp. were further subjected to species level identification on the basis of their cultural, morphological characteristics after which these isolates were subjected to various physiological and biochemical tests according to Bergey's manual to which the results obtained were compared (Table-3).

Incidence of *Lactobacillus* spp in raw milk samples was highest for *Lactobacillus plantarum* (29.72%), followed by *Lactobacillus fermentum* (24.32%), and *Lactobacillus acidophilus* (18.92%) while least 13.52% was found in *Lactobacillus casei* subsp. *casei* and *Lactobacillus brevis* (Table-2 and 3, Fig 2).

In curd samples, highest incidence was 25.51% *Lactobacillus bulgaricus*, followed by *Lactobacillus acidophilus* (21.43%), *Lactobacillus plantarum* (17.35%), *Lactobacillus fermentum*, (17.35%), *Lactobacillus casei subsp. casei* (9.18%), *Lactobacillus brevis* (5.10%), and least was *Lactobacillus casei subsp. rhamnosus* (4.08%) (Table-2 and 3, Fig 2).

Incidence in paneer samples was found to be highest for *Lactobacillus plantarum* (50.00%), followed by *Lactobacillus fermentum* (28.57%), and least 21.43% was *Lactobacillus casei subsp. casei*. (Table-2 and 3, Fig 2).

In dosa batter samples, incidence was highest for *Lactobacillus casei subsp. casei* (43.08 %), followed by *Lactobacillus plantarum* (29.23%), and least was for *Lactobacillus fermentum* (27.69%). (Table-2 and 3, Fig 2).

The statistical analysis of the data obtained revealed significant difference species of bacteria as well as food and dairy samples.

Table 2: Incidence of *Lactobacillus* spp. in food and dairy samples

Sample	Total no. of *Lactobacillus* isolates	Isolates of *Lactobacillus* spp.						
		Lactobacillus casei subsp. casei	*Lactobacillus plantarum*	*Lactobacillus fermentum*	*Lactobacillus brevis*	*Lactobacillus acidophilus*	*Lactobacillus bulgaricus*	*Lactobacillus casei subsp. rhamnosus*
Raw Milk	37	5 (13.52%)	11 (29.72%)	9 (24.32%)	5 (13.52%)	7 (18.92%)	-	-
Curd	98	9 (9.18%)	17 (17.35%)	17 (17.35%)	5 (5.10%)	21 (21.43%)	25 (25.51%)	4 (4.08%)
Paneer	42	9 (21.43%)	21 (50.00%)	12 (28.57%)	-	-	-	-
Dosa	65	28 (43.08%)	19 (29.23%)	18 (27.69%)	-	-	-	-
Total	242	51	68	56	10	28	25	4

Table 3: Identification of *Lactobacillus* spp. isolates in food and dairy samples

Characteristics		***Lactobacillus* isolates in Food and Dairy products**						
		Lactobacillus acidophilus	***Lactobacillus plantarum***	***Lactobacillus casei subsp. Casei***	***Lactobacillus fermentum***	***Lactobacillus delbrueckii subsp. bulgaricus***	***Lactobacillus brevis***	***Lactobacillus casei subsp. rhamnosus***
Colony morphology		Circular, Large, Smooth, Glistening	Circular, White, Glistening, convex	White and creamis, Smooth, Mucoid, Convex	Circular, White, Glistening, convex	White, creamish Smooth Mucoid Convex	Circular, White, Glistening, convex	White and creamish, Smooth, Mucoid, Convex
Gram reaction		+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cell morphology		Rods in pairs and chains	Short rods	Single rods and in chain	Short rods in small chains	Single rods or in chain	Short rods, (in pairs and chains)	Single rods (in chain)
Motility		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Catalase reaction		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase reaction		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Indol production		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Methyl Red test		+ve	+ve	+ve	+ve	+ve	+ve	+ve
Voges proskauer		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate utilization		-ve	-ve	-ve	-ve	-ve	-ve	-ve
Growth	15 ^{0}C	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	37 ^{0}C	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	45 ^{0}C	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose		A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^+G^-
Galactose		A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^-G^-	D	A^+G^-
Maltose		A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^-G^-	A^+G^-	A^+G^-
Manitol		A^-G^-	A^+G^-	A^+G^-	A^-G^-	A^-G^-	A^-G^-	A^+G^-
Xylose		A^-G^-	D	A^-G^-	D	A^-G^-	D	A^+G^-
Sorbitol		A^+G^-	A^+G^-	A^+G^-	A^-G^-	A^+G^-	A^-G^-	A^-G^-
Sucrose		A^+G^-	A^+G^-	A^+G^-	A^+G^-	A^-G^-	D	A^+G^-
Lactose		A^+G^-	A^+G^-	D	A^+G^-	A^+G^-	D	A^+G^-

