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Isolation and Molecular Identification of a Novel Tyramine-producing Bacterium, *Rummeliibacillus pycnus*

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Abstract: Decarboxylation of tyrosine through tyrosine decarboxylase enzyme produces tyramine which may represent a serious threat to public health, as it may cause severe toxicological effects. Intake of tyramine will invariably result in acute symptoms as it can rapidly gain access to the bloodstream and to various organs where it can act as a vasoactive agent through interaction with the sympathetic noradrenergic nerve terminals innervating cardiac and vascular smooth muscle tissues. This research is focusing on tyramine as one of the indicators of inappropriate food storage and processing conditions of milk products. In the current study, 25 dairy product samples were collected from local markets in Alexandria, Egypt, and were analyzed for bacterial contamination and prevalence of tyramine-producing bacteria by PCR using degenerate primers (DEC5/DEC3). While cream was the most contaminated dairy product, balady yogurt samples were the least contaminated ones. Different types of tyraminogenic bacteria have been isolated from the collected dairy products. Cream samples were contaminated with the widest variety of tyraminogenic bacteria among the isolated samples including *Bacillus pumilis, Escherichia coli, Enterococcus faecium, and Proteus mirabilis*. A total of 35 strains harboring tyrosine decarboxylase gene were detected with the identification of a novel tyramine-producing strain: *Rummeliibacillus pycnus*. These results indicate the promising application of degenerate primers (DEC5/DEC3) to detect tyramine production in dairy products, a goal that has been regarded as a challenge by manufacturers.

Keywords: Tyramine, Dairy Products, Tyrosine Decarboxylase, PCR, HPLC

1. Introduction

Different food safety organizations are directing their efforts nowadays towards an increasing exploration of metabolites that could adversely affect the human health. Among these compounds are the biogenic amines (BAs), the presence of which in various food products is a public concern for the food industry. Fermented, pickled food, and dairy products can accumulate BAs, with concentrations varying from just traces to more than 1000 mg kg^{-1} [1]. The existence of BAs in a food product is influenced by several factors including the availability of precursor amino acids, the presence of amino acid-decarboxylating microorganisms, adequate pH, temperature, salt content, and water activity [2].

Tyramine is the most common BA generated by the decarboxylation of tyrosine amino acid through tyrosine decarboxylase (TDC) enzymes derived from the bacteria present in food [3]. Under normal conditions, tyramine ingested with food is rapidly detoxified by the action of amine oxidases, but if the detoxification process is interrupted, or excessive amount of tyramine is present in food, tyramine can accumulate in blood resulting in an intoxication designated as “cheese reaction”. This severe acute effect is characterized by symptoms including migraine, nausea, vomiting, accelerated respiration, increased cardiac output and a hypertensive crisis [4, 5]. For unmedicated adults, the ingestion of 200–800 mg of dietary tyramine is

reported to induce a mild (30 mm Hg) rise in blood pressure [6] but the situation is more problematic for susceptible individuals including those prescribed antidepressant drugs classed as monoamine oxidase inhibitor drugs or those with an impaired detoxification system [7].

Tyramine-producing bacteria, especially those present in dairy products, have received particular attention with many bacterial isolates being reported. Within the genera Enterococcus, *Enterococcus faecalis* and *Enterococcus faecium*, are recognized as the most efficient tyramine producers [8]. Lactobacilli, such as *Lactobacillus curvatus* and *L. brevis* have demonstrated ability to produce tyramine especially when grown in cheese [9]. Other Gram-positive bacteria such as *Leuconostoc*, *Lactococcus* [10] and *Staphylococcus* may also have a role in the production of tyramine [11].

The tyramine decarboxylase pathway is encoded by a locus consisting of four genes [4]. The first gene (*tyrS*) encodes an aminoacyl transfer RNA (tRNA) synthetase; the second gene (*tdc*) corresponds to the tyrosine decarboxylase, the third gene (*tyrP*) codes for the tyrosine/tyramine exchanger, while the last gene (*nhaC-2*) is a Na^+/H^+ antiporter, the involvement of which in the biosynthesis of tyramine remains unknown [1, 12].

