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Exploring Indigenous *Lactobacillus* Species from Diverse Niches for Riboflavin Production

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ABSTRACT

Background: Riboflavin has received rather less attention in the past years, but interest is increasing because it is recognized as an essential component of cellular biochemistry, it is one of the vital vitamins required by human, bacteria, animals and plants. Today, bio production of riboflavin is competing with chemically synthesized one. Lactobacilli are well reckoned for their technological and probiotic significance and their exercise in dairy products for trade and industry. **Materials and Methods:** The present study was carried out to isolate and characterize riboflavin lactobacilli from various dairy and non-dairy sources of Indian origin. The isolates were subjected to phenotypic and genotypic characterizations followed by partial 16S rDNA sequencing. **Results:** As many as 40 isolates were identified as Lactobacilli and among them 14 were identified as *L.plantarum*, 5 as *L. fermentum*, 5 as *L. delbrueckii subsp. bulgaricus*, 4 *L. brevis*, 2 *L. pentosus* and 1 isolate as *L. mucosae.* Further isolates were screened for riboflavin production by chemical assay method and among all the tested isolates, 2 isolates viz. KTLF1 (*L.fermentum*) and KTLP13 (*L.planatrum*) have been observed to produce appreciable amount of riboflavin in MRS and RAM. **Conclusion:** These riboflavin producing lactobacilli can be used as a model for the development of strains that have the potential to produce an essential vitamin *in situ* which would contribute significantly to the functional value of certain fermented foods.

Key words: Health benefits, Lactobacilli, Nutrition, Probiotic, Riboflavin, Vitamin.

INTRODUCTION

These natural sources of vitamins represent a consumer-friendly alternative to fortification using chemically synthesized pseudo-vitamins, and it would allow the production of vitamin enriched foods.[1] Despite of the presence of most of the vitamins in a variety of foods, nevertheless human vitamin deficiency persists in many countries, due to insufficient food intake and unbalanced diet.[2] Metabolically, riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both of which act as electron carriers in oxidation-reduction reactions, functioning as coenzymes for hundreds of FMN- or FAD-dependent enzymes called flavoproteins. The majority of flavin-enzyme interactions involves the ribityl side chain, which represents the position where FMN and FAD differ, thus explaining the specificity of enzymes for either of these two cofactors.

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Lactobacilli are the most vital groups of acid producing bacteria in the food industry for their use as starter culture for dairy products as well as are gaining increasing attention in the area of probiotics due to their potential health benefits to consumer.[3] Being the most dominant microflora of fecal samples, LAB may exist as commensal organisms, adapted to intestinal environment.[4] After the discovery of beneficial effects of LAB on human health the new doors have been opened for health and pharmaceutical industries to explore the beneficial effects of LAB. Intestinal microbiota has also been shown to produce short chain fatty acids (SCFA), conjugated linoleic acid (CLA), essential amino acids, group B vitamins and vitamin K, contributing to the well-being of a host.[5] LAB find enormous applications at industrial level for the biosynthesis of a number of compounds as metabolic end products or secondary metabolites (Figure 1.0). They produce a range of metabolites including B vitamins such as riboflavin (B2), folate (B11), and cobalamine (B12), and low-calorie sugars such as mannitol and sorbitol, exopolysaccharides, diacetyl, and L-alanine. The fermentation based methods are masking the area of chemical synthesis for vitamin production due to economic and environmental consideration which is a growth enhancing factor in the area of nutrition. Riboflavin has been traditionally synthesized for food and feed fortification by chemicals means but past decade has witnessed a surge in information about commercial biotechnological processes. Hence this project was aimed at the isolation, identification and characterization of riboflavin producing lactobacilli from various niches. In the present study, we have targeted *Lactobacillus* species from dairy and non-dairy sources for their phenotypic and genotypic characterization followed by screening for riboflavin production which holds the promise for their possible future applications as novel functional strains.

MATERIALS AND METHODS

Cultivation Conditions and Phenotypic and genotypic characterization

Human faeces, sour dough, (dosa) batter, fermented bamboo shoots and samples of household curd (dahi) were collected from diverse niches of India. The samples were enriched in MRS broth (Himedia Laboratories Pvt. Ltd, Mumbai, India) followed by streaking and pour plating on MRS agar under anaerobic conditions. Isolates were phenotypically assigned to the genus level on the basis of: cell morphology, Gram staining, catalase reaction and carbohydrate fermentation patterns (as determined using API 50 CHL system). Isolates were phenotypically assigned to the genus level and were further subjected to molecular typing methods. *Lactobacillus* genus-specific and species specific primer were used for the confirmation of putative *Lactobacillus* isolates. The purified amplified PCR products (20 µL) were sent for sequencing using primer 7F[6] to obtain partial sequence of the 16S rDNA followed by the construction of phylogenetic dendrogram as shown in Figure 1 by using unweighted pair-group method with arithmetic averages (UPGMA) of MEGA4.0 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Arizona State University, Phoenix, AZ, USA).

