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Evaluation of some lactic acid and probiotic bacteria for their potential for biogenic amine production

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Abstract

The potential capability for biogenic amine (BA) production should be taken into account in the selection and implementation of starter and protective cultures to reduce hygienic and toxicological risks. Therefore, this work aimed to study the production of BA from lactic acid bacteria **(LAB)** used in food products and others isolated from breast-fed infants. The analytical protocol involves the use of PCR and TLC techniques to determine the ability of some LAB to form BA. the results indicated that there are some strains that selected have the ability for BA formation. Six strains from the thirty two examined strains gave positive results for putrescine production and the maximum level was 14.65 mg/kg. On the other hand, six strains yielded positive results for histamine production and the maximum level was 31.77 mg/kg and demonstrated positive indications for the present of histamine decarboxylase gene hdc. Seven strains produced positive results for tyramines production and the maximum level was 2.85 mg/kg and proved positive for the presence of tyrosine decarboxylase gene (*tdc*). Finally, eight strains out of thirty two showed positive results for agamantine production and the maximum level was 174.50 mg/kg and gave positive signs for the existence of dihydrolase (deiminase) gene which is responsible for agmatine formation. Based on this, it could be concluded that the microorganisms used in food and dairy production should be carefully screened for potential BA production. This is due to the possible horizontal transfer as origin of strain to strain variation of the BA production before and during using in food and dairy industry.

Key words: Biogenic amines (BA), Lactic acid bacteria, Histamine, Tyramine decarboxylase, Arginine Putrescine.

Introduction

Biogenic amines (BA) defined as a naturally occurring low molecular weight compounds involved in various biological activities in most living organisms. However, consuming foods containing high concentrations of BA can trigger human health problems leading to palpitations, hypertension, vomiting, headaches and flushing (**Ten Brink et al., 1990; Shalaby, 1996; Silla Santos, 1996; Lonvaud-Funel and Jogeax 1994; Elsanhoty et al., 2009**).

Several research groups support the view that BA are formed in wine making mainly by lactic acid bacteria (LAB) due to the decarboxilation of free amino acids (**Coton et al., 1998; Moreno-Arribas et al., 2000; Landete et al., 2008; Constantini et al., 2006; Lucas et al., 2008)**. In the case of fermented foods, some LAB are able to convert available amino acid precursors into BA via decarboxylase or deiminase activities during or following the fermentation process. For this reason, amino acid catabolism by LAB can affect both the quality and safety of fermented foods (**Silla Santos, 1996; Lonvaud-Funel and Jogeax 1994; Verges et al., 1999).**

Food possesses very rich, diverse and complex microbiota that invades invests them with their organoleptic properties and affects it's their quality. This microbiota is initially derived from starter bacteria, the main function of which is to produce lactic acid at an appropriate rate. However, during manufacture and ripening a complex secondary microbiota (non-starter lactic acid bacteria [NSLAB]) develops (**Beresford and Williams, 2004**).

BA contamination of food takes place during malolactic fermentation **(MLF)** due to the presence of lactic bacteria strains. The decarboxylases activities of such strains convert amino acids into BA. In addition agmatine (produced by arginine decarboxylation) is delaminated to putrescine. Assessing the potential risk of BA accumulation in food at an early stage of production will assist in better managing the fermentation process in order to reduce the spoilage. The proposed method detects microorganisms that have amino acids decarboxylases and agmatine deiminase. The result cannot indicate the final BA concentrations, but the risk of BA spoilage is linked to the presence of the genes in the bacteria population **(Lucas et al., 2008**).

Many publications reported that LAB can produce BA in many fermented products including meats (**Bover-Cid et al., 2001; Suzzi and Gardini, 2003**; **Ruiz-Capillas and Jimenez- Colmenero, 2004; Aymerich et al., 2006, Arena et al., 2007**), many different cheese varieties (**Burdychova and Komprda, 2007**; **Fernández et al., 2007a Fernández et al., 2007b**), wine (**Coton et al., 1998**; **Lonvaud-Funel and Jogeax 1994 ; Moreno-**

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Arribas et al., 2003; Moreno-Arribas and Polo, 2008; Izquierdo Canas et al., 2009) and cider (**Del Campo et al., 2000; Garai et al., 2006, 2007**). Decarboxylase activities exhibited by LAB in acidic environments encountered during wine and cider fermentation may play a role in providing an additional mechanism for energy generation as well as acid stress tolerance (**Konings, 2002**, **coton et al., 2010**).