The molecular knowledge of the genes involved in tyramine production has led to the development of molecular methods for the detection of bacteria able to produce tyramine. A rapid and simple method has been used in the present study for the detection of the ability of bacteria to produce tyramine in order to evaluate the potential risk of tyramine biosynthesis in different dairy products.

2. Materials and Methods

2.1. Dairy Product Samples

A total of 25 samples of different types of soft dairy products were collected from local markets in Alexandria, Egypt, over a period of 6 months (from February to August 2019) and were stored at 4°C until usage. The samples comprised the following: two commercial white creamy cheese, three Feta cheese, five processed cheese, two cottage cheese, one Mozzarella cheese, one Kiri cheese, two cream, two commercial milk, two balady yogurts and five manufactured yogurts.

2.2. Microbiological Methods

A sample of one g of each of the dairy product sample was transferred aseptically into 5 mL of Luria-Bertani (LB) broth (prepared by adding 10 g tryptone, 10 g NaCl and 5 g yeast extract to 1 L of distilled water) and homogenized (using a homogenizer; T 25 digital ULTRA-TURRAX® IKA, labortechnik, Staufen, Germany) for 90 s. Decimal dilutions of the homogenized samples were performed, in duplicates, plated on LB agar and incubated for 24 hr at 37°C. The total viable count was expressed as the number of colony forming units per millilitre per gram of dairy product (CFU/mL/g). Subsequently, the morphologically different types of bacteria were separated and purified by streaking on LB agar plate and incubated aerobically at 37°C for 24 hr [13].

2.3. Detection of Tyrosine Decarboxylase (tdc) Gene

Total genomic DNA was extracted from the isolated bacteria using phenol/chloroform extraction method [14]. The isolated DNA was used as a template for the amplification of *tdc* gene with forward primer DEC5 5' CGTTGTTGGTGTTGTTGGCACNACNGARGARG 3' and reverse primer DEC3 5'CCGCCAGCAGAATATGGAAYRTANCCCAT 3' [15]. An initial denaturation at 94°C for 5 min was followed by 5 cycles of denaturation at 94°C for 30 s then, an annealing step at 47°C for 90 s and an elongation step at 72°C for 90 s. This was followed by 30 cycles starting with denaturation at 90°C for 30 s, then annealing at 50°C for 60 s and elongation at 72°C for 60 s with a final extension step at 72°C for 7 min in a Thermocycler (Bio-Rad, Singapore). The PCR products were then separated in 2% agarose gel electrophoresis (Bio-Rad, Singapore) set at a voltage of 100 V for 30 min [15].

2.4. Identification of Tyraminogenic Bacterial Isolates

Preliminary characterization of the PCR positive isolates was performed by Gram staining. These isolates were then identified by Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (Bruker daltonik MALDI biotyper, USA). Using this technique, the bacteria within the sample were identified to the most related spectra contained in the database [16]. The isolates were confirmed consequently using sequencing analysis of 16S rRNA where PCR was used to amplify an amplicon of about 1000 bp corresponding to 16S rRNA gene for the tested isolates using the 16S rRNA forward primer; 5' AGAGTTTGATCMTGGCTCAG 3' and the 16S rRNA reverse primer 5' TACGGYACCTTGTTACGACTT 3' The following temperature cycling profiles were applied: initial denaturation step at 95°C for 5 min, then followed by 35 cycles of denaturation at 90°C for 30 s, annealing at 52°C for 60 s and extension at 72°C for 60 s, with a final extension step at 72°C for 7 min in the Thermocycler [17].

2.5. Sequencing Analysis

The obtained PCR products of the *tdc* gene bands fractioned in the gel were excised using a surgical blade and transferred onto a wool glass filter laid at the bottom of a 500 µL-centrifuge tube inserted in a 1.5 mL-Eppendorf tube. The Eppendorf tube was centrifuged at 10,000 rpm for 10 min. The aqueous fraction containing DNA was collected in the Eppendorf tube for sequencing [18]. Partial sequences were generated at the sequencing facility, Institute of Genetic Engineering, City of Scientific Research and Technological Applications, Borg El-Arab, Alexandria, Egypt, using the 16S rRNA and the DEC5 and DEC3 forward and reverse primers following the conditions described earlier. The obtained gene sequences were then compared to those

available on the public databases using BLAST search in the National Centre for Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov) and identified according to the closest relative.

