ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM NATURALLY FERMENTED FOOD SAMPLES HAVING ANTIBACTERIAL ACTIVITY *

R. Geetha,[1] **C.T. Sathian**[2] **and A.J. Flamy**[3]
Department of Dairy Science
College of Veterinary and Animal Sciences
Mannuthy-680 651, Thrissur, Kerala

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Abstract

Lactic acid bacteria (LAB) prevailing in natural environment is usually considered as a powerful tool for commercial purpose, related to food technology. In this study various genera of LAB existing in naturally fermented food materials and showing antibacterial activity against Streptococcus agalactiae (S.agalactiae), a common mastitis pathogen, were isolated and identified. Among the 42 LAB isolates screened, 12 LAB isolates showed antibacterial efficiency (C-2, C-4, C-5, D-4, T-5, T-6, CR-1, CR-2, CR-3, CU-2, CU-3 and M-2) against S.agalactiae. A significantly high antimicrobial efficiency against test pathogen was shown by treatments T-5, CR-2 and CR-3, isolated from tomato and carrot. The LAB isolates were found to be belonged to five different genera viz., Streptococcus, Lactobacillus, Enterococcus, Pediococcus and Leuconostoc.

Keywords: *Lactic acid bacteria, fermented food*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*

Lactic acid bacteria are a group of Gram positive bacteria, facultatively anaerobic, non-spore forming, cocci or rods, which produce lactic acid as the major end product due to fermentation of carbohydrates. This group of bacteria is usually seen associated with nutrient rich habitats containing simple sugars. The strains of LAB existing in its natural environment are extremely diverse and are considered to be more powerful in their prospective especially in biotechnological aspects as bacteriocin producers and nutritional characteristics as probiotics. They can be exploited commercially to develop new antimicrobial formulations. But reports are negligible regarding the biodiversity of various genera of LAB and their antimicrobial efficiency. Hence the present study has been undertaken.

Materials and Methods

A total of 52 samples of naturally fermented food items such as curd, dosa-dough, partially decayed vegetables and fruits (tomato, carrot, cucumber, green chilli, banana and mango) collected from various house-holds and local markets of Thrissur, Kerala, were screened for the study. Partially decayed vegetables and fruits were washed in lukewarm water and peeled. Ten gram each of the above items were aseptically grated, emulsified in sterile peptone water (one per cent) and used for further screening. Serial dilutions of all the samples were prepared by adding one gram of the sample to nine ml of sterilized peptone water. One ml aliquot of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}dilutions were used for preparing the pour plates. For the isolation of LAB from the samples, the method described

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1. Assistant Professor
2. Professor & Head
3. Teaching Assistant

by Marth and Steele (2001) was followed. de Man, Rogosa and Sharpe (MRS) agar was the selective media used for culturing LAB. The plates were incubated anaerobically at 37°C for 18 h. in anaerobic jars. Isolates were further purified by streak plating on MRS agar plates. Primary screening based on cultural, morphological and biochemical characteristics was performed on the selected 52 bacterial isolates according to the methods described by Holt *et al*. (1994), for primary identification of LAB. The lactic acid bacterial isolates were tested for their antimicrobial efficiency against clinical isolates of *S.agalaciae,* using filtered cell free supernatants of LAB, containing the extracellular metabolites. The supernatant was filtered through a 0.22 µm pore-size Poly Ether Sulfone (PES) membrane syringe filter for bacterial sterilization and inhibitory spectrum of crude culture supernatants were determined by Agar-Well diffusion assay as described by Tagg *et al*. (1976). Those isolates showed positive test results were subjected to secondary identification process (Holt *et al*., 1994) *viz*., growth at differential temperatures (10°C and 45ºC) in MRS agar, carbohydrate fermentation patterns, arginine hydrolysis, aesculine hydrolysis, salt tolerance, growth at acidic or alkaline pH and various sugar fermentation test to detect the biodiversity of LAB existing in their natural environment.

Results and Discussion

Among the samples screened (Fig.1) for isolating LAB, different colony morphologies were observed. a) smooth white coloured colonies, 0.1 to 0.5mm in diameter b) convex and smooth colonies with one to two millimeter diameter and with entire colony margins c) pinpoint colonies in the subsurface layer of the media. Picture depicting colony morphology of LAB is given in figure 2.

Out of the 52 cultures screened, 42 isolates were identified as belonging to the group of LAB based on preliminary identification tests. Primary tests recognized LAB as Gram positive cocci or rods (Fig.3) microaerophilic, where growth was observed just below the surface of the media. Isolates were non-motile in nature, non spore forming, catalase negative, oxidase negative and showed fermentative reaction for OF test. Nine out of 42 isolates were bacilli and the remaining isolates were cocci. Isolates were grouped based on their morphological features and cell arrangements. Results of primary identification tests are presented in Table1.

