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Growth and Biogenic Amine (Histamine and Tyramine) Potential of Probiotic *Lactobacillus casei* in Skim Milk

¹W.M. Deepika Priyadarshani and ²Sudip Kumar Rakshit

¹Food Engineering and Bioprocess Technology, School of Environmental Resources and Development, Asian Institute of Technology, P.O. Box 4, Klong Luang, 12120, Pathumthani, Thailand ²Bio-Refining Research Institute, Department of Chemical Engineering, Lakehead University, Thunder Bay, Ontario, Canada

Corresponding Author: W.M. Deepika Priyadarshani, Food Engineering and Bioprocess Technology, School of Environmental Resources and Development, Asian Institute of Technology, P.O. Box 4, Klong Luang, 12120, Pathumthani, Thailand

ABSTRACT

The growth and biogenic amines (histamine and tyramine) potential of decarboxylase positive probiotic *Lactobacillus casei* (TISTR 389) was studied in skim milk. Fermentation was followed for 48 h at 37°C and samples were analysed for pH, log CFU mL⁻¹, percentage titratable acidity, proteolytic activity and histamine and tyramine. The maximum viable cell count 9.18 log CFU mL⁻¹ was observed after 24 h of incubation. The past reduction of pH was observed with concomitant increased in acid production during the fermentation. Proteolytic activity was fairly constant between 18-24 h and showed increase after 48 h of incubation. Histamine to a level of 6.87±0.091 mg L⁻¹ was detected after 24 h while tyramine level of 6.08±0.042 mg L⁻¹ was detected after 48 h of incubation. Our study reveals that careful screening for amino acid decarboxylase activity is essential before selecting lactic acid bacteria as appropriate starter or probitic strains in dairy industry.

Key words: Biogenic amines, histamine, tyramine, probiotic, Lactobacillus casei

INTRODUCTION

The contemporary trend today is using probiotic cultures in fermented dairy products and the demand for probiotic dairy products has been remarkably increased over the last two decades (Saarela et al., 2000). Probiotics are used as starter cultures and for their positive effect on human health (Lee and Salminen, 1995; Salminen et al., 1998; Saarela et al., 2000). Among the documented probiotics, Lactobacillus casei strains have been extensively studied and are widely use in dairy products. However, with the pronounced market success of probiotic products throughout the world potential health risk of consuming some of these products are raised and argued by researchers and consumers. Biogenic Amines (BA) are considered as undesirable metabolic product of probiotic or functional starter organisms with potential heath risk to consumers (Ammor and Mayo, 2007; Holzapfel et al., 1995; Leroy and De Vuyst, 2004).

Biogenic amines are organic basic compounds commonly present in living organisms. They can be naturally occurred in non fermented foods such as fruits, vegetables, meat, milk and fish. BA can also be produced in high amounts by microorganisms through the enzymatic activity of amino acid decarboxylase (Santos, 1996; Suzzi and Gardini, 2003). Histamine and tyramine are the most

extensively studied types of 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; Santos, 1996; Zaman *et al.*, 2009).

Biogenic amine formation in milk or milk products such as cheese is mainly affected by the availability of free amino acids and the presence of microorganisms possessing decarboxylase activity. In addition conditions favourable for microbial growth and decarboxylation are also important, such as pH, temperature, water activity, fermentation time, amine catabolism, presence of suitable cofactors (Fernandez-Garcia et al., 2000; Roig-Sagues et al., 1998; Stratton et al., 1991). Amino acid formation in milk is mainly occurred through the proteolytic activity of the starter bacteria and the rennet which contribute to the breakdown of milk protein, casein (Joosten, 1988).

Microorganisms possessing decarboxylase activity starter organisms (Fernandez-Garcia et al., 2000) or contaminating microorganisms arising in the production process (Roig-Sagues et al., 2002). Several types of starter cultures possessing amino acid decarboxyalse activity have been reported (Joosten, 1988; Stratton et al., 1991). Various species of Lactobacillus such as L. bulgaricus, L. casei and L. acidophilus are reported as histamine formers (Edwards and Sandine, 1981; Stratton et al., 1991) in milk where as Lactococcus lactis was observed to produce both histamine and tyramine (Chander et al., 1989). According to De Llano et al. (1998), Halasz et al. (1994) and Straub et al. (1995) amine forming ability should be one of the concern in selecting starter cultures. However, biogenic amine potential of starter or functional probiotic cultures in milk and milk products have not been studied extensively. Knowledge of the potential for production of amines in strain level at particular food system is essential to avoid their inclusion in starter or functional cultures.