2.6. Nucleotide Sequence Submission

The *tdc* fragment as well as 16S rRNA sequences obtained were submitted to the GenBank databases and accession numbers were acquired.

2.7. Evaluation of Tyramine Production by High Performance Liquid Chromatography (HPLC)

HPLC (Agilent 1100, Germany) was performed to qualitatively evaluate tyramine production [19] from the previously unreported tyraminogenic isolates in parallel with two well reported tyramine producing strains; *Enterococcus faecalis* and *Bacillus thuringiensis* [4]. Tyramine standard was prepared by adding 8 mg of tyramine HCl (HPLC grade, Sigma, USA) to 2 mL of mobile phase (0.02 M disodium hydrogen phosphate and acetonitrile pH 8.4 adjusted by 10% phosphoric acid). The standard tyramine solution was directly solubilized in the mobile phase and injected into HPLC without derivatization [4]. For sample preparation, an overnight subculture of each of the tested isolates in tyramine-producing medium, TPM, was acidified by 1M HCl to reach a pH of 5.5 [20], sonicated for 10 min, centrifuged at 3000 rpm for 20 min and the supernatant was dried by lyophilization. TPM was prepared by adding 8 g of meat extract, 5 g of tryptone, 4 g of yeast extract, 3 g of tyrosine, 1.5 g of glucose, 1 g of fructose, 0.5 g of tween 80, 0.2 g of $MgSO_4$, 0.1 g of $CaCO_3$, 0.05 g of $MnSO_4$, 0.04 g of $FeSO_4$, and 4 g of vitamin B_6, as a modification from pyridoxal 5' -phosphate, to 1 L of distilled water [21].

Subsequently, each sample was solubilized into 1 mL mobile phase, filtered using micro-syringe filter and then introduced into the HPLC without derivatization. A reversed-phase C18 column (Phenomenex Luna) with a pore size of 10 µm and dimensions of 250 × 4.60 mm was used with its temperature adjusted at 35°C. The elution program consisted of a gradient system with a flowrate of 0.5 mL/min. The resultant peaks were detected by Agilent 1100 UV–Vis spectrophotometer (HP/ Agilent 1100 Series G1310A, Canada) adjusted at 278 nm.

3. Results

3.1. Determination of the Total Viable Count

The average bacterial count of the different soft dairy product samples ranged from 0.02×10^9 to 4.85×10^9 CFU/mL/g of dairy product. Balady yogurt displayed the lowest count (0.02×10^9 CFU/mL/g) followed by the cottage cheese (0.45×10^9 CFU/mL/g). The cream samples demonstrated the highest count (4.85×10^9 CFU/mL/g) among the tested samples "Figure 1". Upon purification of the bacterial mixture obtained from each dairy sample, 35 morphologically different isolates were obtained from the tested dairy products, with each sample providing one or more morphologically different isolates.

