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GROWTH AND BIOGENIC AMINE POTENTIAL OF *Lactococcus lactis* subsp. *lactis*, isolated FROM TURMERIC (*Curcuma longa* Linn.), WITH PROBIOTIC CHARACTERISTICS IN SKIM MILK

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ABSTRACT

The growth and biogenic amines, (BA), (histamine and tyramine) potential of *Lactococcus lactis* subsp. *lactis* with potential probiotic characteristics isolated from turmeric (*Curcuma longa* Linn.) was studied in skim milk. Probiotic characteristics were determined in terms of acid and bile tolerance, hydrophobicity and antimicrobial activity. Fermentation of skim milk was followed for 48 hrs at 37 ºC determining pH, cell counts and histamine and tyramine with qualitative screening for proteolytic activity. Isolate showed good acid and bile salt tolerance (>90 %) with acceptable adhesion and antimicrobial activity. The maximum viable cell count (8.35 log cfu mL^{-1}) was observed after 48 hrs of incubation in skim milk with corresponding low pH of 4.06. Histamine or tyramine was not detected throughout 48 h of incubation as determined by HPLC. These properties suggest *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric is a safe potential probiotic in terms of BA production in milk.

Keywords: Biogenic amines, Histamine, Tyramine, Probiotic, *Lactococcus lactis*

INTRODUCTION

There is an increasing trend today in food industry to use probiotic starter cultures to promote the health and well being of the consumers. Probiotics are live microbial feed supplements which beneficially affects the host by improving the indigenous micro flora (**Fuller, 1989**). Lactic acid bacteria (LAB) play a major role as probiotic or functional starter organisms in food and beverage industry and are considered to have a safe history of application and consumption in fermented products (**Wood *et al.*, 1995; Caplice *et al.*, 1999; Leroy *et al.*, 2004**). *Lactobacillus* and *Lactococcus* species are mainly used as probiotics in fermented milk products. Since *Lactococcus* strains are widely used as starter cultures, several works showed their application as probiotics in fermented dairy products (**Grahn *et al.*, 1994; Kimoto *et al.*, 1999; Pianpumepong *et al.*, 2012; Enan *et al.*, 2013**).

Strains with probiotic features were isolated from natural habitats or from fermented products (**Oberman *et al.*, 1998**). The recent trend shows the isolation of wild-type of strains from traditional products to be used as starter or probiotic cultures in food fermentation (**Beukes *et al.*, 2001**). LAB isolated from various kinds of fermented plant products show potential use as starter cultures with improved quality of the end products (**Mourad *et al.*, 2004; Prachyakij *et al.*, 2008**). However there is no reported evidence of potential probiotic bacteria isolated from turmeric rhizomes and use in milk fermentation. New or potential probiotic strains should be evaluated not only for their beneficial health effects, but also for their harmful metabolites. Biogenic amines (BA) are considered as undesirable metabolic product of probiotic or functional starter organisms with potential heath risk to consumers (**Holzapfel *et al.*, 1995; Leroy *et al.*, 2004; Ammor *et al.*, 2007**).

Biogenic amines, histamine and tyramine are the most extensively studied amines in milk and milk products due to their toxicological effects. Histamine has been reported as the causative agent of histamine intoxication, while tyramine has been reported to affect the hypertensive crisis in the individuals being administrated monoamine oxidase (MAO) inhibitor drugs (**Anderson *et al.*, 1993; Silla Santos 1996; Zaman *et al.*, 2009**). The high amount of histamine and tyramine can be formed during storage or processing of fermented foods by the activity of bacterial decarboxylase enzyme (**Halász *et al.*, 1994**). Accumulation of biogenic amines in fermented milk or cheese can be affected by the availability of free amino acids and the presence of decarboxylase positive microorganisms (**Fernández-Garcia *et al.*, 2000**). Microorganisms possessing decarboxylase activity can be non-starter lactic acid bacteria and other spontaneous microflora (**Roig-Sagués *et al.*, 2002**) and starter microorganism (**Fernández-Garcia *et al.*, 2000**).