The main of BA produced by LAB species are histamine such as histidine decarboxylase (hdc) (**Lonvaud-Funel and Joyeux, 1994; Coton et al., 1998**), tyramine via tyrosine decarboxylase (tyrdc) (**Lucas et al., 2003**) and putrescine via ornithine decarboxylase (odc) (**Arena and Manca de Nadra, 2001**) or agmatine deiminase (agdi) pathway (**Lucas et al., 2007**). Amongst the prevalent BA, putrescine can also enhance the toxic effects of histamine and tyramine and also react with nitrites to form nitrosamines (**Wathersen et al., 1975).**

Marcobal et al. (2006) developed methods for detecting tyramine-producing microorganisms. However, these are commonly long and tedious, as they require the isolation of microorganisms, and do not allow the identification of microorganisms without several extra microbiological or enzymatic tests being performed. Fortunately, the increasing number of sequences and genomes deposited in public databases, especially those regarding the tyrosine decarboxylation genes of LAB, has allowed the development of molecular methods to detect their presence (**Coton et al., 2004, Landete et al., 2007, coton et al., 2008**). These culture-independent methods are specific, sensitive and rapid, and are subject to less variability than phenotypic characterization. Nevertheless, their main drawback is that they are not quantitative, but recently a quantitative a approach devised.

Ladero et al. (2010) described the real Time PCR (qPCR) assay that allows the quantification of tyramine-producing microorganisms in cheese based on the specific amplification of the tdc gene, for the detection, quantification and identification of bacteria with the ability to produce tyramine. This method was found to be specific and showed a wide dynamic range, thus allowing the quantification of these tdc bacterial groups among the complex microbiota of cheese. Until now, few studies have been carried out on cider LAB and BA production. Amongst LAB species isolated from Spanish ciders, only few *L. diolivorans* strains appeared to be histamine-producers as well as to a lesser extent a *L. collinoides* strain (**Garai et al., 2007)**. In another study, *six L. spp*. as well as *O. oeni* strains were shown to produce histamine in ciders **(Del Campo et al., 2000**).

In a survey of 118 wines randomly chosen in different wine-producing areas of Southwest France**, Coton et al. (1998)** found that the presence of histamine-producing bacteria is not rare. Almost half of the tested wines possessed bacteria carrying the histidine decarboxylase gene wherein all of strains belonged to *O. oeni*. In contrast, formation of histamine was not observed in any species that may be involved in malolactic fermentation (**Straub et al., 1995**).

Gonza´lez de Llano et al. (1998) described two strains of Leuconostoc from of dairy origin showing tyrosine decarboxylase activity. These results suggest that Leuconostoc may be responsible for tyramine production in wines, in addition to *L. brevis* strains previously described **(Moreno-Arribas et al., 2000).** Several BA-forming species are of importance in food fermentations. In cheese, the role of contaminating strains of *L. brevis* and *L. buchneri* in the formation of tyramine and histamine has been concretely established. It can be derived from investigations of **Straub et al. (1995)** that some strains of *L. buchneri* may also contribute to the formation of putrescine and cadaverine.

Garai et al. (2007) showed that one cider strain belonging to the *L. diolivorans* species can produce tyramine. This results suggests that the main BA-producers amongst cider LAB appear to be histamine-producers and that BA-production levels are low.

Martin-Alvarez et al. (2006) indicated that the choice of starter cultures is fundamental to guarantee the quality of the final product. For this reason, the inability to form BA should be an important criterion in the selection of starter cultures for the production of fermented foods and beverages. Inoculation with starter cultures which are unable to produce BA is a viable option for the control of these compounds in foods. Before using LAB in food production, a quantitative analysis of BA is very essential to confirm the bacterial production. Therefore, this work aimed to detect specific lactic bacteria strains that have coding genes for the enzymes involved in BA production isolated from some food products of different geographical origins. Then, these were screened for potential BA-producers using both molecular methods and thin layer chromatography **(TLC)**.