Chemical assay method for screening of riboflavin producing *Lactobacillus* species

The riboflavin producing ability of isolates was estimated as described.[7] In brief, 0.8 ml of culture broth was added to 0.2 ml of 1 M NaOH. A 0.4-ml vol. of the resulting solution was neutralized with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0), and absorbance at 444 nm was measured. The riboflavin concentration was calculated by using an extinction coefficient of 1.04×10^{-2} $M^{-1}cm^{-1}$. Standard curve was plotted using known concentration of riboflavin (Sigma). The MRS and Riboflavin assay medium (RAM) (Chemically defined medium) were used for the screening of riboflavin producing ability of lactobacilli isolates. As riboflavin producing ability of isolates is strain specific and the level of riboflavin production varies from strain to strain. The isolates were screened for riboflavin production after 24 hrs.

RESULTS

Isolation, identification and characterization of *lactobacillus species*

Initially screened on the basis of microscopic examination and catalase test, 40 isolates appeared to belong to the *Lactobacillus* genus and were further characterized by biochemical and molecular methods. All these isolates conformed to the general phenotypic characteristics of genus *Lactobacillus* by API method. These isolates were observed to be invariably rod shaped cells, gram-positive, catalase-negative, non motile, facultative anaerobic bacteria and were able to grow at 15°C, 2%, and 4% NaCl. The generic status was further confirmed by PCR as all the isolates showed amplification product of expected size (250 bp) using genus-specific primers. Further, partial sequencing of 16S rDNA was performed for representative isolates after screening for in vitro probiotic properties. The sequences obtained from the isolates were compared to those of reference strains held in GenBank. Sequence

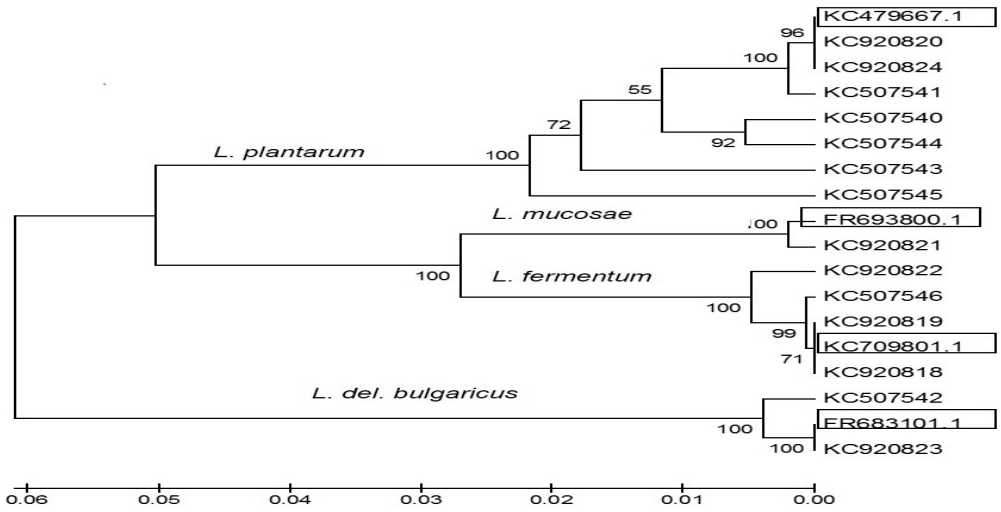

Figure 1: Phylogenetic analysis of all isolates with reference strains of *Lactobacillus* using 16S r DNA sequences along with standard sequences obtained from database constructed using the UPGMA method by Mega 4.0 software. The boxed accession numbers are taken as a reference from NCBI genbank Database for similarity check