Therefore, the main aim of the present study was to investigate the growth and formation of bioactive amines, histamine and tyramine by the probiotic *Lactobacillus casei* strain TISTR 389 in skim milk. In addition to this proteolytic activity of the strain in milk was also analyzed which might give further information on the formation of biogenic amines.

MATERIALS AND METHODS

Lactobacillus casei strain: The L. casei strain TISTR 389 identified as probiotic was obtained in freeze-dried form from Thailand Institute of Scientific and Technological Research (TISTR), Pathumthani, Thailand.

Preparation of inocula: Freeze dried culture was activated by growing it in de Man-Rogosa-Sharpe (MRS) (Merck, Darmstadt, Germany) broth for 24 h at 37°C. 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 and Holzapfel, 1999).

Preliminary qualitative investigation for biogenic amine production: The biogenic amine potential of probiotic *Lactobacillus casei* strain TISTR 389 was screened using the synthetic decarboxylase medium reported by Bover-Cid and Holzapfel (1999). The pH of the medium was adjusted to 5.3 by adding HCl and NaOH. Medium was autoclaved for 10 min at 121°C and aseptically poured to sterile plastic Petri dish. Decarbooxylase medium plates prepared without

amino acids were used as control. Subcultured strains (about 0.01 mL) were streaked in duplicate on the decarboxylase plates prepared with and without amino acids and incubated for 4 days at 37°C. Decarboxylase activity was identified based on the colour change or clear hallow around the tyrosine precipitates occurred.

Sample preparation: Powdered skim milk (Himedia, Mumbai, India) was reconstituted with distilled water (10%), sterilized at 121°C for 5 min according to the manufacturer's specification and analyzed for microorganisms, pH, titratable acidity and biogenic amines histamine and tyramine. The sub cultured strain (6×10⁷ CFU mL⁻¹) was aseptically added to the milk at the rate of 1% (v:v) and incubated at 37°C for 48 h. Samples were taken at 6 h interval within first 24 h and then after 24 h interval and analyzed for pH, titratable acidity, colony forming units and biogenic amines, histamine and tyramine. Two replicates were done for the analysis.

Sample analysis

Viable microorganisms: Samples of the sterilized reconstituted skim milk were analyzed for mesophilic bacteria (APHA, 1992). Mesophilic counts were obtained for 10° and 10⁻¹ dilutions on plate count and MRS agar after 48 h of incubation at 37°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, India) and inverted plates were incubated at 37°C for 48 h. Petridishes with 30-300 separate colonies with white, smooth appearance were selected for the enumeration and number of colony forming units was recorded mL⁻¹ of sample.

Chemical analysis: The pH was measured during fermentation 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. The percentage titratable acidity was determined by standard titration method with 0.1 N NaOH using phenolphthalein as an indicator. Percentage lactic acid was calculated according to the AOAC (2000):

Lactic acid (%) = [N of NaOH×mL of NaOH×Eq. wt of lactic acid/(mL of sample×10)]×100

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°C until BA analysis. The amines were separated and quantified by HPLC, following the procedure optimised in this laboratory (Priyadarshani and Rakshit, 2011) with a little modification, using the similar equipment and chromatographic conditions. The pre-column derivatization of acid extract was done similarly to the conditions described by Priyadarshani and Rakshit (2011) 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. Standard curves were prepared with recovery data obtained by spiking known amounts (0.1-200 mg L⁻¹) of standard histamine and tyramine to the milk samples followed by extraction and HPLC analysis. All the samples and replicates were injected at least in duplicate to the HPLC column. The quantity of each amine was expressed in mg L⁻¹ milk.

Measurement of proteolytic activity: The proteolytic activity of the strain TISTR 389 was determined by the Hull method (Hull, 1947). The method is based on the reaction of tyrosine and tryptophan released from the milk protein with phenol reagent. Reaction of amino acids with phenol reagent yielded the blue colour and absorbance was measured at 650 nm using a spectrophotometer (Model UV2, UV/Vis spectrometer, UNICAM, UK). Extent of proteolysis (mg tyrosine L⁻¹ milk) was calculated from a calibration curve obtained from series of tyrosine diluted in distilled water.

