

## Riboflavin Producing Probiotic Lactobacilli as a Biotechnological Strategy to Obtain Riboflavin-enriched Fermented Foods

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With the aim to find new riboflavin producing starter cultures, selected strains were inoculated in milk and whey based media and evaluated for technological attributes. The study of new probiotic strains for their technological relevance and use in dairy products is important for trade and industry. Eight lactobacilli strains isolated from dairy and non-dairy sources were selected for milk coagulation, percent acidity, exopolysaccharides production, proteolytic activity and antifungal activity. The tested strains displayed appreciable starter activities. Among the tested strains, *Lactobacillus fermentum* KTLF1 and *Lactobacillus mucosae* KTLF5 were the prolific riboflavin producers in milk (1.5mg/l) and whey (0.83mg/l). These riboflavin producing isolates displaying starter properties may be extended to a wide range of dairy-based, cereal based foods, feed, and beverages that that could in turn be used as an alternative to fortification with the controversial synthetic chemical riboflavin.

**Key words:** Riboflavin, Lactic acid bacteria, dairy food, Health, probiotic.

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Fermented dairy products are widely accepted across the world because of their health benefits and valued components of food diets (Georgieva *et al.*, 2008). The incorporation of riboflavin producing probiotic bacteria as adjuncts in different fermented products can be important strategy to naturally fortify the products with this vitamin which will result into beneficial industrial and commercial consequences. Riboflavin is a water soluble vitamin which belongs to the B group. Being as a precursor of two essential coenzymes: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fischer and Bacher, 2005). It is necessary for the activity of many flavoenzymes involved in different redox reactions (Fraaije and Mattevi, 2000). Riboflavin deficiency is common in many parts of the world,

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including both industrialized and developing countries (O'Brien *et al.*, 2001). The vitamin producing ability of bacteria has attracted many research groups since human lacks the biosynthetic capacity for most vitamins and they are dependent on dairy and cereal based food, plants, fungi, and bacteria for dietary supplementation (LeBlanc *et al.*, 2010). In recent years, the use of Lactic acid bacteria (LAB) was proposed as these microorganisms are able to synthesize B-group vitamins particularly riboflavin to obtain fermented bio-enriched food (Thakur *et al.*, 2015a; Capozzi *et al.*, 2011; Laino *et al.*, 2012; Vaesken *et al.*, 2012). LAB besides their starter and probiotic nature, have been shown to be ideal cell factories as they also produce a range of metabolites that are collectively termed as 'nutraceuticals', which include B vitamins like riboflavin (B<sub>2</sub>), folate (B<sub>11</sub>) and cobalamine (B<sub>12</sub>), low calorie sugars like mannitol and sorbitol, exopolysaccharides, diacetyl

and L-alanine (Stiles, 2004). Recent studies have reported on the selection of riboflavin producing strains for potential food and dairy applications, for example, the manufacture of riboflavin enriched dairy and cereal based products (LeBlanc *et al.*, 2011). The metabolic exploitation of such virtuous microbes has a relevant importance in preparation of various dairy fermented foods. In the present study, riboflavin producing lactobacilli were well characterized for their starter properties, in the direction to develop riboflavin enriched fermented food in the future.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The lactobacilli (Thakur *et al.*, 2015b) used in this work were previously described putative riboflavin producing *Lactobacillus* strains using spectrophotometric assay, polymerase chain reaction and microbiological assay method and high performance liquid chromatography (Thakur and Tomar, 2015c). The riboflavin producing isolates have displayed probiotic attributes (Thakur *et al.*, 2015d; Thakur *et al.*, 2015e) which led us to study the technological properties of the selected strains in direction to develop riboflavin enriched fermented foods in future. All the strains stored previously were at -80°C in MRS supplemented with glycerol (20% v/v) were routinely cultured on de Man-Rogosa -Sharp (MRS) medium (Sigma-Aldrich (St. Louis MO USA) for this study.

### Riboflavin production in milk and whey based medium

The selected isolates were inoculated in skim milk and whey based media and the vitamin produced was estimated after 12 hrs and 24 hrs. The riboflavin production was evaluated by the method described by Ashoor *et al.*, (1983).

### Acidity and milk coagulation

To determine starter activity of all isolates, tubes containing 20 mL of heat treated (90°C, 10 min) reconstituted skimmed milk 11.5% (Modern Dairies, Karnal, India) in test tubes that were inoculated with active culture (3% v D v) for starter activity and incubated at 37°C for 4 h. For acid production, tubes were inoculated with (1% v D v) active culture in two sets and were further incubated at 37°C for 6 and 24 h. The percentage of lactic acid formed was estimated by titrating

curd with 0.1 N NaOH to an end point of pale pink using phenolphthalein as indicator, and the pH of acidified milk was measured using a pH meter (Thermo Electron, Madison, WI, USA) in triplicate.