-ve = (90 – 95 % strains Negative), +ve = (90 – 95 % strains Positive), D = (11 – 89 % strains positive)
A+ = Acid Positive, A^- = Acid Negative,
G+ = Gas Positive, G^- = Gas Negative

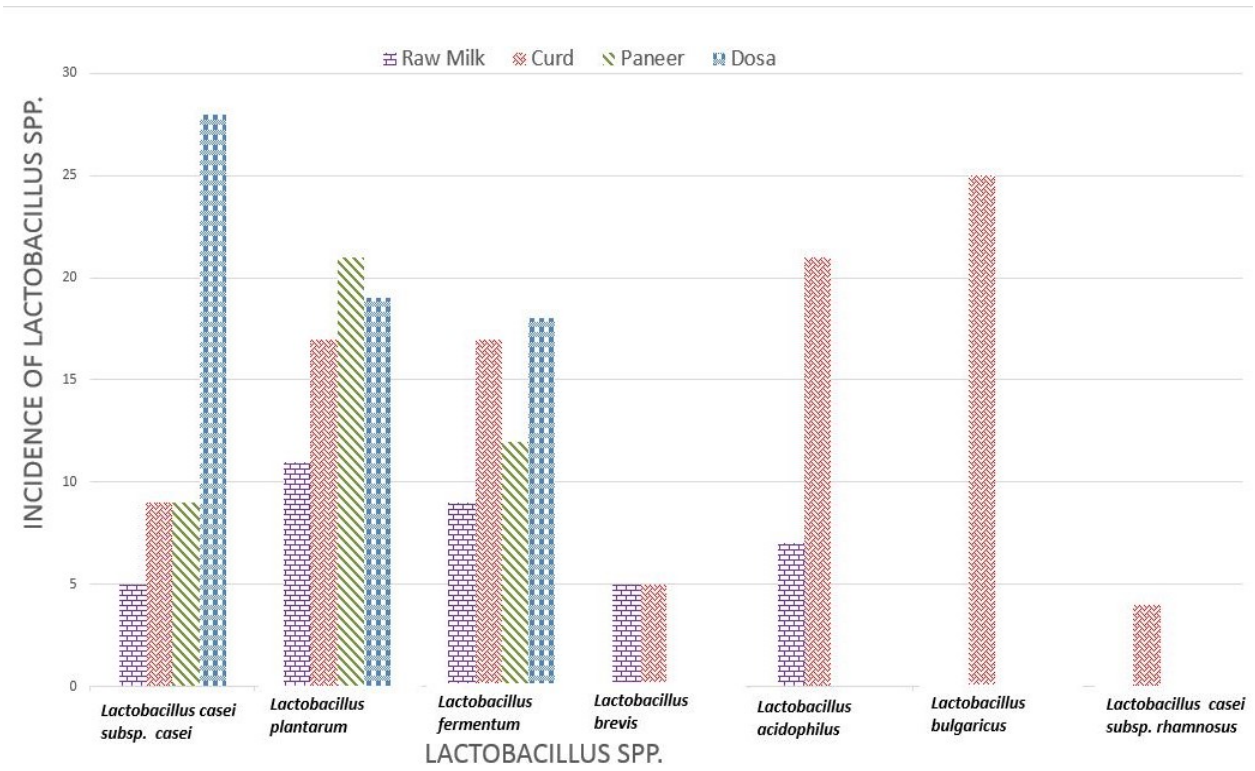

Fig. 2: Incidence of isolated *Lactobacillus* spp. food and dairy samples

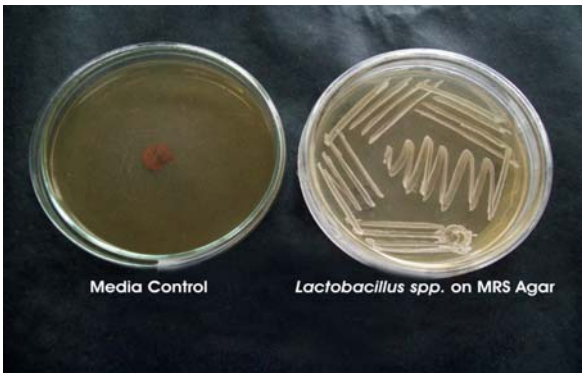

Plate 1: Growth of isolated *Lactobacillus* spp. on MRS Agar media

Incidence percentage of *Lactobacillus* spp.

Other researchers have also reported isolation of *Lactobacillus casei*, *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* from butter, paneer, milk, curd, yoghurt, yoghurt milk and pharmaceutical products (Anas *et al*., 2008; Sarany and Hemashenpagam 2011, Zdolec *et al*., 2011 and Nama *et al.,* 2013) [2, 17, 29, 12].

Other researchers also reported the incidence of *Lactobacillus acidophilus* between the range of 12-16%, similar to the present study (Aziz *et al.,* 2009; Singh and Sharma, 2009; Tambekar and Bhutada, 2010) [3, 18, 20]. Whereas, higher incidence 26.09% of *Lactobacillus acidophilus* is also reported from yoghurt, yoghurt milk and pharmaceutical products. (D'Aimmo *et al.,* 2007) [26].

Similarly*, Lactobacillus acidophilus* was also isolated, from the various type of samples like Paneer, Cheese, yoghurt, milk sausage, pickle, beer, fermented olives, chicken and mutton and environment waste materials. Malini Maria and Janakiraman (2012) [10]

Higher incidence percentage of *Lactobacillus delbrueckii* subspp. *bulgaricus* (47.82%) was reported from yoghurt, yoghurt milk and pharmaceutical products (D'Aimmo *et al.,* 2007) [26]. Another, study reports isolation of *Lactobacillus delbrueckii* from yoghurt (fresh curd). Morani Devi *et al*., (2013) [11].

