JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2016. Vol. **10**(1), p. 161-166

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

Kiran Thakur[1], Vaibhao Kisanrao Lule, Rajni C.S., Narendra Kumar, Surajit Mandal[1], Santosh Anand[1], Vandna Kumari and Sudhir Kumar Tomar[1]*

National Collection of Dairy Culture, [1]Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, India.

(Received: 06 November 2015; accepted: 30 December 2015)

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, exoploysaccharides production, proteolytic activity and antifungal activity. The tested strains displayed appreciable starter activities. Among the tested strains, *Lactobacillus fermentum* KTLF1 and *Lactobacillus muocase* KTLF5 were the prolific riboflavin producers in milk (1.5mg/lt) and whey (0.83mg/lt). 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.

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, 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_2), folate (B_{11}) and cobalamine (B_{12}), low calorie sugars like mannitol and sorbitol,

* To whom all correspondence should be addressed.
E-mail: sudhirndri@gmail.com

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 -80ºC 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 (90ºC, 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 37ºC for 4 h. For acid production, tubes were inoculated with (1% v D v) active culture in two sets and were further incubated at 37ºC 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.

Exoploysaccharides production

For exoploysaccharides (EPS) production, the active MRS cultures (30°C/24hrs) were streaked on sucrose agar plates and incubating the plates at 30°Cfor 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 30ºC 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 30ºC for 18 h. After growth of test cultures, 10mL of spores of were added. Plates were then incubated in aerobic conditions at 25ºC, 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