### Exopolysaccharides production

For exopolysaccharides (EPS) production, the active MRS cultures (30°C/24hrs) were streaked on sucrose agar plates and incubating the plates at 30°C for 24 hours. The formation of mucoid or viscous growth on sucrose agar was taken as positive for EPS production (Garvie, 1984). EPS production ability of isolates was also screened by copper sulphate staining. The isolates were microscopically examined for capsule formation.

### Proteolytic activity

In order to determine the proteolytic activities of isolates, the identified isolates were cultivated on reconstructed agar plates (10%) containing skim milk medium and were heated at 30°C for 18-20 h. Colonies having a transparent ring around them were considered as strains indicating proteolytic activity (Swearingen *et al.*, 2001).

### Antifungal activity

Antifungal activity of test cultures was checked in MRS plates using the overlay technique (Magnusson and Schnurer, 2001). Test cultures were placed on the surface in 10-mm lines on 90-mm plates containing 25mL of MRS agar. Plates were incubated in anaerobic conditions at 30°C for 18 h. After growth of test cultures, 10mL of spores of were added. Plates were then incubated in aerobic conditions at 25°C, and, after the growth of fungi, were evaluated for inhibition halos around the areas of growth of test cultures.

## RESULTS AND DISCUSSION

### Riboflavin production in milk and whey based media

There are number of reports for development of riboflavin enriched fermented products and their *In vivo* manifestations in suitable animal models (LeBlanc *et al.*, 2006; LeBlanc *et al.*, 2005; Valle *et al.*, 2014; Burgess *et al.*, 2004; Capozzi *et al.*, 2011). Guru and Viswanathan, (2013) have concluded that, whey is better fermentation medium compared to skim