Materials and Methods

Materials

1. Strains and media

Tables 1 indicate the strains under investigation. The probiotic strains candidate: *Lactobacillus acidophilus* P *2, 4, 5, 6, 7, 8, 9, 112*, 106; *Lactobacillus plantarum p1*; *Lactobacillus brevis p102; Lactobacillus pentosus p160* and *Enterococcus faecium p187 BL bifidobacterium longum* were identified by **Mahrous (2006).** Strains were isolated from healthy, breast-fed infants (15-30 days old) and used after the selection had been done according to Bergey's Manual of Determinative Bacteriology (**Holt *et al*., 1994**) with confirm the identification by

SDS-PAGE technique and API System. The strains were tested for their probiotic characteristic, e.g., gastric acid resistance, bile salt tolerance, antibacterial activity, adhesion to human mucus. *Lactobacillus* strains were cultivated in MRS (de Man Rogosa Sharpe) broth (Lab M, IDG, UK) and incubated at 37 °C in BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) provided with disposable BBL gas generating pack (CO_2 system envelopes, Oxoid, Ltd., West Heidelberg, Victoria, Canada). All LAB strains under investigation were grown at 30 °C in MRS broth supplemented with or without 2.5 g/l L-tyrosine disodium salt (Sigma-Aldrich, USA), 2.5 g/l L-histidine mono hydrochloride, 2.5 g/l L-ornithine monohydrochloride, 2.5 g/L L-lysine mono hydrochloride and/or 1 g/L agmatine sulfate salt (Sigma, USA). Cultures were incubated without agitation for 24 h to 8 days according to the species.

2. Chemicals and reagents

Amines (histamine, putrescine, cadaverine, and tyramine) as their crystalline hydrochlorides, dansyl chloride (5-dimethylaminonaphtalene-1- sulphonyl) and TLC plates (20 × 20 cm aluminium sheets coated with 0.20 mm silica gel G-60) were obtained from Merck company. All chemicals used were of analytical grades.

2.1. Detection of Biogenic amines (BA) producing.

2.1.1 DNA extraction from bacterial culture

DNA was extracted from pure cultures cells of 2 mL of culture were harvested by centrifugation at 13000 g for 15 min. The pellet is then suspended in 600 µL TE buffer (Tris-HCl 10 mM, EDTA 1mM) containing lysozyme (10 mg/mL) and incubated at 37 °C for 30min. The extraction was continued according to the methods described by **De et al. (2010)**. The final pellet obtained was dissolved in 50 µl Tris–EDTA (10:1, pH 8.00) and stored frozen at −20 °C till further analysis.

2.2.2. Quantification of genomic DNA

Genomic DNA concentration was determined by recording the absorbance at 260 nm (A_{260}) using a Nanodrop spectrophotometer (Wilninggto, DE, USA, and spectrophotometer 2000 according to the Manufacturer's producer's instructions). The purity of the DNA was determined from the A_{260}/A_{280} ratio to investigate the DNA quantity (**Sambrock, et al. 1989**). DNA purity was measured using the appropriate ratio of OD260:OD280 (1.65–1.85). Concentrations (ng/lL) and A260/ A280 readings were recorded for each sample. The quality of the isolated DNA was also evaluated by (0.9% agarose) gel electrophoresis using 2 µl of isolated DNA. The type of band pattern indicates the quality of the isolated DNA. A known amount of bacteriophage lambda DNA was used to compare the intensity and approximate size of the isolated DNA. The extracted DNA concentration was measured and adjusted by dilution to 20–25 ng/lL prior to PCR using bi-distilled, deionised and sterilized water (Fluka, Germany).