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

Decarboxylase activity of the strain *Lactobacillus casei* strain TISTR 389: The decarboxylase activity was observed in the probiotic *Lactobacillus casei* strain TISTR 389 on the screening medium plates after 4 days of incubation at 37°C. The positive reactions were observed when a purple colour occurred on the medium plates.

Characteristics of the sterilized reconstituted skim milk: The microbial counts of the sterilized skim milk are shown in Table 1. Very low count was detected showing their very little influence on amine accumulation during fermentation. The pH of the milk samples were varied between 6.6 to 6.8 and titratable acidities (as lactic acid) from 0.18 to 0.195%. 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.

Growth characteristics of *Lactobacillus casei* strain TISTR 389 in skim milk: The growth study showed that the probiotic *L. casei* strain TISTR 389 grow well in skim milk. Growth of the strain is shown in Fig. 1a. The initial viable cell count was 7.8 log CFU mL⁻¹. The strain attained maximum viable cell number of 9.2 log CFU mL⁻¹ after 24 h of incubation. The strain showed marked reduction in viable cell number after 24 h of incubation.

The pH of the skim milk decreased from 6.6 initially to 3.7 after 48 h of incubation (Fig. 1b). The significant (p<0.05) change in pH and percentage titratable acidity (Fig. 1c) was observed in milk inoculated with probiotic *L. casei*. A sharp reduction in pH was observed with concomitant increase in percentage titratable acidity in milk samples.

Table 1: Colony forming units of mesophilic bacteria in sterilized skim milk samples

	Colony forming units (mL ⁻¹)				
	Total plate count agar		MRS agar		
Milk samlpe	10°	10 ⁻¹	10°	10^{-1}	
1	0.5	0	0	0	
2	0.0	0	0	0	
3	0.0	O	0	0	

Values are mean value for duplicate analysis

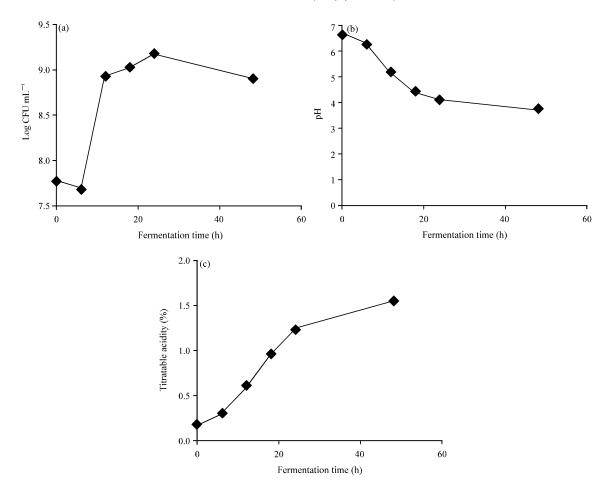


Fig. 1(a-c): Total colony forming units (a) Log CFU mL⁻¹ (b) pH and (c) Percentage titratable acidity of reconstituted and sterilized skim milk fermented with probiotic *Lactobacillus casei* strain TISTR 389 at 37°C. Results are the average of duplicate treatments

Table 2: Correlation coefficients and linear regression equations between recovered BA and peak areas in HPLC analysis

	Linear equation			
Biogenic amine	а	b	Correlation coefficient (R2)	
Histamine	460333	69183	0.9929	
Tyramine	303311	46289	0.9948	

y = a+bx, where, y is peak height and x is injected amount of amine (0.5-200 mg L⁻¹)

Biogenic amine (histamine and tyramine) formation by *L. casei* strain TISTR in skim milk: Quantification was done by exploration of the standard calibration curves prepared with 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 2. The correlation coefficient for both histamine and tyramine were found to be 0.99.