Several species of lactic acid bacteria have been identified as biogenic amines formers in milk. Several species of *Lactobacillus bulgaricus*, *L. casei* and *L. acidophilus* are reported as histamine formers (**Edwards *et al.*, 1981; Stratton *et al.*, 1991; Petridis *et al.*, 1996**). According to **Chander *et al.*, (1989)** *Lactococcus lactis* was found to produce histamine, tyramine and tryptamine. As reported by **González de Llano *et al.*, (1998)**; **Halász *et al.*, (1994)**; and **Straub *et al.*, (1995)** amine forming ability should be one of the concerns in selecting starter cultures. Thus testing for BA formation by commonly used or intended to use starter or probiotic LAB strains is essential. However sufficient progress has not yet been made in the optimal strains selection in terms of BA formation and information relevant to the BA formation by probiotic *Lactococcus lactis* subsp. *lactis* in milk system is still scarce.

Therefore, the aim of this study was to study the growth characteristics and biogenic amine potential of non dairy based *Lactococcus lactis* subsp *lactis* isolated from fresh turmeric rhizomes during the fermentation of skim milk.

MATERIAL AND METHODS

Isolation and Identification of Lactic Acid Bacteria (LAB)

Fresh turmeric rhizomes were purchased from Thakun, Surathani Province, Thailand. Lactic acid bacteria were isolated and identified and characterized as previously reported (**Adnan *et al.*, 2007; Pianpumepong *et al.*, 2010**). In brief, 10 g of chopped turmeric rhizomes was mixed with 90 mL of sterile distilled water and extracted for 2 min using stomacher. Serial dilutions in sterile NaCl (0.85 %) were prepared with the 1 mL of extracted suspension. Diluted suspension (0.1 mL) was spread on MRS agar (Himedia, Mumbai, India) plates containing 0.5 % (w/v) calcium carbonate solution and incubated anaerobically at 37 ºC for 24-48 hrs. Acid producing bacteria were recognized by the appearance of clear zone around the colonies. The purity of the colonies was examined on MRS agar plates containing 0.06 g L^{-1} bromocresol purple as an indicator. The

medium colour changed from purple to yellow as a result of pH reduction indicating the production of a lot of lactic acid during the log phase of bacterial growth. Isolates were tested for catalase production by placing a drop of hydrogen peroxide solution (3 % (v/v) in sterile distilled water) on bacterial cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Following the catalase test isolates were further analyzed and identified by using API kits (bioMérieux, Inc., Durham, NC, USA).

Probiotic Characterization

Probiotic characteristics of the strain *Lactococcus lactis* subsp. *lactis* were investigated as acid and bile salt tolerance, hydrophobicity, and antimicrobial activity. Acid tolerance was tested as reported by **Pianpumepong *et al.*, (2010).** Cell suspension was added to MRS broth maintained at pH 3.0 and a control at pH 6.4 and incubated for 2 h at 37 ^{o}C. At the end of 2 h incubation viable cell count was determined by growing them in MRS agar for 48 hrs at 37 ^{o}C. Acid tolerance was measured as percentage of cell survival calculated by comparing the initial bacterial cell count to the count after 2 h incubation at pH 3.0. Bile salt tolerance was observed as reported by **Pianpumepong *et al.*, (2010).** Bacterial cell count was compared to the cell count in MRS broth with added bile salt and without bile salt after 24 h of incubation. Antibacterial activity was tested against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp. and hydrophobicity and antimicrobial activity were calculated as reported by **Pianpumepong *et al.*, (2010).**
Lactococcus lactis subsp. *lactis* with potential probiotic characteristics was further studied for their growth characteristics and biogenic amine potential in skim milk.

Preparation of Inocula

The strain *Lactococcus. lactis* subsp. *lactis* was maintained along with other isolated bacterial strains at the culture stocks of Biotechnology Laboratory of the Asian Institute of Technology, Pathumthani, Thailand. Prior to the inoculation the strain was subcultured ten times in MRS broth containing 0.1 % histidine (Serva, Heidelberg, Germany), 0.1 % tyrosine di-sodium salt (Biochemica, Hessen, Germany) and 0.005 % pyridoxal-5-phosphate (Sigma Aldrich, Buchs, Switzerland) according to the previously described method (**Bover-Cid *et al.*, 1999**) in order to enhance amino acid decarboxylase activity.

Preparation of Milk Samples

Powdered skim milk (Himedia, Mumbai, India) was reconstituted with distilled water (10 %), sterilized at 121 ^{o}C for 5 min according to the manufacturer's specification and analyzed for microorganisms, pH and biogenic amines histamine and tyramine prior to the inoculation. The sub cultured strain was aseptically added to the milk at the rate of 1 % (v:v) and incubated at 37 ^{o}C for 48 hrs. Samples were taken at 6 hrs interval within first 24 hrs and then after 24 hrs interval and analyzed for pH, titratable acidity, colony forming units and biogenic amines, histamine and tyramine. Two replicates were done for the analysis.