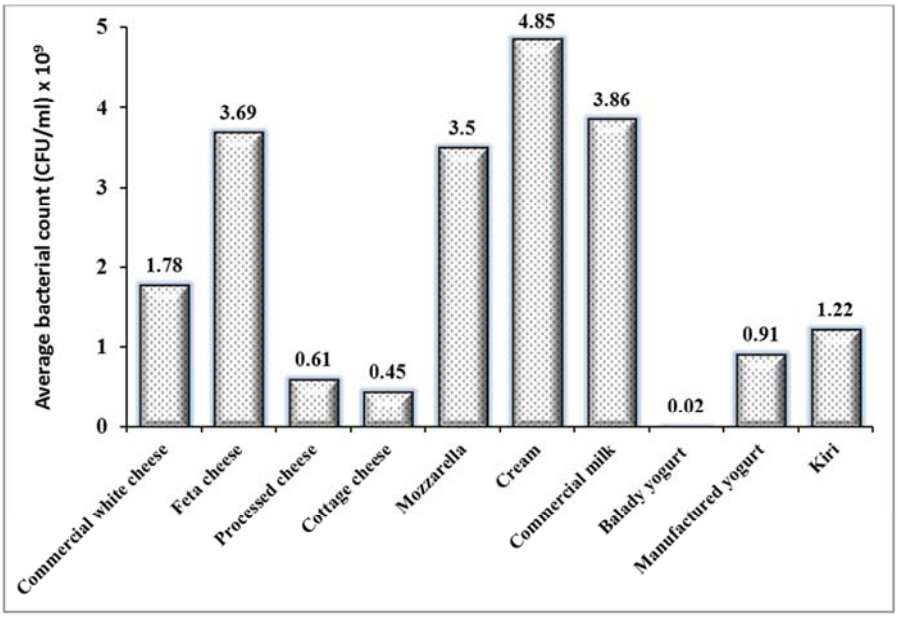

Figure 1. *Average bacterial count (CFU/mL/g) among the collected dairy product samples.*

3.2. Molecular Detection of Tyrosine Decarboxylase Gene

The total genomic DNA extracted from the 35 isolates provided templates for the amplification of *tdc* gene using PCR to identify the ability of the isolates obtained from different dairy samples to produce tyramine. Previously described PCR primers [15] were used to amplify an amplicon of 350 bp corresponding to a fragment of *tdc* gene. Out of 35 isolated strains, 9 were found to possess *tdc* gene and thus were confirmed to be tyraminogenic bacteria. Some of the samples provided more than one tyraminogenic species

"Figure 2".

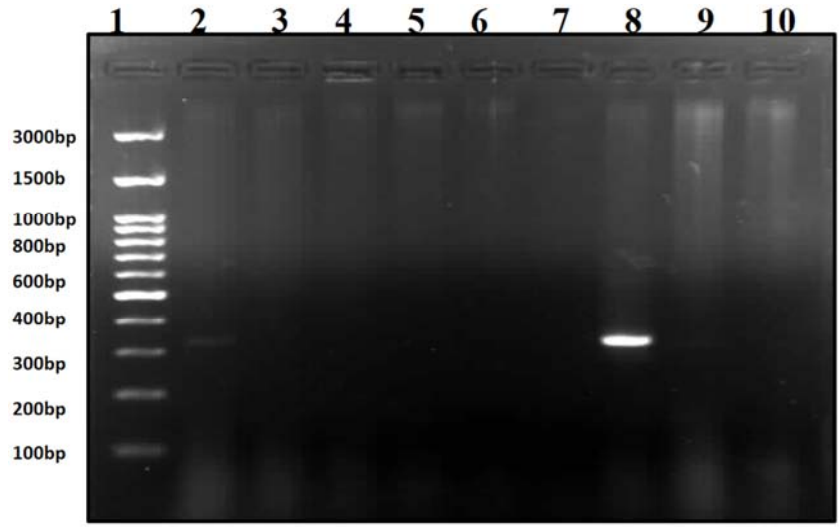

Figure 2. *Agarose gel showing PCR amplicons of tdc gene with a size of 350 bp. Lane 1 is loaded with 100 bp DNA ladder. Positive result is shown in lane 8.*

The analysis of *tdc* gene prevalence in bacteria isolated from soft dairy products revealed that 100% of the cream samples contained tyramine producers "Figure 3". On the contrary, the collected samples of Feta, Mozzarella, Kiri and processed cheese, as well as samples of commercial milk were not contaminated with any tyraminogenic bacteria. Half of the commercial white cheese, cottage cheese and balady yogurt samples were found to contain tyramine producers. Only 20% of manufactured yogurt samples contained tyraminogenic strains. Overall, 24% of the collected soft dairy product samples contained tyraminogenic isolates.

