Isolation and Identification of lactobacilli from raw milk samples obtained from Aarey Milk Colony

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Abstract- Milk and milk products have been used by human population since ancient ages and have been a well known source of lactobacilli. The present study is directed towards the study of prevalence, isolation and identification of Lactobacillus species in milk. Milk samples were collected from the cow and buffalo sheds of a local dairy in the Aarey Milk Colony which is a major supplier to the city of Mumbai. A total of 40 milk samples were obtained and 163 colonies were isolated from them. These colonies were subjected to characterization by standard microbiological methods. On the basis of physiological tests and sugar utilization pattern, all the 163 isolates were confirmed to belong to the genus Lactobacillus: L.fermentum (48%), L.acidophilus (34%), L.viridescens (8%), L.brevis (5%), L.gasseri (4%) whereas two isolates could not be identified upto the species level. The results indicate that L.fermentum is predominant in the milk obtained from this sector.

Index Terms- Characterization; Isolation; Identification; *Lactobacillus fermentum;* Milk samples

I. INTRODUCTION

The genus *Lactobacillus* consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic organisms.[7]Among lactic acid bacteria (LAB), lactobacilli present a diverse group of homofermentative and heterofermentative species which can produce a variety of substances such as lactic acid, ethanol, formic acid, acetone, hydrogen peroxide, diacetyl etc.[15] Additionally they also produce a variety of exopolysaccharides (EPS). The EPS vary in composition, structure and size with the producing organism. Although tasteless, the EPS from LAB increases the residence time the milk products spend in the mouth and hence, imparts an enhanced perception of taste.[1] Additionally, exopolysaccharides of microbial origin are gaining importance because of their potential applications in food industries as texturizers, viscosifiers, emulsifiers and syneresis-lowering agents, for their pseudoplastic rheological behavior and water binding capacity.[1, 6]

Lactobacilli possess two major advantages in that they are GRAS and are probiotics[2]. The LAB are widely used in manufacturing fermented food products and can also be safely used for medical and veterinary applications [3].

Lactic acid bacteria (LAB) and physiologically related group of gram-positive bacteria have also been known to produce a variety of antimicrobials called bacteriocins. Bacteriocins are generally defined as extracellular peptides or proteins exhibiting bactericidal activity against species closely related species or strains. Although bacteriocins may be found in many Gram positive and Gram-negative bacteria, those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives [4]. Lactic acid bacteria and their metabolites have been shown to play an important role in improving microbiological quality and shelf life of many fermented food products and provide a good example of bio preservation [5].

In the food industry, LAB is widely used as starter cultures and has been cited to be part of human microbiota. In raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer as they strongly determine the flavor, texture and the nutritional value of the food and feed products.

From the health point of view, ingestion of live cells of certain strains of lactobacilli in adequate amounts is believed to confer several beneficial physiological effects on the host amongst these is the maintenance of a healthy and equilibrated intestinal flora and enhanced resistance to intestinal infections.[7] Due to the immense amount of varied products and their diversified applications, LAB pose as an avenue which can be exploited with both health and economic benefits.

The present study deals with the isolation and identification of lactobacilli from various milk samples obtained from Cows and Buffaloes harboured in the sheds of the Aarey Milk Colony and which provide an important channel of milk supply throughout the eastern and the western suburbs of Mumbai.

II. MATERIALS AND METHODS

COLLECTION OF MILK SAMPLES

Milk samples were collected in sterile containers at the sheds and were transported to the lab in temperature controlled boxes. Further analyses of the samples were carried out at the lab.

ISOLATION AND IDENTIFICATION OF LACTOBACILLUS

Appropriate dilutions of the collected milk samples were made in normal saline and pour plated on MRS agar and incubated at 37°C anaerobically for 24 to 48 hours[9]. At the end of 48 hours, when the colonies became predominant, morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking. Colonies showing typical characteristics of lactobacilli on agar surface were picked up randomly and transferred into MRS broth for further enrichment. Further, their purity was checked on MRS agar.

The pure isolates were subjected to identification as per the Bergey's Manual of Determinative bacteriology and open source softwares named PIBWin and IDENTAX. Macroscopic appearance of all the colonies was examined for cultural and morphological characteristics. Size, shape, colour and texture of the colonies were recorded.