When antibacterial efficiency of crude culture supernatant was tested against clinical isolates of *S.agalactiae,* only 12 LAB isolates showed positive test results among the 42 isolates screened (Table.2). When the overall performance of all the 12 isolates was statistically analysed, a significant difference was observed in the activity spectrum against *S.agalactiae*. The samples T-5, CR-2 and CR-3 obtained from crude culture supernatants of

Table1. Primary identification tests for various LAB isolates

Sources	No. of isolates	Grams reaction	Motility	Spore forming ability	Catalase test	Oxidase test	OF test
Tomato	5	+ve	-ve	-ve	-ve	-ve	F
Carrot	4	+ve	-ve	-ve	-ve	-ve	F
Cucumber	5	+ve	-ve	-ve	-ve	-ve	F
Green chilli	2	+ve	-ve	-ve	-ve	-ve	F
Banana	5	+ve	-ve	-ve	-ve	-ve	F
Mango	4	+ve	-ve	-ve	-ve	-ve	F
Curd	11	+ve	-ve	-ve	-ve	-ve	F
Dosa-dough	6	+ve	-ve	-ve	-ve	-ve	F

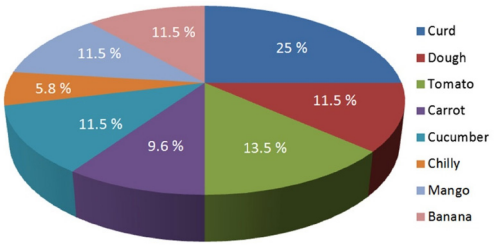

Fig.1 Data on samples screened

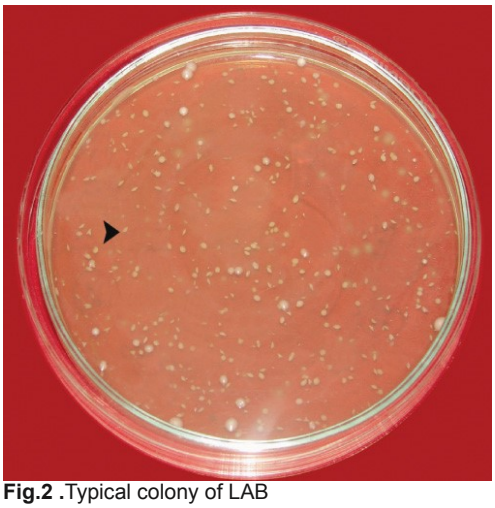

Fig.2 .Typical colony of LAB

Isolate code	***S. agalactiae*** Zone of inhibition (mm diameter) *Mean±SE
C-2	8.66±0.66 abc
C-4	8.00±0.00 abcd
C-5	7.66±0.33 abcd
T-5	9.33±0.66a
T-6	5.66±0.66 e
CR-1	7.00±0.57 bcde
CR-2	9.00±0.57ab
CR-3	9.00±0.57 ab
D-4	7.66±0.88 abcd
M-2	6.33±0.33 de
CU-2	6.33±0.33 de
CU-3	6.36±0.33cde
LL (control)	6.33±0.33de

Table 2. Antimicrobial spectrum of crude extr act from 12 selected LAB isolates
The difference is significant (p≤0.05);
** Mean of six replications*

LAB from tomato and carrot showed maximum antimicrobial spectrum with a zone of inhibition of 9.33±0.66 mm, 9.00±0.57mm and 9.00±0.57 mm diameter respectively. Findings from this assay proved that, inhibitory substance secreted by different LAB isolates has their specific inhibition spectrum. Since the CCE obtained from three different LAB strains, designated as T-5, CR-2 and CR-3 showed remarkable antimicrobial potential against *S.agalactiae*, they were considered as probable antimicrobial substance producers. The 12 isolates which showed positive test results were differentiated up to the genus level on the basis of biochemical tests (Table 3a and 3b).

Isolates of bacilli which were unable to grow at 10ºC, but competent to grow successfully at 45ºC were identified as *Lactobacillus,* since the only bacilli belonging to the group of LAB that can grow at 45ºC is *Lactobacillus.* The genera, *Leuconostoc* were separated from other isolates of cocci by their heterofermentative nature and their inability to produce ammonia from arginine, since important characteristic used in the differentiation of LAB genera is the mode of glucose fermentation. According to Sharpe (1979), LAB can be grouped into two, the homofermentatives, converting glucose to lactic acid and the heterofermentatives, fermenting glucose to lactic acid, ethanol and CO_2.