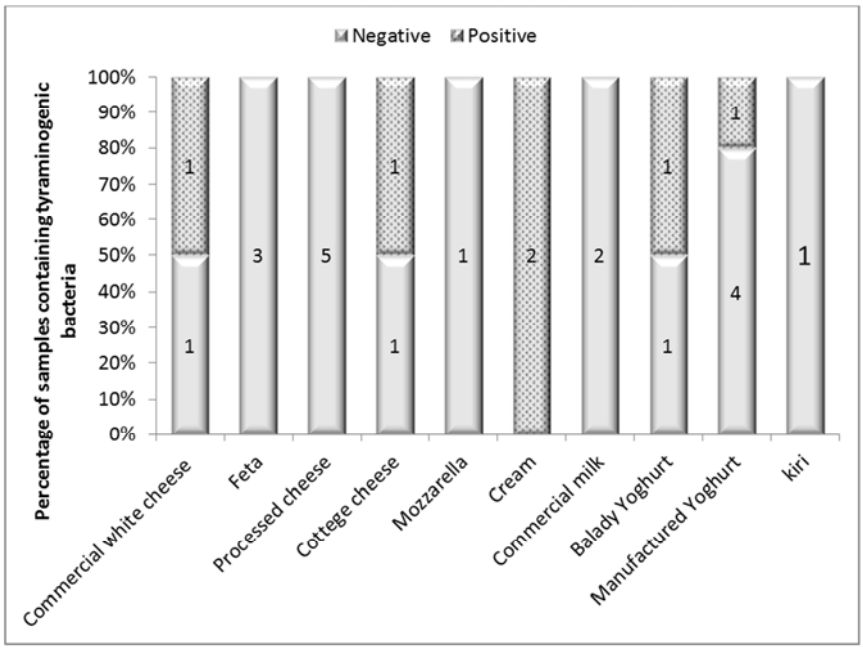

Figure 3. *Dairy product samples containing tyraminogenic bacteria.*

3.3. Phenotypic and Species-level Identification of Tyramine Producers

The isolates were segregated by MALDI-TOF-MS into 7 Gram positive and 2 Gram negative tyramine producers (Table 1). The Gram positive species comprised: *Enterococcus faecium* (2 isolates), *Lactococcus lactis* (1), *Rummeliibacillus pycnus* (1), *Bacillus pumilis* (2) *and Bacillus thuringiensis* (1). While the Gram negative species were confined to: *Proteus mirabilis* (1) and *Escherichia coli* (1). Analysis of MALDI-TOF-MS results revealed that cream was the dairy product with the highest frequency content of different types of tyramine-producing strains followed by the manufactured yogurt samples. On the other hand, balady yogurt, commercial white cheese and cottage cheese samples were associated with the lowest frequency of different types of tyramine producers as shown in "Figure 4".

Table 1. *Types of bacteria contaminating the tested dairy product samples.*

Dairy product sample containing tyraminogenic bacteria	Identified organisms
Cream	*Bacillus pumilis* + *Escherichia coli* + *Enterococcus faecium* + *Proteus mirabilis*
Balady yogurt	*Bacillus thuringiensis*
Manufactured yogurt	*Enterococcus faecium* + *Bacillus pumilis*
Commercial white cheese	*Rummeliibacillus pycnus*
Cottage cheese	*Lactococcus lactis*

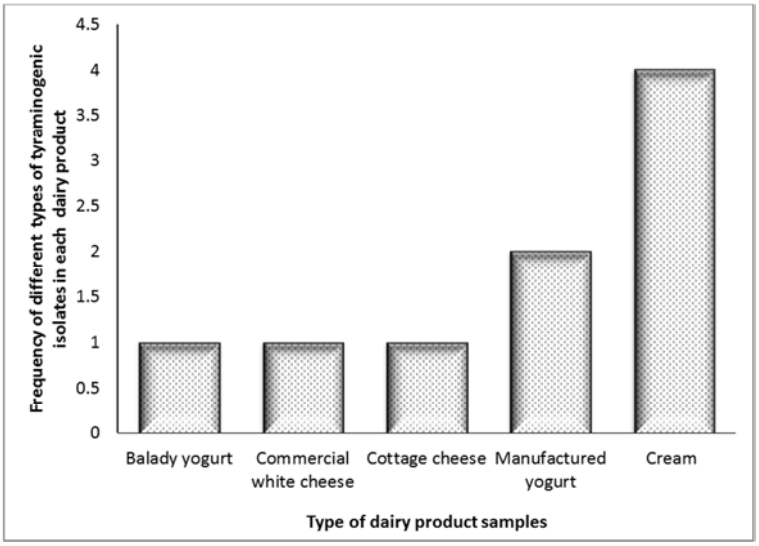

Figure 4. *Frequency of the tyraminogenic bacteria among tested dairy product samples.*