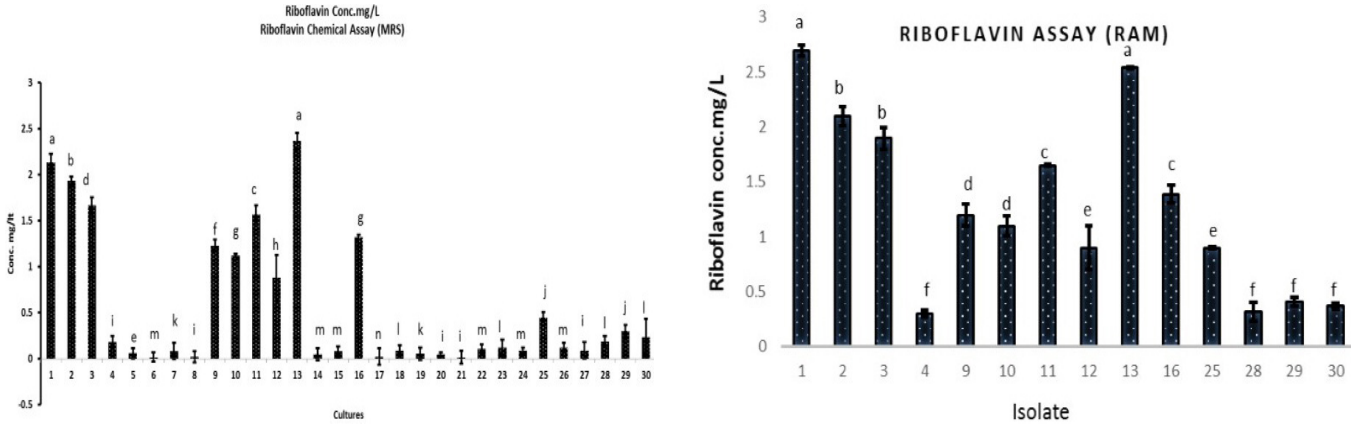

Figure 2: Graphical representation of comparison of Riboflavin producing ability of different *Lactobacillus* isolates in MRS and RAM respectively (Figure 2.1 and Figure 2.2)

data was generated for selected isolates on the basis of their origin, probiotic and technological properties were submitted to the GenBank. The phylogenetic analysis (Figure 1) together with species specific PCR profile has showed the diversity in *Lactobacillus* species with respect to their source of origin. Among all the isolates obtained from various sources, *L. plantarum* was the predominant flora. The presumptive *Lactobacillus* isolates were initially identified on the basis of their Analytical profiling index (API) by using API-50CHL kit .The ability of fermenting various sugars is a major biochemical characteristic for differentiating *Lactobacillus* species from other closely related species.[8] For the screening of riboflavin producing isolates, lactobacilli were grown in MRS and RAM (devoid of riboflavin) and, after the entry in the stationary phase, the supernatant was analyzed for riboflavin content. Only those isolates which harbour the riboflavin biosynthesis genes can grow in RAM which is devoid of riboflavin. Although a requirement for riboflavin is very rare among bacteria, it is known that it is an essential growth factor for *Enterococcus faecalis, Streptococcus pyogenes, Listeria monocytogenes*, and some lactobacilli.[9] The biosynthetic deficiency correlates with the absence of riboflavin biosynthetic genes in the genomes of these organisms.[10] Out of 40 isolates, only 14 isolates were able to survive in RAM medium which is devoid of riboflavin. The isolates were found to accumulate riboflavin into the medium to different extents from 0.2 mg to 2.7 mg as shown in Figure 2.1 and Figure 2.2. The levels of riboflavin in the remaining strains were also assayed after 24 hours and the best riboflavin producing strains were identified as isolates viz. KTLF1 (*L. fermentum*) and KTLP13 (*L. planatrum*) belonging to the genus *Lactobacillus* sp. The strain KTLF1 and KTLP13 produced 2.13 mg/L, 2.36 mg/L of riboflavin in MRS and 2.71 mg/L, 2.54 mg/L

Table 1: Riboflavin production (mg/L) by different lactobacilli strains

Nomenclature	Isolates	A_{600} at 24 h	A_{444} at 24 h	Concentration of riboflavin in mg/L at 24 h (MRS)	Concentration of riboflavin in mg/L at 24 h (RAM)
1	KTLF1	0.6745	0.747	2.132±0.097	2.71±0.05
2	KTLF2	0.586	0.664	1.934±0.045	2.14±0.09
3	KTLF3	0.469	0.618	1.665±0.087	1.90±0.11
4	KTLF4M	0.5795	0.282	0.183±0.067	0.33±0.03
5	KTLF5	0.386	0.589	0.058±0.056	No growth
6	KTLF6	0.775	0.159	0.015±0.05	No growth
7	KTLF7	0.5645	0.191	0.082±0.09	No growth
8	KTLF8	0.473	0.2845	0.023±0.06	No growth
9	KTLF9	0.529	0.564	1.23±0.067	1.21±0.10
10	KTLF10	0.548	0.4345	1.12±0.02	1.10±0.09
11	KTLF11	0.435	0.646	1.568±0.097	1.65±0.01
12	KTLF12	0.48	0.3525	0.879±0.25	0.97±0.21
13	KTLF13	0.634	0.734	2.365±0.087	2.54±0.01
14	KTLF14	0.423	0.15	0.045±0.067	No growth
15	KTLF15	0.321	0.133	0.08±0.056	No growth
16	MTCC8711	0.6025	0.46	1.32±0.025	1.39±0.08
17	KTLF17	0.3835	0.1015	0.023±0.09	No growth
18	KTLF18	0.4745	0.1885	0.087±0.06	No growth
19	KTLF19	0.562	0.1915	0.056±0.067	No growth
20	KTLF20	0.631	0.2985	0.049±0.02	No growth
21	KTP21	0.505	0.2825	0.015±0.07	No growth
22	KTP22	0.599	0.1505	0.108±0.045	No growth
23	KTP23	0.4285	0.164	0.123±0.087	No growth
24	KTP24	0.497	0.1555	0.089±0.032	No growth
25	KTLF25	0.6015	0.224	0.45±0.056	0.980±0.01
26	KTLF26	0.3975	0.1535	0.123±0.05	No growth
27	KTLF27	0.504	0.2785	0.09±0.09	No growth
28	KTLF28	0.6135	0.1735	0.187±0.06	0.32±0.09
29	KTLF29	0.514	0.245	0.303±0.067	0.41±0.04
30	KTP30	0.6615	0.165	0.234±0.2	0.37±0.03