Determination of Viable Cell Count

Samples of the sterilized reconstituted skim milk were analyzed for mesophilic bacteria (APHA, 1992). Mesophilic counts were obtained for 10^{0} and 10^{-1} dilutions on plate count and MRS agar after 48 hrs of incubation at 37 ^{o}C. Milk samples were serially diluted by 10 fold in 0.5% sterile peptone water. Serial dilutions (sample volume of 0.1 mL) were plated on MRS agar (Himedia, Mumbai, India) and inverted plates were incubated at 37 ^{o}C for 48 hrs. Petridishes with 30- 300 separate colonies with white, smooth appearance were selected for the enumeration and number of colony forming units was recorded per mL of sample.

Determination of pH

The fermented milk samples with L. *lactic* subsp. *lactis* were tested for pH. The pH was measured using an electronic pH meter (Model Jenway 3310, Stone, Staffordshire ST15, OSA, UK). The pH meter was calibrated using standard buffer solutions (Merck, Darmstadt, Germany) of pH 4.0 and 7.0 prior to the analysis.

Screening for Proteolytic Activity

Proteolytic activity of the selected strain was qualitatively screened in MRS agar plates supplemented with 10% skim milk. Medium plates were bored with sterile cork borer. Strains (500 µl) were inoculated into the holes and incubated at 37 ^{o}C for 4 days. Proteolytic activity was recognized by the clear halo around the colonies.

Determination of Biogenic Amines, Histamine and Tyramine

Biogenic amines in milk samples were extracted according to the previously described method of **Santos *et al.*, (2003).** The filtered supernatants were stored at -20 ^{o}C until BA analysis. The amines were separated and quantified by HPLC, following the procedure optimized in this laboratory (**Priyadarshani *et al.*, 2011**) with a little modification, using similar equipment and chromatographic conditions. The pre-column derivatization of acid extract was done similarly to the conditions described by **Priyadarshani *et al.*, (2011)**, but with addition of 200 µl of NaOH instead of 20 µl. The identification of amines was performed by comparison of retention times of amines in samples to standard solutions spiked to milk. Quantification of histamine and tyramine was done by using external calibration lines prepared with recovery data obtained by spiking known amounts (0.5 – 200 mgL^{-1}) of standard histamine and tyramine to the milk samples followed by extraction and HPLC analysis. The linear regression equations between recovered BA and peak areas are y= 460333+69183x and y=303311+46289x for histamine and tyramine respectively with corresponding correlation coefficient of 0.9929 and 9948. All the samples and replicates were injected at least in duplicate to the HPLC column. The quantity of each amine was expressed in mgL^{-1} milk.

Statistical Analysis

Statistically significant differences were evaluated by one-way analysis of variance with Fisher's LSD test at a 95 % significance level using Minitab (version 14) statistical software.

RESULTS

Screening for Lactic Acid Bacteria (LAB)

The MRS medium was used to isolate the lactic acid bacteria from fresh turmeric rhizome. The LAB was selected from the colonies on MRS plus bromocresol purple agar. The colour of the media changed to yellow indicating the acid production. The isolates were found Gram positive and catalase negative (data not shown). The isolates were further specifically identified at species level by API 50 CHL media. A strain of *Lactococcus lactis* subsp. *lactis* was used for further tests.

Probiotic Characterization

Probiotic characterization was determined by acid and bile salt tolerance, hydrophobicity, and antimicrobial activity. The LAB strain, *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes showed important probiotic characteristics as shown in the Table 1.

Table 1 Probiotic characteristics of *L. lactis* subsp. *lactis* isolated from fresh turmeric rhizomes

Probiotic characteristic	**Experimental results**
Acid tolerance (% Survival rate)*	95.00 ±1.13
Bile tolerance (% Survival)*	90.56 ±2.83
Hydrophobicity (%)**	58.87
Antimicrobial activity against indicator bacteria	
Escherichia coli	+++
Salmonella spp.	+++
Staphylococcus aureus	+++

* Values are expressed as mean ± standard deviation
** The cell surface hydophobicity (%) was calculated according to the **Gusils *et.al.* 2002** using H (%) = $((A_o - A)/ A_o) * 100$, where *Ao* = Optical density of cell suspension; *A* = Optical density of lower aqueous phase.
+++, inhibition zone of more than 10 mm

Characteristics of Sterilized Reconstituted Skim Milk

Microbial counts on sterile skim milk are shown in Table 2. The initial pH of the milk samples were varied in between 6.6 to 6.8. The variation could be expected due to the variation of pH in the distilled water used to prepare milk samples and different lots of powdered milk used in the same brand. No histamine or tyramine was detected in the sterilized reconstituted skim milk samples.