EF	ACGCGATGAATTAATGAAAGACGGTATTTACTATTATGTACACGTGGATG	50
RP	**********G**********	50
EF	CTGCTTATGGTGGTTATGGACGTGCCATCTTCTTAGACGAAGACAACAAC	100
RP	**********	100
EF	TTCATCCCTTACGAAGATTTACAAGATGTTCACGAAGAATACGGTGTCTT	150
RP	**********	150
EF	CAAAGAGAAAAAAGAACACATTTCAAGAGAAGTGTATGATGCATATAA	200
RP	**********	200
EF	AGCAATCGAATTAGCAGAATCAGTAACAATTGACCCTCATAAAATGGGT	250
RP	**********	250
EF	TATATCCCTTATTCAGCTGGTGGTAT	276
RP	**********T**********	276

Figure 5. *Sequence alignment of the tdc gene amplicon (284 bp) of R. pycnus (RP) with Enterococcus faecalis (EF). Asterisks and dashes, introduced to maximize similarity, indicate nucleotides identity and gaps, respectively.*

From the literature review, it was confirmed that a single strain, *Rummeliibacillus pycnus*, was not previously reported as tyraminogenic bacteria and hence, was identified as a novel tyramine-producing strain. Further identification by sequencing analysis of the 16S rRNA gene of *R. pycnus* was performed. Sequences of the 16S rRNA and the *tdc* gene fragment generated by *R. pycnus* were then submitted to the GenBank databases and accession numbers were acquired; SAMN09924829 and MH796760 for *tdc* gene and 16S rRNA gene fragment, respectively. The amplified *tdc* fragment obtained from *R. pycnus* showed 99% identity to the *tdc* FN392111 sequence of *E. faecalis* strain CNRZ1535 "Figure 5".

3.4. Evaluation of Tyramine Production by HPLC

The tyramine-producing ability of *R. pycnus* was confirmed

by HPLC using a column temperature of 35°C, a flow rate of 0.5 mL/min and the UV detector being adjusted at 278 nm. Under these conditions, a peak was obtained at a retention time of 7 min identical to that of the standard tyramine sample "Figure 6". The tyramine content was deduced by calculating area under the curve (AUC) of the tyramine standard peak and comparing it with the AUC of the sample. *R. pycnus* yielded a high level of tyramine (1294 mg/L).