The isolates were stained by Gram's method and examined under microscope for purity and those isolates readily identified as Gram positive rods and catalase negative were included for further characterization[13] which included cytochrome oxidase; growth at 15°C and 45°C; acid production from carbohydrates (1 % w/v) - L-arabinose, D-fructose, cellobiose, , esculin, lactose, maltose, D-galactose, melezitose, melebiose, mannitol, D-mannose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose in MRS broth devoid of glucose and beef extract with phenol red as indicator; production of acid and gas from 1 % glucose (MRS broth without beef extract); methyl red and Voges-Proskauer test in MRVP medium; production of ammonia from arginine; nitrate reduction in nitrate broth; indole production in tryptone broth and growth on acetate agar.[10,11,12]

MEDIA

The strains were maintained on MRS medium with the following composition: peptone 1%, yeast extract 0.5%, beef extract 0.5%, glucose 2%, dipotassium phosphate 0.2%, dibasic ammonium citrate 0.2%, sodium acetate 0.5%, magnesium sulphate 0.01%, manganese sulphate 0.005%, Tween 80 (0.1%) and agar 3% (for solid medium).

Catalase test was performed with the help of hydrogen peroxide.(3% H_2O_2 reagent grade).

Carbohydrate utilization was checked in 1% (w/v) MRS broth containing the specific sugar and devoid of glucose and beef extract containing phenol red as indicator

Acid and gas production from glucose was checked with the help of inverted Durham's tube containing 1% (w/v) glucose in MRS broth without beef extract.

MR-VP tests were performed in MRVP broth. For MR test, methyl red was used as a reagent whereas for VP test alpha naphthol and KOH were used as reagents.

Nitrate test was performed in Nitrate broth and tested with alpha- naphthylamine and sulphanilic acid.

Indole test was performed in tryptone broth and tested with the help of Kovac's reagent.

Citrate test was performed on Simon's Citrate agar.

III. RESULTS

IDENTIFICATION

The preliminary investigations included macroscopic analysis, microscopic analysis (Gram-positive bacilli), lactic acid biosynthesis, endospore test, milk coagulation activities and the negative catalase reaction permitted the classification of the working bacterium into the *Lactobacillus* genus. Microscopically they were Gram-positive rod shaped, non- motile, catalase negative and absence of endospore. The isolates were tolerant to a range of salt concentrations (1-9%) and also have the ability to coagulate milk. All these key characteristics helped to classify the isolates as lactobacilli.

BIOCHEMICAL TESTS

Identification upto the species level was carried out with the help of biochemical tests given in Bergey's Manual of Determinative bacteriology whereas open source softwares like IDENTAX and PIBWin were used to aid the identification and help develop an identification scheme[8,11,14](Table 1)

No	*Lactobacillus* spp	Morphology	Growth at			Acid and gas from glucose	NH_3 from arginine	Sugar fermentation													
			15 °C only	45 °C only	15 and 45 °C			Arabinose	Cellobiose	Mannitol	Mannose	Melebiose	Raffinose	Ribose	Salicin	Lactose	Melezitose	Rhamnose	Sorbitol	Xylose	Trehalose
1.	*L. plantarum*	SR	+	-	+	-	-		+	+	+	+	+	+	+						
2.	*L. brevis*	SR	+	-	+	+	+	+						+		+	-	-	-	+	
3.	*L. divergens*	SR	+	-		+	+		+		+	-	-		+	-				+	+
4.	*L. gasseri*	SR	-	+		-	-	-	+		+			-	+			-	+		+
5.	*L. rhamnosus*	SR	-	-	+	-	-		+	+	+	-			+	+	+		+	+	+
6.	*L. fermentum*	SR			+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
7.	*L. viridescens*	SR			+			-	+	+	+	-	-	-	-	+	+	-	-	-	+
8.	*L. farciminis*	SR			+		+	-	+	+	+	+	-	+	+	-	+	-	+	+	+
9.	*L. buchneri*	SR	+			+	+	-	+	-	-	+	-	+	-	+	-	-	-	+	-
10.	*L. acidophilus*	SR	-	-				+	+	+	+	-	-	-	+	+	+	-	-	+	+
11.	*L. alimentarius*	C	+					+	+	+	+	+	-	+	+	-	+	-	+	+	+
12.	*L. animalis*	C	-	-				+	+	+	+	+	+	-	+	+	-	+	+	-	+
13.	*L. reuteri*	R			+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	+	-

Table 1: Scheme used for the identification of lactobacilli[8]