Apart from the above stated findings other results were isolation of isolation of *Lactobacillus* spp., along with *Streptococcus thermophilus* and *Bifidobacterium* spp. from yoghurt, yoghurt milk and pharmaceutical products (D'Aimmo *et al.,* 2007) [26].

Similarly to the present results, another study by Anas *et al*. (2008) [2] also reported isolation and identification of *Lactobacillus* spp. namely *Lactobacillus paracasei* subspp. *Paracasei*, *Lactobacillus rhamnosus, Lactobacillus delbrueckii* subspp. *Lactis, Lactobacillus paraplantarum* and *Lactobacillus sakei* subspp. *sakei.* from Raw Goat's milk.

Similarly, in other studies, reported isolating *Leuconostoc* spp. along with *Lactobacillus paracasei* and *Lactobacillus curvatus; Pediococcus* spp. *Bifidobacterium* spp. and *Lactococcus* spp., *Lactobacillus delbrueckii, Lactobacillus rhamnosus, Lactobacillus pentosus* from the various type of samples like Paneer, Cheese, yoghurt, milk sausage, pickle, beer, fermented olives, chicken and mutton and environment waste materials.. (Zdolec *et al*., 2011; Malini Maria and Janakiraman 2012; Morani Devi *et al*., 2013 and Ahmed *et al.,* 2014) [10, 11, 29, 30].

Conclusion

Lactic acid bacteria are the natural microflora in food and dairy products. The results obtained during the present study reveals the incidence of lactic acid bacteria are present as the microflora in food and dairy products of. Lactic acid bacteria are the group of heterogeneous bacteria which are capable of undergoing homofermentative and hetro fermentation yielding lactic acid alone or lactic acid along with alcohol. These bacteria are used as starter culture in food and dairy industry. Apart from these major products it also produces some compounds like organic acids, hydrogen peroxide, diacetyl, bacterions which inhibits the growth of pathogenic and spoilage causing bacteria..

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