Table 2 Colony forming units of mesophilic bacteria in sterilized skim milk samples

Milk Sample	Colony forming Units per mL			
	Total Plate Count Agar		MRS agar	
	10^0	10^{-1}	10^0	10^{-1}
1	0.5	0	0	0
2	0	0	0	0
3	0	0	0	0

Values are mean value for duplicate analysis

Growth Characteristics of *Lactococcus lactis* subsp. *lactis* in Skim Milk

The growth study showed that the probiotic *Lactococcus lactis* subsp. *lactis* grow well in skim milk. Growth of the strain is shown in Figure 1A. The initial viable cell count was 7.15 log cfu mL^{-1}. The strain attained maximum viable cell number of 8.35 log cfu mL^{-1} after 48 hrs of incubation. The pH of the skim milk inoculated with *L. lactis* subsp. *lactis* decreased from 6.8 initially to 4.06 after 48 hrs of incubation (Figure 1B).

The analysis of proteolytic activity on skim milk agar plates revealed that tested strain of *L. lactis* subsp. *lactis* did not show clear proteinase activity as clear hallo around the colonies after 4 days of incubation was not observed (Figure 2).

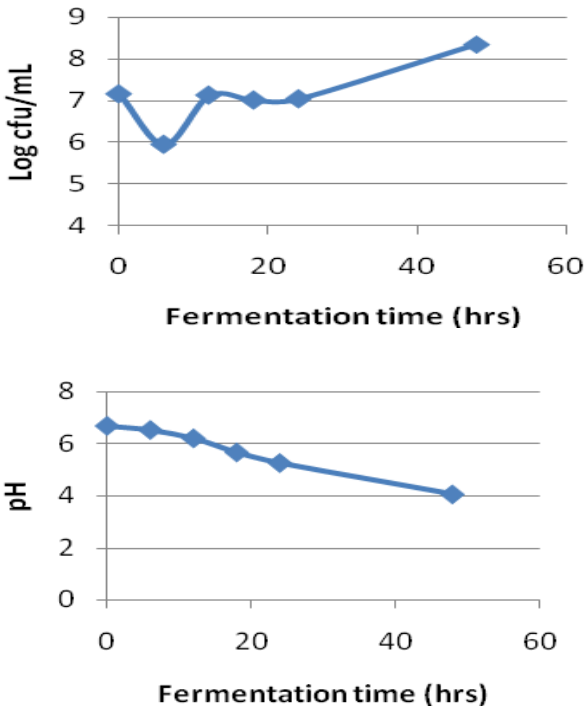


Figure 1 (A-B): A: Total colony forming units Log CFU mL^{-1}; B: pH of reconstituted and sterilized skim milk fermented with *Lactococcus lactis* subsp. *Lactis*, isolated from turmeric rhizomes. Results are the average of the duplicate treatments

Biogenic Amine (Histamine and Tyramine) Formation by *L. lactis* subsp. *lactis* in Skim Milk

Quantification was done by exploration of the standard calibration curves prepared from peak areas obtained by recovered amines spiked to skim milk. Calibration lines for both histamine and tyramine were constructed separately by plotting peak height vs. amount of amine. Linear regression analysis was performed for obtained data. The results of regression analysis were shown in the Table 3. The correlation coefficient for both histamine and tyramine were found to be 0.99. Therefore it is revealed that there is a linear relationship between amount of amine and detector response. This further indicated that the method applied for derivatization and HPLC analysis in this study was satisfactory. Potential to produce histamine and tyramine in milk during fermentation was not observed for the probiotic *Lactococcus lactis* subsp. *lactis* (Figure 3) as none of the amines were detected.