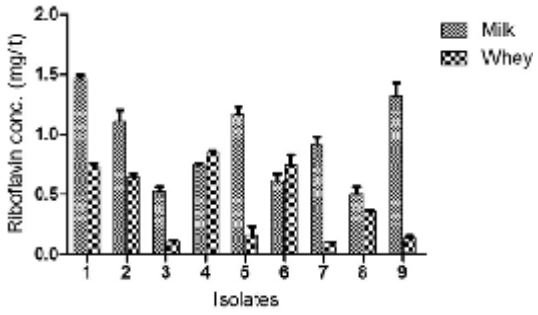

Fig.1. Riboflavin production by lactobacilli in milk and whey based media

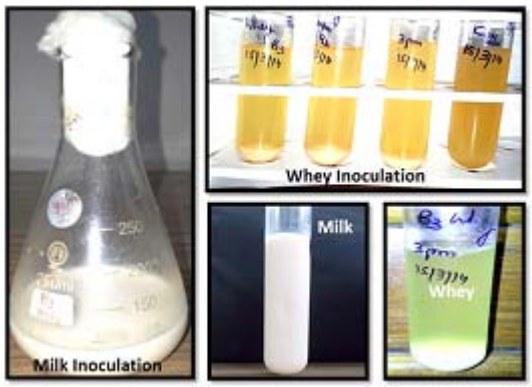

Fig.2. Growth of riboflavin producing isolates in milk and whey based media

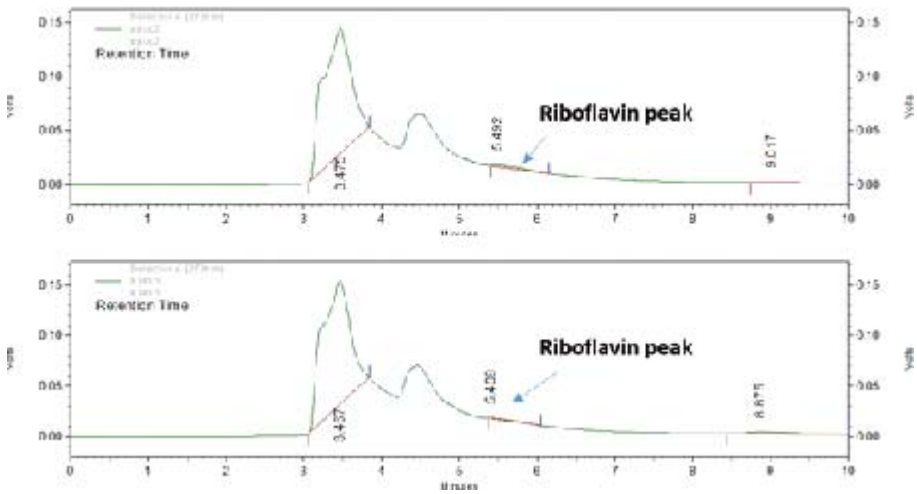

Fig. 3. Quantification of riboflavin by HPLC

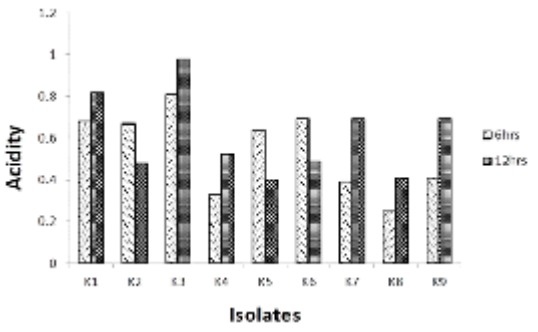

Fig. 4. Acidity profile of riboflavin producing isolates

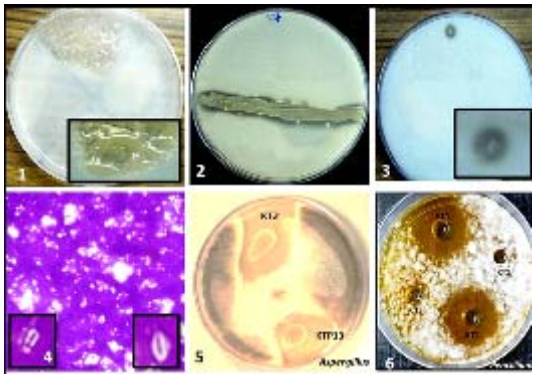

Fig. 5. Techno functional properties of riboflavin producing isolates.1. Exoploysaccharides production (mucoid growth), 2. & 3. Proteolytic activity (Transparent zone and transparent ring), 4. Capsule staining, 5. & 6., Antifungal activity of riboflavin producers

better fermentation medium compared to skim 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 CuSO4 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 *Pencillium 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, exoploysaccharides 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.

ACKNOWLEDGMENTS

The authors thankfully acknowledge the support extended by Indian Council of Agricultural Research (ICAR), New Delhi and the Director, National Dairy Research Institute (NDRI Karnal, India).

REFERENCES

1. Arendt, E., K., Ryan, L., A., M. and Dal Bello, F., Impact of sourdough on the texture of bread, *Food. Microbiol*. 2007; **24**: 165-174.

2. Ashoor, S., H., Saperich, G., J., Monte, W., C., and Welty, J., HPLC determination of riboflavin in eggs and dairy products, *J. Food. Sci*. 1983; **48**: 92-94.
3. Burgess, C., O'Connell-Motherway, M., Sybesma, W., Hugenholtz, J. and van Sinderen, D., Riboflavin Production in *Lactococcus lactis*: Potential for In Situ production of vitamin-enriched foods. *Appl. Environ. Microbiol.* 2004; **70**: 5769-5777.
4. Capozzi, V., Menga, V., Digesu, A., M., De, Vita, P., van, Sinderen, D., Cattivelli, L., Fares, C., and Spano, G., Biotechnological production of vitamin B2-enriched bread and pasta. *J. Agr. Food. Chem.* 2011; **59**: 8013–8020.
5. Dick, J., C., Berg, V., D., Smits, A., Pot, B., Ledeboer, A., M., Kersters, K., John, M., A. and Verrips T., Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. *Food. Biotechnol*. 1993; **7**: 189-205.
6. Fischer, M. and Bacher, A., Biosynthesis of flavocoenzymes. *Nat. Prod. Rep*. 2005; **22:** 324-50.
7. Fraaije, M. W. and Mattevi, A., Flavoenzymes: Diverse catalysts with recurrent features. *Trends. Biochem. Sci*. 2000; **25**: 126-132.
8. Garvie, E., I., Separation of species of the genus *Leuconostoc* and differentiation of the leuconostots from other lactic acid bacteria, In: T. Bergan (Ed.), Methods in Microbiology, 1984; **16**: Academic Press, London, 147-178.
9. Georgieva, R. N., Iliev, .I N., Chipeva V A, Dimitonova S P, Samelis J, and Danova S T., Identification and *in vitro* characterization of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. *J. Basic. Microb.* 2008; **48**: 234–244.
10. Guru, V. and Viswanathan, K ., Riboflavin production in milk whey using probiotic bacteria- *Lactobacillus acidophilus* and *Lactococcus lactis. Ind. J. Fund. Appl. Life.* Sci. 2013; **3:** 169-176.
11. Hebert, E., M., Raya, R., R., Tailliez, P. and de Giori, G. S., Characterization of natural isolates of Lactobacillus strains to be used as starter cultures in dairy fermentation. *Int. J. Food. Microbiol*. 2000; **59**: 19–27.
12. Laino, J., E., Juarez, del, Valle, M., Savoy, de, Giori, G. and LeBlanc, J., G., J., Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT Food. Sci. Technol.* 2012; **54**: 1-5.
13. LeBlanc, J., G., Burgess, C., Sesma, F., Savoy de Giori, G. and van Sinderen, D., Ingestion of milk fermented by genetically modified *Lactococcus lactis* improves the riboflavin status of deficient rats. *J. Dairy. Sci*. 2005; **88**: 3435–3442.
14. LeBlanc, J., G., Laino, J., E., del, Vall, M., J., Vannini, V., van Sinderen, D., Taranto, M., P., de Valdez, G., F., de Giori, G., S., and Sesma, F., B-group vitamin production by lactic acid bacteria – current knowledge and potential applications. *J. Appl. Microbol.* 2011; **111**: 1297-1309.
15. LeBlanc, J., G., Rutten, G., Bruinenberg, P., Sesma, F., de Giori, G., S. and Smid, E., J., A novel product fermented with *Propionibacterium freudenreichii* improves the riboflavin status of deficient rats. *Nutrition*. 2006; **22**: 645-651.
16. LeBlanc, J., G., Taranto, M., P., Molina, V., and Sesma, F., B-group vitamins production by probiotic lactic acid bacteria. In: Mozzi F, Raya R, Vignolo G (eds) Biotechnology of lactic acid bacteria: novel applications, Wiley-Blackwell, Ames, 2010.
17. Magnusson, J. and Schnurer, J., *Lactobacillus coryniformis subsp. coryniformis* strain si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl. Environ. Microbiol.* 2001; **67**: 1–5.
18. O'Brien, M., M., Kiely, M., Harrington, K., E., Robson, P., J., Strain, J., J., and Flynn, A., The North/South Ireland Food Consumption Survey: vitamin intakes in 18–64-year-old adults. *Public. Health. Nutr.* 2001; **4**: 1069–1079.
19. Pepe, O., Blaiotta, G., Anastasio, M., Moschetti, G., Ercolini, D., and Villani, F., Technological and molecular diversity of Lactobacillus plantarum strains isolated from naturally fermented sourdoughs. *Syst. Appl. Microbiol.* 2004; **27**: 443-453.
20. Sawadogo-Lingani, H., Diawara, B., Traore, A., S., and Jakobsen, M., Technological properties of *Lactobacillus fermentum* involved in the processing of dolo and pito, West African sorghum beers, for the selection of starter cultures. *J. App. Microbiol*. 2007; **104**: 873-882.
21. Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S., Navarinie, L., Boscoe, M. and Cescuttif, P., Exopolysaccharide-producing lactic acid bacteria strains from traditional Thai fermented foods: isolation, identification and exopolysaccharide characterization. *Int. J. Food. Microbiol.* 1999; **51**: 105-111.
22. Stiles, M., E., Biopreservation by lactic acid bacteria. Antonie. Van. Leeuwenhoek. 2004; **70**: 331-345.