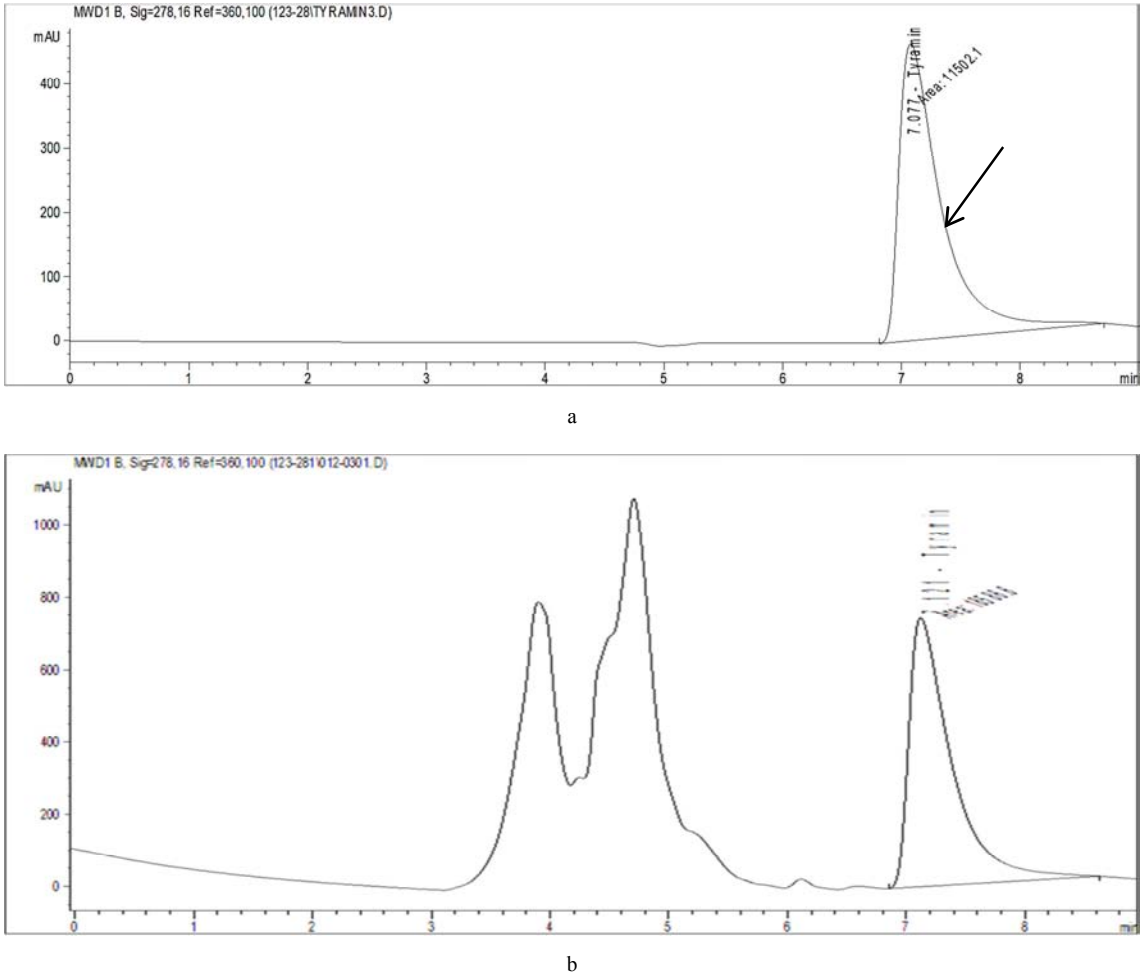

Figure 6. *(a). HPLC chromatogram of standard tyramine (b). HPLC chromatogram of R. pycnus showing an obvious tyramine peak (arrow) at 7 min.*

4. Discussion

Different dairy products can provide an adequate medium for the growth of many microorganisms, including those producing toxic metabolites and they have been frequently associated with foodborne intoxications due to their contamination with preformed toxins of microbial origin, including BAs [22]. Although, cream should be exposed to elevated temperatures through manufacturing process to increase its shelf life [23], both of the cream samples collected from the local markets in this study contained the highest average bacterial count. Following cream, commercial milk samples contained the second highest number of bacteria as compared to the other dairy products. These organisms could have been present as contaminants in raw milk or might have contaminated milk at various points during production or processing or might have existed due to inadequate packaging system or improper temperature control [24].

Karish or cottage cheese, a soft acid cheese made from skimmed cows' milk, buffalo milk or buttermilk, was found to be contaminated with a relatively low bacterial count reaching 0.45×10^9 CFU/mL/g. As indicated in the literature, brucella spp., *E. coli*, and *Listeria monocytogenes* can survive during the ripening and storage of soft cheese such as raw milk cheese, cottage, and Queso-fresco cheese [25]. The observed low bacterial content may be explained by the short ripening time required during its production, brief storage period, short-term expiry date and the fact that this type of cheese is expected to reach the consumer directly after manufacturing without any delay in the transportation process.

White cheese, or Domiati cheese, is a traditional soft white cheese pickled in brine found in the Middle East, especially in Egypt. It was found to contain a relatively high bacterial count of 1.78×10^9 CFU/mL/g. Contamination might have occurred during the transfer of the cheese to the tins filled with brine or developed from the non-hygienic process of cutting and weighing the cheese before delivering it to the consumer. Feta cheese contained a considerable number of

bacteria which could have entered this semi-hard cheese during the production process. The low water activity in both Domiati and Feta cheese is supposed to act as a limiting factor for bacterial growth, nevertheless, surface contamination could happen during the shelf-life resulting in high microbial count [26].