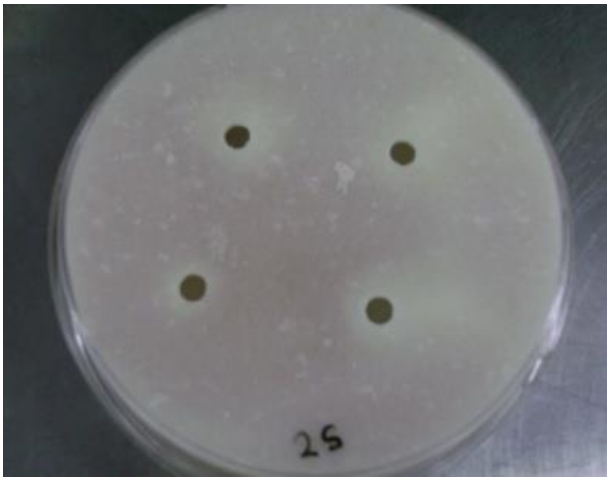
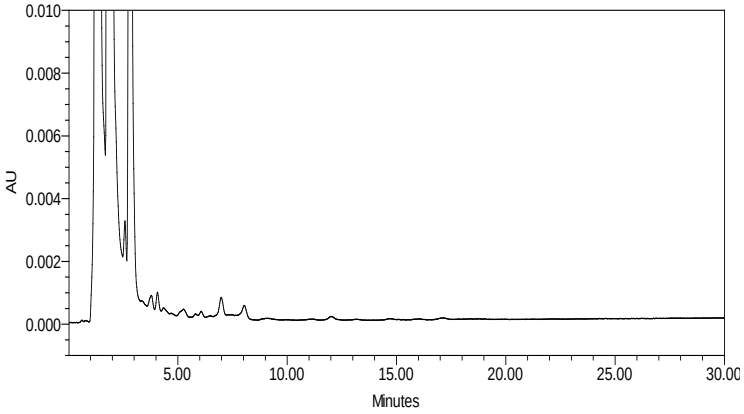
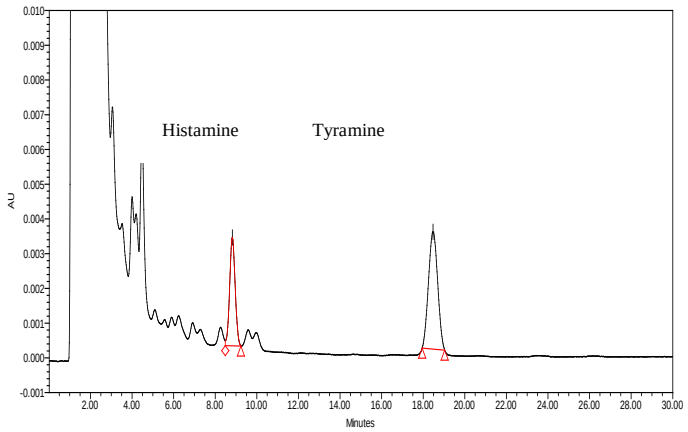


Figure 2 Proteolytic activity of strain *Lactococcus lactis subsp. Lactis* isolated from fresh turmeric rhizomes after 4 days incubation at 37 ^oC on skim milk



A



B

Figure 3 Representative HPLC chromatograms of skim milk A: inoculated with *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes after 48 hrs of incubation at 37 ^oC. B: spiked with authentic biogenic amines, histamine and tyramine 5 mgL^{-1}

DISCUSSION

A non-dairy based *Lactococcus lactis* subsp. *lactis* was isolated from fresh turmeric rhizomes using MRS medium. Isolate was found Gram-positive and catalase negative. The strain was specifically identified by API 50 CHL media at species level. The probiotic isolate was selected based on acid tolerance, bile salt tolerance, hydrophobicity and antimicrobial activity. The ability to resist low pH is an important selection criterion for probiotic microorganisms as acidic gastric juice in stomach destroys most of the ingested microorganisms. As reported by **Pianpumepong *et al.*, (2010)**, *L. lactis* subsp. *lactis* was able to tolerate 2 h of acid exposure at pH 3 and with good survival rate at 0.3 % bile salt. The ability of LAB to survive the passage through acidic media is variable and strain dependant. The survival rate of approximately 85 % is considered to be very

significant for the probiotic field (**Fernádez *et al*., 2003; Pennacchia *et al*., 2004**). The isolated strain showed >90 % of survival rate at both low pH and bile salt.

Adhesion to intestinal surfaces is an important property of probiotic bacteria. The hydrophobicity test was used to demonstrate adhesion capacity of the culture to the intestinal epithelium of the host. Several studies revealed the possibility of *Lactococcus* strains to be present in the human or animal gastrointestinal tract (**Gruzza *et al*., 1992; Klijn *et al*., 1995**). According to **Kimoto *et al*., (1999)** highest adhesion rate is observed with *Lactococcus lactis* subsp. *lactis* NIAI527. The turmeric derived *L. lactis* subsp. *lactis* studied in present study showed >50 % adhesive capacity.