of riboflavin in RAM respectively after 24 hrs of growth as shown in (Table 1).

DISCUSSION

In this study, 40 isolates of *Lactobacillus* isolated from diversified niches were identified by a combination of conventional and molecular techniques as shown in figure 1. The isolates from curd possessed homofermentative and heterofermentative characteristics as reported previously by different authors.[11,12] *L. pentosus* and *L. plantarum* were prevalent in Bamboo shoot and dosa batter. There are few reports on isolation of *L. plantarum, L. pentosus, L. Brevis* and *L. fermentum,* from fermented bamboo shoot products.[13,14] The phylogenetic analysis has shown the close relationship between *L. mucosae* and *L. fermentum*. They can be regarded as closely related species on the basis of similarity index also reported.[15] The microflora varies in different stages of the host's life. Furthermore, this microflora is also influenced by geographical conditions.[16] There are reports mentioning the screening of riboflavin producing LAB from Vellore region of India.[17] As described by author efficient riboflavin producing bacterium *L.fermentum* MTCC 8711 showed 2.29 mgl/l of riboflavin in chemical defined media after 24 h. Guru and Viswanathan, (2013) have reported riboflavin producing probiotic *L.acidophilus sp.* obtained from curd and cheese samples.[18] After the discovery of beneficial effects of LAB on human health the new doors have been opened for health and pharmaceutical industries to explore the beneficial effects of LAB. In market, probiotic cultures are used for the manufacturing of a number of products such as yoghurt, cheese, ice cream, chocolates pharmaceutical tablets, infant formulas and dietary supplements.[19] Riboflavin is synthesised by many bacteria and its biosynthetic pathway has been studied extensively in *B. subtilis* and *E. coli*.[20,21] This study highlights the microbial diversity of riboflavin producing strains isolated from various sources. The findings suggest that the Lactobacilli

isolated from human feces and fermented bamboo shoots have shown maximum riboflavin production as compared to isolates of dairy origin. The data generated in this study suggests that isolates of human and plant origins were more likely to produce riboflavin. These findings suggest that the isolates surviving in human intestine or of plant origin where riboflavin needs are fulfilled by exogenous sources only, have acquired the riboflavin biosynthesis gene through horizontal gene transfers. It is well known that the riboflavin is in abundance in dairy sources. This might be a reason for isolates not having complete riboflavin operon since they are adapted to riboflavin uptake from surrounding environment. These isolates can be of more interest for further charactization of riboflavin biosynthesis genes in both riboflavin producers and non producer strains.

CONCLUSION

The focus of this study was to evaluate the riboflavin producing lactobacilli isolated from dairy and non-dairy sources as well as plant sources. This research also highlights the microbial diversity of strains isolated from various sources. The two isolates (KTLF1 and KTP13) were recognized as prolific riboflavin producers on the basis of riboflavin production. The partial 16sRNA sequences of riboflavin producing isolates have been submitted to NCBI Genbank. This would pave the way as an initiative of analysing the composition of unexplored microbiota of Indian population. Further research is required for further exploration of these isolates endowed with appreciable riboflavin producing ability for industrial use as novel and native starter cultures to produce an essential vitamin *in situ* which would contribute significantly to the functional value of certain fermented foods. Microbial metabolites synthesis could be an effective strategy for their delivery as a functional bio-ingredient through foods to meet the daily recommended intake of human population. Bacteria producing even small amount of metabolite will be better choice to be used as a starter for the formation of fermented products than consuming bacteria. The Proper selection & exploitation of functional metabolite producing LAB will be an interesting strategy to produce novel food with enhanced nutritional and health promoting properties without increasing the cost of production & by reducing the *in situ* fortification step.

CONFLICT OF INTEREST

We all authors declare there is no conflict of interest.

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