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SCREENING OF SOME FERMENTED MILK FOR DECARBOXYLASE AND BIOGENIC AMINES PRODUCING MICROORGANISMS AND THEIR PUBLIC HEALTH SIGNIFICANCE

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ABSTRACT

The objectives of this study were based on screening for presence of decarboxylase producing microorganisms (Enterobacteriaceae, Enterococci and Lactobacilli) in sour milk, yoghurt and labenh, collected from supermarkets in Ismailia City. The isolated strains were tested for decarboxylase activity and the levels of histamine, tyramine, putrescine and cadaverine were determined in the examined samples. The results revealed that all the examined samples were contaminated with Enterobacteriaceae and Enterococci at variable counts. All the isolated strains of Enterobacteriaceae, Enterococci and *Lactobacillus* spp. have the potential to release decarboxylase enzymes with biogenic amines formation. The examined samples were found to contain the estimated amines with variable levels, expect yoghurt that didn't contain tyramine. In conclusion, there was a positive correlation between the type of microorganisms present in fermented milk and the type of biogenic amines formed. The prevention of biogenic amines formation in fermented milks could be achieved by following good hygienic measures and careful screening of lactic acid bacteria for amino acid decarboxylase activity before selecting as starter or probiotic strains in dairy industry.

Key words: *Decarboxylase bacteria, fermented milk, biogenic amines*

INTRODUCTION

Fermented milk products such as buttermilk, sour cream, yoghurt, sour milk and labenh are popular dairy products. The high nutritive value of the fermented milk, its attractive taste and extended shelf-life at low temperature and pH which reduce the multiplication of pathogenic microorganisms makes it a valuable food (Costa *et al.*, 2015). The addition of probiotics to these products increases their potential functional benefits with health, as they produce substances benefit for human health. However, other kinds of metabolites, such as biogenic amines may also be produced by starter culture and probiotic strains in fermented milk (Kongo *et al.*, 2006).

Biogenic amines can be formed in food primarily due to the release of specific amino acids and by the action of decarboxylases enzymes produced by certain microorganisms such as Enterobacteriaceae and Enterococci (Priyadarshani and Rakshit, 2011). The production property and storage period of fermented milk help the formation of biogenic amines by enhancing the activity of proteolytic microorganisms that increases the amount of free amino acids (Linares *et al.*, 2011). The levels of existence of biogenic amines are related to numerous factors, such as the composition and availability of free amino acids, water activity, storage time, storage temperature, pH of the product and the presence of decarboxylase-positive microorganisms. The pH is consider an important factor for fermentation and formation of biogenic amines. The amino acid decarboxylase enzyme is highly active in an acidic environment, with optimum pH around 5.0 (Schirone *et al.*, 2012). Ingestion of foods containing high levels of tyramine and histamine causes numerous outbreaks of food intoxication because they have vasoactive, psychoactive and toxicological properties. In addition, putrescine and cadaverine may potentiate the toxicity of these biogenic amines (Ȏzdestan and üren, 2010). Therefore, this study aimed to isolate and identify the decarboxylase producing microorganisms, and to determine their ability to release decarboxylase enzymes. Also, the levels of the most prevalent biogenic amines such as histamine, tyramine, putrescine and cadaverine in the fermented milk samples were estimated.

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MATERIALS AND METHODS

Collection of samples

A total of 45 samples (15 each) of sour milk, yoghurt and labenh were collected from supermarkets in Ismailia City, Egypt. The samples were transported promptly in an ice-box to the laboratory for microbial and biogenic amines estimation.

Microbiological and decarboxylase assessment

Enterobacteriaceae was enumerated using violet red bile glucose agar (OXOID) (Kornacki and Johnson, 2001). Enterococci count was performed using Kanamycin Escculin Azide agar (OXOID) according to Hartman *et al.* (2001). The isolated strains were identified in accordance with the protocol followed by Manero and Blanch (1999). Lactobacilli were isolated using DEMAN Rogosa Sharpe agar (MRS) (HiMedia) according to Gupta *et al*. (1996).