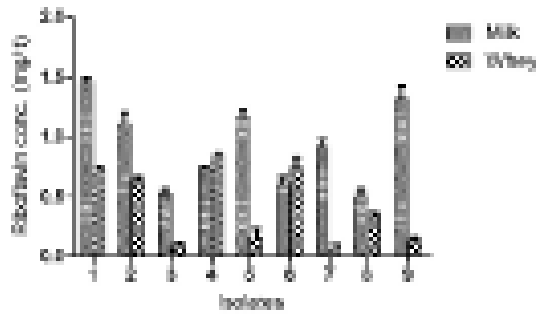


Fig. 1. Riboflavin production by lactobacilli in milk and whey based media



Fig. 2. Growth of riboflavin producing isolates in milk and whey based media

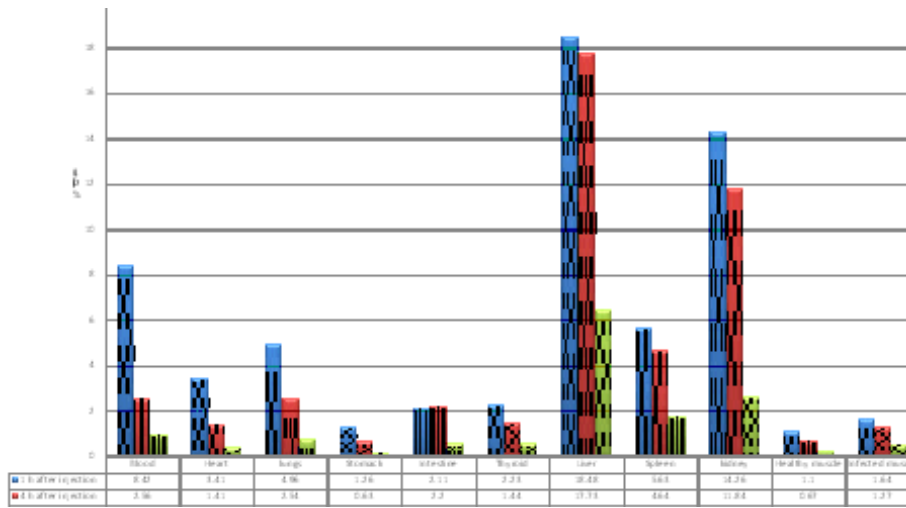


Fig. 3. Quantification of riboflavin by HPLC

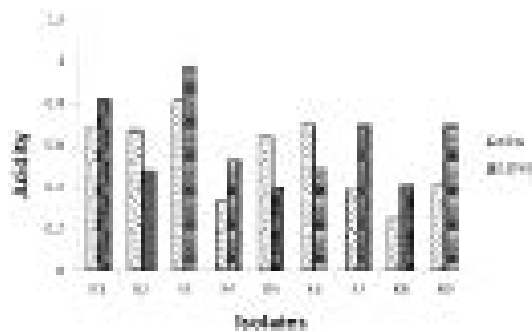


Fig. 4. Acidity profile of riboflavin producing isolates

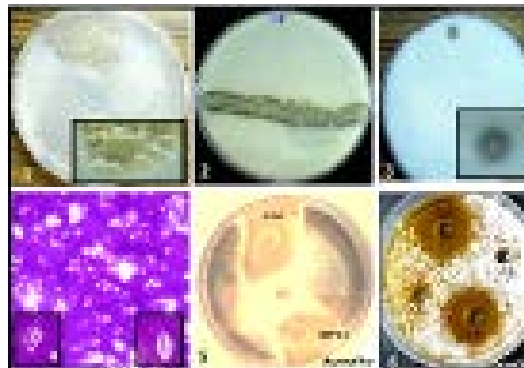


Fig. 5. Techno functional properties of riboflavin producing isolates. 1. Exopolysaccharides production (mucoid growth), 2. & 3. Proteolytic activity (Transparent zone and transparent ring), 4. Capsule staining, 5. & 6., Antifungal activity of riboflavin producers

milk for riboflavin production. Bacteria producing even small amounts of riboflavin will be a better choice to be used as a starter for the formation of fermented products rather than traditional starters which consume riboflavin (Thakur *et al.*, 2015c). In our study, we have found that riboflavin production was higher in milk at 12 hrs but it declined at 24 hrs (Fig. 1, Fig. 2 & Fig. 3). In case of whey based medium, the trend of riboflavin production was opposite to skim milk.

#### **Techno functional attributes of riboflavin producing isolates**

The riboflavin producing *Lactobacillus* strains were tested for proteolytic activity, EPS production and acidification properties. The acidification properties of these *Lactobacillus* strains were determined in milk and decrease in pH was observed after 6h and 12h (Fig. 4). The milk coagulation ability was also observed and it was found that 3 isolates (KTLF1, KTP11 and KTP30) out of 7 isolates, screened were able to coagulate the milk after 12h. One isolate KTLF2 was able to produce EPS and CuSO<sub>4</sub> staining has shown the presence of capsules (Fig. 5). The isolates KTLF3 and KTP13 have shown proteolytic activity by forming transparent zones and rings (Fig. 5). The antifungal activity was shown by most of the isolates against *Penicillium notatum* and *Aspergillus niger* (Fig. 5) with typical inhibition halos around the test isolates. EPSs produced by lactobacilli can affect dough rheology, water absorption of the dough, loaf volume and bread staling (Arendt *et al.*, 2007; Zotta *et al.*, 2008). Proteolytic activity of *Lactobacillus* strains are also reported to affect the mechanical properties of the dough and bread quality (Pepe *et al.*, 2004). A large number of *Lactobacilli* such as *L. plantarum*, *L. brevis*, *L. fermentum*, *L. sakei*, *L. casei*, *L. paracasei*, *L. hilgardii* and *L. helveticus* have also been known as EPSs producers (Sawadogo-Lingani *et al.*, 2007). As all technological properties were estimated only qualitatively, no relationship could be established among different technological traits. The proteolytic activity of LAB has a role in cheese maturation (Hebert *et al.*, 2000). Hebert *et al.*, (2000) reported that in the *Lactobacillus* strains they studied, some represented high proteolytic ability while others represented poor activity. Different EPS screening methods have been reported for LAB. The visual inspection of

bacterial colonies on agar plates is most probably the easiest method, but it is insensitive and indicative. This method is unable to detect LAB strains that produce low amounts of EPS, unless they are very ropy (Smitinont *et al.*, 1999; Dick *et al.*, 1993). In our study, none of the isolates presented a ropy phenotype, but a more apparent mucoid phenotype than the other isolates analysed. The inhibitory activity in the fermentative phase, as presented in some strains of *Lactobacillus*, has interesting technological possibilities for a variety of fermented food products, for example dry fermented sausages and cheese. In fact, this lactic acid bacteria is often used as a starter to guide fermentation, and its behaviour towards moulds can be considered to be one of the main selection characteristics.

The present study have estimated the production of riboflavin in milk and whey based media. The technologically relevant properties of eight candidate riboflavin producing probiotic lactobacilli strains have been reported. Their capacity to acidify and coagulate milk, exopolysaccharides production, proteolytic activity and antifungal activity make them appropriate for diverse food applications and highlight on their capability to be incorporated in dairy products. The study has generated the data for further exploration of these isolates endowed with appreciable starter activities 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. From this study, it is clear that milk and whey can be utilized to supply riboflavin to consumers.

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