Processed cheese is produced by blending shredded natural cheese with emulsifying agents and different spices, then heating the blend under a partial vacuum with constant agitation until a homogeneous mass is obtained. Since the usual heat treatment during processing is relatively mild, processed cheeses are not sterile [27]. This explains the bacterial content of the collected processed cheese samples throughout this study to be as high as 0.61×10^9 CFU/mL/g.

During the manufacturing of Mozzarella cheese, the curd should reach a pH of 5.2, then it is subjected to kneading, plasticizing and molding processes [28]. In the present study, the Mozzarella cheese sample contained one of the highest bacterial counts; 3.5×10^9 CFU/mL/g. This type of cheese is sold mainly as shredded cheese, a factor that increases the surface area of the cheese, allowing high levels of contamination. The pH used during the manufacturing process of Mozzarella cheese could account for its contamination as well, being a value favored by different types of microorganisms.

The present study revealed that balady yogurt samples contained the least bacterial count, a fact that could be explained by the short shelf life of this product, a high temperature during its production process and the brief fermentation period required.

The ability to detect the presence of tyramine-producing microorganism in raw materials or in the early stages of manufacture could reduce tyramine accumulation in the final product. Several analytical and microbiological methods have been developed to detect the presence of tyramine or tyramine-producing microorganisms in different food products [29, 30]. Molecular characterization of *tdc* gene was performed using DEC5/DEC3 primers for screening 35 DNA samples isolated from bacteria contaminating the collected 25 dairy products samples. PCR screening results revealed that about 24% of the selected dairy products samples were contaminated by tyraminogenic strains, a percentage lower than the one reported by Ladero, *et al*. who detected tyramine in 56.1% of Spanish cheese samples [31]. The collected samples of Feta cheese, processed cheese, commercial milk, Mozzarella and Kiri were not contaminated by tyramine-producers. In accordance with our results, a study investigating the effect of the hygienic quality of milk on changes in microbial count and BA content reported that raw milk did not contain any detectable level of tyramine [32]. Similarly, the low pH and high salt content of Feta cheese was reported by Valsamaki *et al*. to create unfavorable conditions for amino acid decarboxylation, keeping the level of tyramine relatively low [33].

On the other hand, cream, balady yogurts, commercial white cheese and cottage cheese samples were associated with a considerable number of tyramine producers. The existence of tyramine-producing bacteria in these samples is highly influenced by the type of milk used, as mostly of these local products are manufactured from unpasteurized milk [34, 35].

Identifying microorganisms producing tyramine early in the dairy product-making process enables manufacturers to uncover and remedy potential sources of contamination quickly and thus minimize the risk of a product recall. MALDI-TOF-MS and 16srRNA sequencing results revealed that enterococcus spp. and bacillus spp. were the most common tyramine producers detected in 16% of collected samples. This observation is consolidated by many other studies reporting that the most abundant tyraminogenic species in dairy products were enterococci [36-38].

Searching the data base, GenBank and the mostly known academic research engines, we could not find a record for the tyraminogenic activity of *R. pycnus* which was hence considered to be a novel tyramine-producing bacterium. Alignment of nucleotide sequence of the target region of *tdc* gene of *R. pycnus* isolated in this work showed 99% nucleotide sequence similarity to the *tdc* gene fragment of *E. faecalis* CNRZ1535.

The *in vitro* tyramine production ability of *R. pycnus* was quantified by HPLC analysis without derivatization and revealed a production of 1294 mg/L of tyramine. Similar results were reported by Guo *et al*. who detected tyramine in rice wine by HPLC without derivatization. A method described by the authors to be a sensitive, precise, and accurate procedure for the quantification of tyramine [19, 39].

5. Conclusion

In conclusion, the use of HPLC analysis without derivatization for tyramine detection used in this investigation is considered to be a rapid, simple and successive method to evaluate the tyramine-producing ability of different bacteria isolated from dairy products. The study, as well, emphasizes the promising application of degenerate primers (DEC5/DEC3) to detect tyramine production in dairy products, a goal that has been regarded as a challenge by manufacturers. In addition, a novel tyramine-producing strain; *R. pycnus,* isolated from the traditional Egyptian Domiati soft white cheese is reported in this study.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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