The isolate was found to produce strong inhibition zones (zone of inhibition of more than 10 mm) against three indicator organisms. The high antagonistic activity was demonstrated against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp. which are considered to be disease causing agents in the lower digestive tract in human. Similar to this study **Enan *et al*., (2013)** shows the antibacterial activity of *Lactococcus lactis* subsp. *lactis* Z_{11} isolated from Zabady (Arabian yoghurt) against *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus.*

The growth and survival of probiotic bacteria in milk and milk products are affected by various factors. Some of these are: acid and hydrogen peroxide formation by yoghurt starter; availability and content of oxygen in milk, package, and temperature. (**Bolduca *et al*., 2006; Shah, 2000a**). Moreover their growth and survival was found to be affected by milk composition (chemical and microbiological), amount of milk solids and nutrient availability (**Shah, 2000b**). Among probiotics *Lactococci* have numerous nutritional requirements for growth, especially nitrogen sources (**Law *et al*., 1976**) and amino acid requirement reported to be strain dependent (**Chopin, 1993**). They utilize peptides or proteins as nitrogen sources through proteolytic enzyme activity (**Leitão *et al*., 2000**). The content of these compounds are low in raw milk. Hence presence of proteolytic system is an important aspect for probiotic lactic acid bacteria which are used in food fermentation (**Kojic *et al*. 1991**). The pH reduction during incubation enhances the proteolysis to liberate short peptides and free amino acids. Presence of proteinase enzymes then enhances the growth in milk. The present study revealed the proteinase negative (Prt^-) characteristics as milk protein degradation was not observed on the skim milk agar plates. Sharp reduction in pH during first 24 h of incubation was not observed in *L. lactis* subsp. *lactis* in milk when compared to other strains studied (data not shown). Similarly to this study **Durlu-Ozkaya, (2001)** reports slow acidifying activity of *L. lactis* subsp. l*actis* strains in milk at 30 ^{o}C. In this study, highest viable count of 8.35 log cfu mL- with concomitant low pH of the medium was observed at the 48 h of incubation.

Many factors are needed to be fulfilled in order to accumulate BA in milk. The factors of concern are: availability of precursor amino acids; presence of decarboxylase positive microorganisms and availability of proper conditions for growth and decarboxylation (**ten Brink *et al*., 1990; Russo *et al*., 2010)**. Various factors are related to such conditions in milk such as milk treatments, use of starter cultures and enzymes, the duration and the temperature of fermentation, the level of proteolysis, the pH, the NaCl concentration, the presence of oxygen, the activity of water and relative humidity, the bacterial density and water activity and synergetic effects (**Gardini *et al*., 2001**). The main producers of BA in milk and milk products are LAB including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Streptococcus* (**Bonetta *et al*., 2008; Calles-Enríquez *et al*., 2010; Linares *et.al*., 2012**). However, biogenic amine production by the *Lactococcus* strains has not been much documented.

de Llano *et al*., (1998) has reported high level of tyramine production by wild dairy *L. lactis* subsp. *lactis* in decarboxylase broth supplemented with amino acid precursors. But no tyramine production was detected in cultures of these strains grown in milk. However, evolution of tyramine production is observed in skim milk supplemented with tyrosine after 18 h of incubation. In contrast, **Durlu-Ozkaya *et al*., (2001)** report decarboxylase negative strains of *L. lactis* subsp. *lactis* isolated from Beyaz cheese made from raw ewes' milk. In the present study no histamine or tyramine production was detected in milk cultured with L. *lactis* subsp. *lactis* strain isolated from turmeric throughout the 48 h of incubation time.

CONCLUSION

This study showed that *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes carries potential probiotic characteristics and does not produce histamine and tyramine in fermented milk. This suggests *Lactococcus lactis* subsp. *lactis* isolated from turmeric as a potential non dairy based functional starter. However results of BA formation by the same strain in laboratory media will not imply the similar behavior in complex food matrixes. This makes the situation more complex and implies that tests on the probiotics for amine production in the food matrix into which it is planned to be applied should be done for positive BA producers. BA production capability should be an important criterion for choice of probiotic and starter cultures and only those strains not producing BA should be used as probiotics or functional starter cultures.

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