The identified Enterobacteriaceae and Enterococci strains were screened for decarboxylase activity using improved screening medium according to Joosten and Northolt (1989). The *lactobacillus* spp. were estimated for decarboxylase activity using MRS agar according to Bover-cid and Holzapfel (1999).

Biogenic amines assay

Twenty-five grams of homogenized samples were used for extraction of biogenic amines using the method of Antila *et al.* (1984). The standard amines, histamine-2HCl, tyramine-2HCl, putrescine-2HCl and cadaverine-2HCl were prepared using dansylchloride (Sigma) according to the method described by Antila *et al.* (1984). Ten µl of each sample extract were spotted onto thin layer chromatography plates (TLC) (Aluminum foil 20x20 cm coated with silica gel G 60) and after being developed in chloroform: benzene: triethylamine (6: 4.5: 1) for 17 cm heights, then dried. The resulted zones were examined and marked under long ultraviolet wavelength (360 nm). The marked areas were determined using CS-9000 dual wavelength flying spot scanning densitometer (SHIMADZU) using wavelength 254 nm. Standard curve of each dansylamine (standard amine) was used in the calculation of the concentration of biogenic amines expressed as mg/100g in the examined samples according to Ayesh *et al.* (1995).

Statistical analysis

Data were analyzed using the two-ways analysis of variance (ANOVA) according to the general linear model procedures. Logarithmic transformations were applied for all microbiological counts. Mean separations were done through Duncan's Multiple Range Test using the Statistical Analysis System, SAS 9.2, (SAS Institute Inc., 2009). Results were considered statistically significant at ($P \le 0.05$).

RESULTS

Table 1: Logarithmic counts of Enterobacteriaceae and Enterococci in examined samples

Microorganisms	Mean (Logarithmic) ± SE			*P*-value
	Sour milk	Yoghurt	Labenh	
Enterobacteriaceae	$3.74443^{b} \pm 0.13$	$3.63357^{b} \pm 0.26$	$4.82556^{a} \pm 0.12$	0.001
Enterococci	$4.19515^{b} \pm 0.13$	$3.59511^{c} \pm 0.19$	$5.13021^{a} \pm 0.11$	0.001

*Log counts with different superscripts showed significant differences at $P \le 0.05$.
*The original counts can be obtained by the antilogarithm (Base 10) of these results.

Table 2: Decarboxylase enzyme producing isolates in the examined samples

Isolates	Sour milk		Yoghurt		Labenh	
	No.	%	No.	%	No.	%
Enterobacteriaceae spp.						
E.coli	8	100	13	100	9	100
Enterobacter aerogenes	7	100	3	100	5	100
Citrobacter diversus	3	100	0	100	0	100
Proteus vulgaris	4	100	2	100	3	100
Klebsiella pnrumoniae	3	100	-	-	1	100
Enterococci spp.						
E.fecalis	12	100	15	100	11	100
E.faecium	3	100	-	-	4	100
**Lactobacillus* spp.	15	100	15	100	15	100

Table 3: Biogenic amines levels (mg/100g) in examined samples

Type of Amine	Sour milk	Yoghurt	Labenh	P-value
	Mean ±SE	Mean ±SE	Mean ±SE	
Histamine	$0.97^{a} \pm 0.13$	$0.56^{b} \pm 0.11$	$1.19^{a} \pm 0.12$	0.034
Tyramine	$1.38^{a} \pm 0.19$	0.000^{c}	$2.28^{b} \pm 0.13$	0.001
Putrescine	$0.92^{a} \pm 0.21$	$0.02^{c} \pm 0.004$	$1.51^{b} \pm 0.10$	0.001
Cadaverine	$0.46^{a} \pm 0.14$	$0.016^{c} \pm 0.002$	$1.44^{b} \pm 0.09$	0.001

*Different superscripts showed significant differences at $P \leq 0.05$.