23. Swearingen, P., A., O'Sullivan, D., J. and Warthesen, J., J., Isolation, characterization and influence of native, nonstarter lactic acid bacteria on Cheddar cheese quality. *J. Dairy. Sci*. 2001; **84**: 50-59.
24. Thakur, K., Tomar, S. K. and De S., Lactic acid bacteria as a cell factory for riboflavin Production. *Microbial Biotechnol* 2015a. doi:10.1111/1751-7915.12335.
25. Thakur, K.,Nanda, D. K., Kumar, N. and Tomar, S. K., Phenotypic and genotypic characterization of Indigenous Lactobacillus Species from Diverse Niches of India. *Curren. Trends. Biotechnol. Pharma*. 2015b; **9**: 222-227.
26. Thakur, K. and Tomar, S., K., Exploring indigenous Lactobacillus species from diverse niches for riboflavin production. *J. Young. Pharmacists*. 2015c; **7**: 122-127.
27. Thakur, K., Gosh, S., Kadam, J. and Tomar, S., K., Adhesion Properties of Riboflavin Producing Lactobacillus Mucosae Isolated from Human Faeces. *J. Cell. Tissue. Res.* 2015d; **15**:5095-5100.
28. Thakur, K. and Tomar, S. K., Invitro study of Riboflavin producing lactobacilli as potential Probiotic. LWT-Food sci Technol.Accepted, 2015e
29. Vaesken, S., M., Aperte, A., E., and Moreiras, V., G., Vitamin food fortification today. *Food. Nutr. Res*. 2012; **56:** 5459. DOI: 10.3402/fnr.v 56i0. 5459.
30. Valle, J., D., M., Lainoa, J., E., G., Savoy, D., G., G. and LeBlanc, J., G., Riboflavin producing lactic acid bacteria as a biotechnological strategy to obtain bio-enriched soymilk. *Food. Res. Int.* 2014; **62**: 1015-1019.
31. Zotta, T., Piraino, P. and Parente, E., Characterization of lactic acid bacteria isolated from sourdoughs for Cornetto, a traditional bread produced in Basilicata (Southern Italy). *World. J. Microb. Biot.* 2008; **24**: 1785-1795.