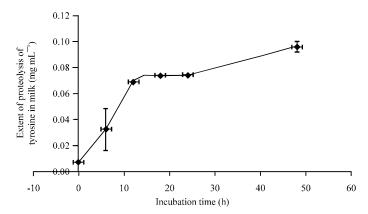


Fig. 2: Proteolytic pattern of *Lactobacillus casei* strain TISTR 389 grown in skim milk at 37°C. Each point indicates the mean and the SEM

Table 3: Biogenic amine production by strain TISTR 389 Lactobacillus casei grown in skim milk

	Biogenic amine content (mg L^{-1} milk)		
Time (h)		Tyramine	
0	ND	ND	
6	ND	ND	
12	ND	ND	
18	ND	ND	
24	6.87 ± 0.091^a	ND	
48	6.86 ± 0.035^{a}	6.08±0.042	

Values are expressed as Mean±Standard devitaion

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 observed for the probiotic *Lactobacilluc casei* strain TISTR 389 (Table 3). Formation of histamine after 24 h of incubation at 37°C was observed. However, tyramine was detected at the 48 h of incubation (Fig. 3). The quantity of histamine produced by the *Lactobacillus casei* strain TISTR 389 was not significantly changed between 24 and 48 h of incubation.

Proteolysis by probiotic *L. casei* grown in skim milk: The proteolytic activity of the strain TISTR 389 grown in skim milk was assessed according to the Hull (1947). The extent of proteolysis was calculated from the standard calibration curve (Fig. 2) obtained for series of dilutions of tyrosine in distilled water. Figure 2 shows the proteolytic activity of strain TISTR 389 grown in milk. The strain showed an increase of proteolytic activity in early stage of the growth and was fairly constant between 18-24 h of incubation and showed increase after 48 h of incubation period. *L. casei* grown in milk at pH 4.0-3.7 showed high proteolytic activity. The maximum growth occurred at 24 h of incubation which coincides to the possible high proteolytic detection after 48 h of sampling.

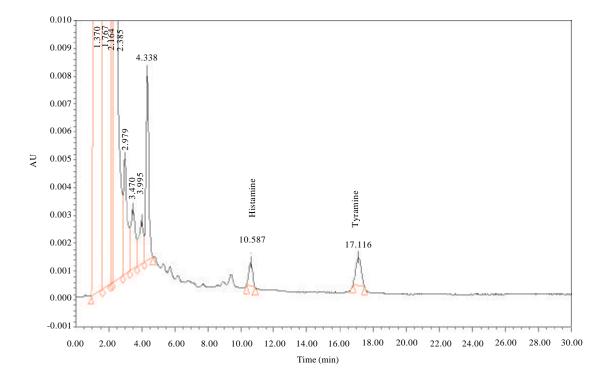


Fig. 3: Representative HPLC chromatogram of skim milk inoculated with *Lactobacillus casei* (TISTR 389) after 48 h of incubation at 37°C

DISCUSSION

As revealed by the results probiotic *Lactobacillus casei* strain TISTR 389 is found to be potential histamine and tyramine formers in the synthetic decarboxylase medium. Many authors have reported both histamine and tyramine potential of different L. casei strains isolated from different sources. Bover-Cid and Holzapfel (1999), in their study revealed the possibility of L. casei to produce tyramine with the reported amount of 3137 mgL⁻¹. However histamine was not detected in their study. In addition, Roig-Sagues et al. (2002) have identified eight decarboxylase positive L. casei strains isolated from Spanish traditional cheese. Seven of those strains were tyramine positive and one was a histamine positive strain. In contrast to the positive records of BA production by L. casei, many non histamine and tyramine producers were reported. Landete et al. (2005) reported 3 strains of non histamine producing L. casei isolated from wine while Moreno-Arribas et al. (2000) has reported L. casei strains of 393, 7469 and 8102 as non tyramine producers. In addition, Kalac et al. (2000), in their study revealed the commercial starter L. casei (CCM 3775) strain as non significant BA producer in sauerkraut. Also, Ergonul and Kundakci (2011), have observed probiotic L. casie to be a minute BA producer in Turkish fermented sausages. However they were considered as safe, because of the low levels of BA formed. As reported by Landete et al. (2007), L. casei do not belong to the common BA producers. The comparison of our preliminary screening results with those reported in literature revealed that the ability to produce BA is strain dependent rather than being related to the specific species. As the ability of the L. casei to produce BA is strain specific, it is important to do the screening on a case by case basis taking into account the product into which it is planned to be incorporated. Even though probiotic *Lactobacillus casei* is widely associated with dairy foods, to date there has been very little information on the role of probiotic *L. casei* in the formation of histamine and tyramine.