Table 4: Correlations between counts of microbial groups and the type of biogenic amines

Type of biogenic amines	Microbial groups	
	Enterobacteriaceae	Enterococci
Histamine	**0.53****	**0.23**
Tyramine	**-0.04**	**0.31***
Putrescine	**0.22**	**0.06**
Cadaverine	**0.10**	**0.12**

-The range < ±0.5 indicates weak correlation
-The range > 0.5 indicates strong correlation
-1.0 indicates perfect correlation
* Correlation is significant at the 0.05 level $P \leq 0.05$.
** Correlation is highly significant at the 0.01 level $P \leq 0.01$.

DISCUSSION

The ability of microorganisms to form biogenic amines was considered as strain specific property rather than a species related property. Amine formation was recognized as a defense mechanism of microorganisms against an acidic environment. Moreover, some strains with decarboxylase activity could overcome or reduce the effects of temperature, sodium chloride and other biological and chemico-physical factors that induce stress responses in the bacterial cells by the production of some biogenic amines (Karovičová and Kohajdov, 2005).

Data illustrated in the Table 1 revealed that all the examined samples contained Enterobacteriaceae and enterococci at variable counts. The highest bacterial counts were recorded in labenh, sour milk and yoghurt, consequently. All bacterial strains of Enterobacteriaceae, enterococci and *Lactobacillus* spp. isolated from the fermented milk samples had the ability to release decarboxylase enzymes with biogenic amines formation (Table 2). These results were consistent with Calles-Enríquez *et al.* (2010) and La Gioia *et al.* (2011) who reported the presence of tyrosine and histamine decarboxylase activity in strains from various starter cultures. Priyadarshani and Rakshit (2011) suggested that amine formation by starter culture is strain dependent and not related to the species in which *Lactobacillus casei* and *Lactobacillus delbrueckii subsp. bulgaricus* were found to produce biogenic amines. While, biogenic amines formation was not released by *Lactobacillus acidophilus*, *Lactobacillus lactis subsp. Lactis*, *Lactococcus lactis subsp. lactis* and *Lactobacillus plantarum* strains.

Concerning the concentration of biogenic amines, high level of histamine was detected in labenh and sour milk then yoghurt, consequently. There was a significant difference of the levels of histamine between sour milk and yoghurt, as well as between labenh and yoghurt (Table 3). Costa *et al.* (2015) detected histamine in fermented cow's milk (average 1.79 mg/100g) and in fermented goat's milk (average 5.3 mg/100g). On the other hand, lower histamine concentration (average 0.31 mg/100g) was detected in sour milk that previously reported by Magwamba *et al.* (2010). Bodmer *et al.* (1999) reported higher level of histamine with a mean value of 1.3 mg/100g in yoghurt. Sömer and Kılıç (2012) couldn't detect histamine in any of the examined labenh samples. The presence of histamine in food represents public health significant and has physiological and toxicological effects. Parente *et al.* (2001) reported that histamine intake in a level of 40–100 mg and higher than 100 mg causes intermediate and intensive poisoning, respectively. Moreover, Histamine poisoning occurs after the consumption of food containing biogenic amines at concentrations higher than 500 ppm and manifests by an allergic reaction (Gonzaga *et al.*, 2009). Yongmei *et al.* (2009) added that the predisposing factors of histamine poisoning are people having deficient in natural mechanisms for detoxifying biogenic amines through genetic reasons or through inhibition by the intake of antidepressant medicines, such as monoamine oxidase inhibitors (MAOIs).

Regarding tyramine, it was prevalent in labenh (average 2.28 ± 0.13 mg /100g), followed by sour milk (average 1.38 ± 0.19 mg/100g), whereas tyramine didn't detected in yoghurt samples. There was a high significant difference (P> 0.001) in levels among all fermented milk products examined (Table 3). Costa *et al.* (2015) estimated higher results (24.5 and 33.3 mg /100g) in sour cow's and goat's milks, respectively. On contrary, Sömer and Kılıç (2012) found that none of the examined labenh samples contained tyramine. Tyramine usually formed in fermented dairy products from free amino acid tyrosine, which is further decarboxylated by lactic acid bacteria. It was considered as initiator of dietary induced migraine and hypertensive crisis. Levels of 100–800 mg tyramine have been reported as toxic doses in food similar in symptoms to histamine poisoning (Silla-Santos, 1996; Ôzdestan and üren, 2010).