According to Daeschel *et al.* (1987) *Leuconostocs* are also important in spontaneous vegetable fermentations, especially sauerkraut, where they often initiate the lactic fermentation. The homofermentative cocci that grew at 10ºC and 45ºC and tolerated pH 9.6 were identified as genus *Enterococcus. Enterococci* are not considered to be of particular significance in food industry, as the normal habitat of many *Enterococci* is the intestine of human and animals. However, cultures of *Enterococcus faecium* and *Enterococcus faecalis* have been used as probiotics (Fuller, 1986). The possibility of the isolates to be *Tetragenococcus* was ruled out as it could not tolerate 18 per cent sodium chloride. Based on homofermentative reaction, tetrad forming ability and observing salt tolerance, LAB belonging to genera *Pediococcus* was identified. Certain LAB isolates appeared as chain in their morphology, unable to grow at 10ºC, but capable to grow effectively at 45ºC, able to tolerate 6.5 per cent salt, could grow at acidic (pH 4.0) were identified to be belonging to the genera of *Streptococcus.*

The results of the current study agree with the observation of Axelsson (2004). According to him, classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations and acid or alkaline tolerance. According to Simpson and Taguchi (1995) *Pediococci* and *Tetragenococci* constitute the tetrad forming LAB, and *Pediococcus* can be demonstrated as the only acidophilic, homofermentative, LAB that divide in two perpendicular directions to form tetrads. According to Garvie (1986) *Tetragenococci* can show extreme salt tolerance (18per cent NaCl), which distinguish them from other LAB. According to Hardie (1986) the thermophilic nature differentiates *S. thermophilus* from most other cocci included under the genera of LAB.

In the present study the 12 LAB isolates which showed antibacterial potential, comprised of five genera (Table 4), three isolates of *Lactobacillus,* four isolates of *Streptococcus,* three isolates of *Leuconostoc* and one isolate each of *Pediococcus* and *Enterococcus.*

Various genera of LAB existing in naturally fermented food materials, having

Table 3a. Secondary biochemical tests for identification of lactic acid bacterial isolates

Morphological & Biochemical Characteristics	C2	C4	C5	T5	T6	CR1	CR2	CR3	CU2	CU3	M2	D4
Shape	C	C	B	B	C	C	C	C	B	C	C	C
Gas production From glucose	-	-	-	+	-	-	-	-	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 10°C	-	-	-	-	-	-	-	+	-	-	-	-
Growth in 6.5 % NaCl	+	+	+	+	+	+	-	+	-	+	-	-
Growth in 18 % NaCl	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH 9.6	-	+	-	-	-	-	-	+	-	-	-	-
Arginine hydrolysis	+	-	-	-	+	+	+	+	-	-	-	-
Aesculine hydrolysis	-	-	-	+	-	-	-	-	-	-	-	-
Citrate utilization	-	+	-	-	-	-	-	-	-	+	+	+
Test for acid tolerance	+	+	+	+	+	+	+	+	+	+	+	+

(+)= Positive reaction, (-) =Negative reaction,

Table 3b. Sugar fermentation tests for identification of LAB

Sugars	C2	C4	C5	T5	T6	CR1	CR2	CR3	CU2	CU3	M2	D4
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	+	+	+	-	-	-	-	+	+	+	+
Fructose	-	+	+	+	-	-	+	-	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	-	+	+	+	+	+	+	+	+	+
Trehalose	-	+	+	-	-	-	-	-	-	+	+	+
Melibiose	+	+	-	+	+	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	-	+	+	+	+
LArabinose	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	-	+	+	+	+	+	+	+	+	+	+

(+)= Positive reaction, (-) =Negative reaction,

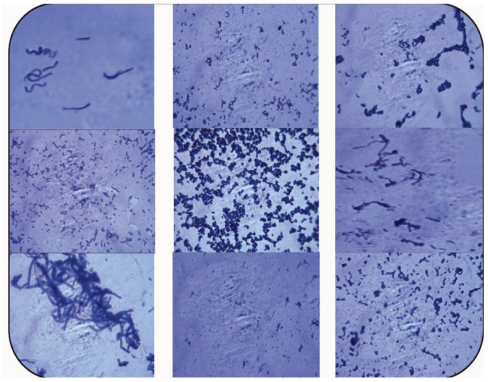

Fig.3. Morphology and Grams reaction of selected LAB

antimicrobial efficiency were isolated in current research. The selected isolates were belonged to five different genera of LAB. Genus *Streptococcus, Lactobacillus* and *Leuconostoc* were the predominant genera existed in the screened samples. The current study indicates that biodiversity of LAB strains existing in natural habitat is a valuable resource for probiotics and bacteriocin producers. The phenotypic characteristics adopted in the present study were useful as a starting point for more sophisticated tests. A combination of phenotypic, chemotaxonomic and genotypic methods can be carried out for more precise identification of isolates at their strain level.

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Sl.No	Samples	Genera of Lactic acid bacteria
1	Curd	*Streptococcus*
2		*Pediococcus*
3		*Lactobacillus*
5	Tomato	*Lactobacillus*
6		*Streptococcus*
6	Carrot	*Streptococcus*
7		*Enterococcus*
8		*Streptococcus*
9	Dosa-dough	*Leuconostoc*
10	Mango	*Leuconostoc*
11	Cucumber	*Lactobacillus*
12		*Leuconostoc*

Table 4. Various genera of Lactic acid bacteria differentiated by secondary identification tests

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