The microbial loads in sterilized milk samples were very low. Therefore, amine formation by these microorganisms would be neglected and irrelevant (Fernandez-Garcia et al., 2000). No histamine or tyramine was detected in the skim milk samples. However, presence of biogenic amines in cow's milk has been reported by many authors. Presence of biogenic amines like spermine, spermidine, cadaverine are reported by Bardocz (1993), Sanguansermsri et al. (1974) and Santos et al. (2003). In addition agmatine was also found in milk. This is anticipated to be present in milk as agmatine is the precursor for putrescine spermine and spermidine (Bardocz, 1993). Presence of histamine and tyramine was not reported and hence its presence in the milk indicates the microbial origin.

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 (Bolduc et al., 2006; Shah, 2000a). Moreover, their growth and survival was found to be affected by milk composition (chemical and microbilogical), amount of milk soilds and nutrient availability (Shah, 2000b). Literature suggested that ability of probiotic bacteria to grow and survive in milk is strain dependant rather than being related to the specific genera (Godward et al., 2000; Phillips et al., 2006). Metabolism of milk protein and milk sugar can vary among different strains of probiotic bacteria. Therefore, different growth patterns can be expected in different strains. However, present study revealed that the probiotic L. casei strain TISTR 389 exhibited good growth in skim milk.

Lactic acid bacteria such as *L. casei* require and able to use only complex organic nitrogen sources, such as amino acids. They also utilize peptides or proteins as nitrogen sources through proteolytic enzyme activity (Leitao *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). Many studies have reported that *L. casei* possesses proteolytic activity and release small peptides and amino acids (Hickey *et al.*, 1983; Khalid and Marth, 1990; Kojic *et al.*, 1991). The proteolytic activity of the strain TISTR 389 grown in skim milk was assessed according to the Hull (1947). Figure 2b shows the proteolytic activity of strain TISTR 389 grown in milk. The strain showed an increase of proteolytic activity in early stage of the growth and was fairly constant between 18-24 h of incubation and showed increase after 48 h of incubation period. As reported by El Soda *et al.* (1986) and Kojic *et al.* (1991), proteinases associated in cell wall of *L. casei* could be responsible for the degradation of protein in milk. Similarly to our results highest proteolytic activity of probiotic *L. casei* grown in skim milk was reported by Ong *et al.* (2007).

Many factors are needed to be fulfilled in order to accumulate BA in foods. 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). Therefore it seems that presence of histamine and tyramine could be expected in skim milk with decarboxylase positive L. casei possessing strong proteolytic activity.

As shown in the Table 3. *L. casei* strain TISTR 389 showed formation of histamine after 24 h of incubation at 37°C where as tyramine was detected at the 48 h of incubation. The amount of histamine was not significantly changed after 48 h of incubation. The formation of histamine and tyramine might be limited by the free histidine and tyrosine available in milk. Fresh milk contain very low amount of histidine, however, milk protein can comprise about 9.6 g of histidine per

100 mL (Jenness and Patton, 1976). Formation of biogenic amines in fermented milk or milk products such as cheese are governed by many factors such as presence of decarboxylase enzymes and activity of bacteria; availability of precursor amino acids; presence of cofactors; proteolysis; existence of better environment as influenced by pH; salt concentration; water availability; ripening and storage temperature; and amine catabolism etc. (Standara et al., 2000; Vale and Gloria, 1997; Vale and Gloria, 1998). Therefore, formation of histamine and tyramine after 24 h of incubation can be coincide with proteolytic activity of the strain and pH of the medium. Strain TISTR 389 reached pH 4.085 after 24 h incubation and was 3.765 at the 48 h of the incubation. This acidic environment could make favourable conditions for the amine formation. Therefore, strain TISTR 389 could be expected to produce higher amount of histamine and tyramine if use in prolong ripening processes such as in cheese.

Although the hazard levels for histamine and tyramine are dependent on the individual's efficacy in detoxification, there are many suggested upper limits for histamine and tyramine in literature (FDA, 2011; Ten Brink et al., 1990; Stratton et al., 1991). Probiotic L. casei strain TISTR 389 could be expected to produce toxicological reactions in individuals if they are added as starter or functional probiotic cultures in milk foods which requires prolong ripening such as cheese.

Referring to reported literature it can be concluded that results of BA formation by the same strain in synthetic laboratory media will not imply the similar behavior in food systems. This makes the situation more complex and implies that tests on the probiotics for biogenic amine production in the food matrix into which it is planned to be applied. Hence, 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.

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