Putrescin and cadaverine were detected in higher levels in labenh, followed by sour milk then yoghurt. There was a high significant difference (P> 0.001) in Putrescin and cadaverine levels among all fermented milk products examined (Table 3). Higher levels of putrescine (3mg/100g) were detected in yoghurt by Eliassen *et al.* (2002). However, Sömer and Kılıç (2012) couldn't detect these amines in all labenh samples examined.

Tyramine and putrescine are vasoactive amines that increase blood pressure leading to heart failure or brain hemorrhage. Presence of putrescine and cadaverine, at high concentrations enhances the toxicity of histamine through the inhibition of histamine oxidizing enzymes (Emborg *et al.*, 2005). Different studies revealed that some strains of lactobacilli and *S. thermophilus* can produce cadaverine in dairy products (Gezginc *et al.*, 2013).

There was a highly significant positive correlation ($P \leq 0.01$) between histamine and Enterobacteriaceae, as well as a significant positive correlation ($P \leq 0.05$) between tyramine and enterococci (Table 4). Enterobacteriaceae and enterococci were found to be active in formation of biogenic amines. These results were supported by Marino *et al.* (2000), who reported that Gram-negative bacteria, mainly Enterobacteriaceae were able to produce histamine, putrescine and cadaverine. Also, Bover-Cid *et al.* (2001) stated that enterococci were considered as an important tyramine producer in association with fermented foods. The presence of biogenic amines in foods has traditionally been used as an indicator of undesired microbial activity. High levels of certain amines have also been reported to indicate the deterioration of food products and/or their defective manufacture (Fernández *et al.,* 2007).

It's clearly evident from the obtained results that despite short storage period of fermented milks, it was found to contain biogenic amines at variable levels that may constitute a public health hazards. In addition, there was a correlation between the type of microorganisms and biogenic amines formation. Therefore, the prevention of biogenic amines formation in fermented milks could be achieved by using temperature control, high-quality raw material, good manufacturing and hygienic practices with careful screening of lactic acid bacteria for amino acid decarboxylase activity before selecting as starter or probiotic strains in dairy industry.

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فحص بعض الألبان المتخمرة للميكروبات المفرزة لأنزيم الديكربوكسيلاز والأمينات الحيوية وأهميتهم الصحية

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تهدف هذه الدراسة الى الكشف عن تواجد الميكروبات المفرزة لأنزيم الديكربوكسيلاز مثل مجموعة البكتريا المعوية والكريات المعوية والميكروبات العصوية في اللبن الرايب والزبادي واللبنة والتي تم جمعها من أسواق مدينة الاسماعيلية. وتم اختبار قدرة الميكروبات المعزولة على افراز أنزيم الديكربوكسيلاز ومستويات الهيستامين والتيرامين والبيوترسين والكادافيرين في العينات التي تم فحصها. وأظهرت النتائج أن العينات كانت تحتوي على البكتريا المعوية والكريات المعوية والميكروبات العصوية بأعداد مختلفة وأن جميع العترات المعزولة كانت لها القدرة على افراز انزيم الديكربوكسيلاز وتكوين الأمينات الحيوية وأن العينات التي تم فحصها كانت تحتوي على الأمينات الحيوية المختبرة ماعدا الزبادي فقد وجد أنه لا يحتوي على التيرامين. وخلصت الدراسة الى أن هناك علاقة ايجابية بين نوع الميكروبات الموجودة في الألبان المتخمرة ونوع الأمينات الحيوية. ولهذا يجب اتباع الاجراءات الصحية الجيدة وفحص البادئات بعناية من حيث قدرتهاعلى افراز انزيم الديكربوكسيلاز قبل استخدامها في تصنيع منتجات الألبان المتخمرة وذلك للسيطرة على تكوين الأمينات الحيوية.