IV.DISCUSSION

Milk and milk products have always been an important source exhibiting a plethora of lactobacilli. A vast majority of efforts are channelized on isolating lactobacilli and exploiting them for health related benefits. The present study was aimed at isolating lactobacilli from raw milk samples obtained from a local dairy in the Aarey Milk Colony which is an important milk supplier to the city of Mumbai. A total of 163 isolates were isolated and identified from the 40 milk samples obtained from the dairy. These isolates were maintained on MRS medium and subcultured as required. After confirming the purity of the isolates, they were subjected to microscopic and biochemical analysis. Those isolates which appeared to be rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic, were confirmed to belong to the genus lactobacillus. These isolates were further subjected to biochemical analysis and sugar fermentation patterns according to the Bergey's Manual of Determinative bacteriology and also with open source softwares like IDENTAX and PIBWin. Out of the 163 isolates obtained, 78 were found to belong to *L.fermentum*, 56 were found to belong to *L.acidophilus*, 12 were found to belong to *L.viridescens*,8 were found to belong to *L.brevis*, 7 were found to belong to *L.gasseri* whereas two isolates could not be identified upto the species level. These results indicate that *L.fermentum* is predominant in milk obtained from this sector followed by *L.acidophilus*(Figure1).

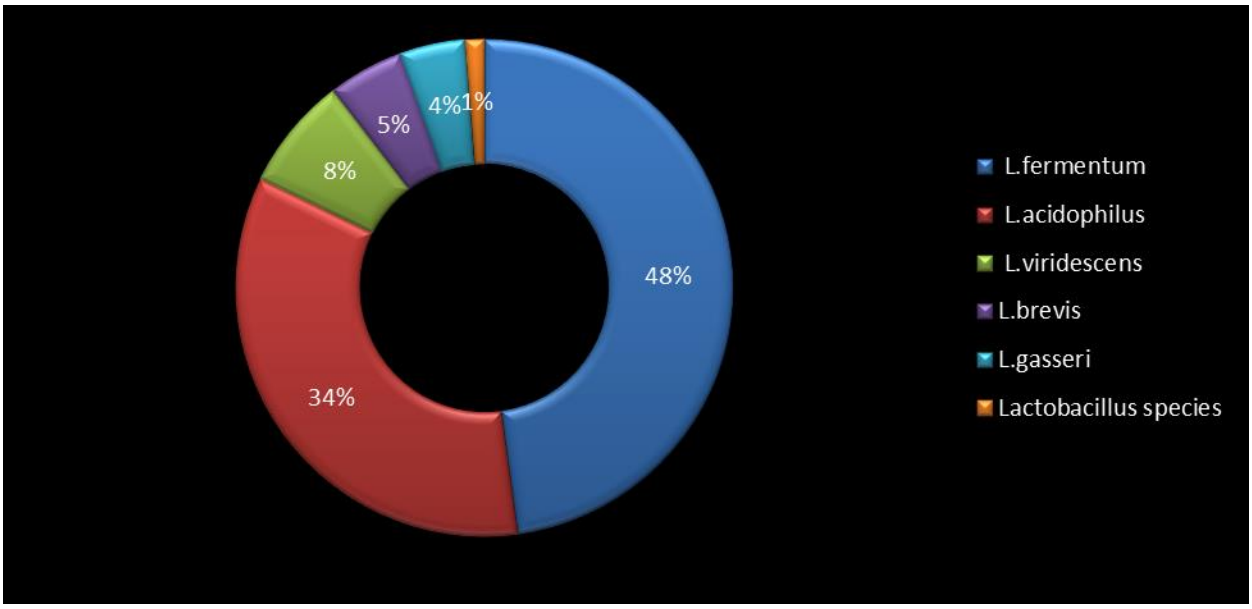

Figure 1: Percentage of lactobacillus species amongst the isolates.

V.CONCLUSION

The results of this study indicate that cow and buffalo milk exhibit a wide diversity of lactobacilli occurring naturally in the milk and can be used as a potential natural source to isolate a variety of strains of lactobacilli. According to the current study, the results indicate that *L.fermentum* is predominant in milk obtained from this sector of the Aarey Milk Colony and may play an important role in the quality of the milk. Since some strains of lactobacilli possess potential probiotic and therapeutic properties including anti-inflammatory and anti-cancer activities,[16,17,18] as well as other features of interest, these isolates can be further screened for their probiotic and related properties and exploited for health and economic benefits.

VI.REFERENCES

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