2.2.3. Oligonucleotide primers

The primers used in this study together with their target specific part of the investigated DNA are listed in Table 2). All primers were synthesized by Biosynthesis (Inc., USA), and obtained in a lyophilized state. All primers were solved before use to obtain a final concentration of 20 pmol/l for each.

2.2.4. DNA amplification and PCR condition

PCR was carried out on thermo cycler (Biometra, T1) using a prepared master mix. Amplification by PCR is performed in 25 µl reaction mixture containing 12.5 ng of template DNA, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 200µM of each dNTP, 1 mM of each primer, and 1 U of DNA polymerase and water was added to reach 25 µl. Oligonucleotide primer sequences for the amplification of internal fragments of the genes coding histidine-, tyrosine, ornithine decarboxylase, and agmatine deiminase by PCR have been designed by a number of research groups (**Coton and Coton. 2005, Laucas et al. 2007 and Laucas et al. 2008**).The PCR reactions were performed according to the cycling parameters given in table 3 (PCR conditions). The PCR products are analyzed by electrophoresis in 1.5 % agarose gel and revealed under UV after being stained with ethidium bromide.

2.3. Detection of specific BA-producing strains

2.3.1. Detection of histamine-producing strains

The methods described by **Coton and Coton (2005),** with the specific primers HDC3/HDC4 were used to detect the capacity of the strains to produce histamine. The hdcA gene was amplified as codes to detect the enzyme histidine decarboxylase.

2.3.2. Detection of tyramine-producing strains

To detect the tyrosine decarboxylase gene in the strains under investigation, tdc gene was used according to the method described in **Marcobal et al., 2005**.

2.3.3. Detection of putrescine-producing strains (via ornithine decarboxylase).

The gene (*odc*) used as code for the conversion of ornithine to putrescine was detected in the strains under investigation according to the method described in **Marcobal et al. (2004).**

2.3.4. Detection of agmatine dihydrolase (Deiminase) gene in the strains

The primer pair AGD1f/AGDIR were used for detection of agmatine dihydrolase (Deiminase) gene according to the methods described by **Laucas et al. (2007)**.

Table 1. Origin and incubation conditions of various bacterial strains used for investigate

Strains	**Origin**	**Growth temperature (C) and O_2 need**
Lactobacillus acidophilus ATCC 20552	1	37°C Anaerobic
Lactobacillus paracasei TISTR 453	2	30 °C Aerobic
Lactobacillus rhamnosus TISTR 541	2	37°C Aerobic
Lactobacillus salivarius TISTR 390	2	37°C Aerobic
Lactobacillus acidophilus TISTR 450	2	37°C Anaerobic
Lactobacillus johnsonii ATCC 33200	3	30°C Aerobic
Lactobacillus casei DSMZ 20011	4	30°C Aerobic
Lactobacillus acidophilus DSM 9126	4	37°C Aerobic
Lactobacillus acidophilus DSM20079	4	37°C Aerobic
Lactobacillus acidophilus DSM20242	4	37°C Aerobic
Lactobacillus sanfrasiscensis DSM20451	4	37°C Aerobic
Lactobacillus plantrum	3	30 °C Aerobic
Bifidobacterium infantis DSMZ 20088	4	37 °C Anaerobic
Bifidobacterium angulatum DSMZ 20098	4	37°C Anaerobic
Lb. delbrueckii subsp *bulgaricus*	3	37°C Aerobic
Lactobacillus acidophilus P 2	5	37°C Anaerobic
Lactobacillus acidophilus P4	5	37°C Anaerobic
Lactobacillus acidophilus P5	5	37°C Anaerobic
Lactobacillus acidophilus P 6	5	37°C Anaerobic
Lactobacillus acidophilus P7	5	37°C Anaerobic
Lactobacillus acidophilus P 8	5	37°C Anaerobic
Lactobacillus acidophilus P 9	5	37°C Anaerobic
Lactobacillus acidophilus P112	5	37°C Anaerobic
Lactobacillus acidophilus P106	5	37°C aerobic
Lactobacillus plantarum p1	5	37°C aerobic
Lactobacillus brevis p102	5	30°C aerobic
Lactobacillus pentosus p160	5	30°C aerobic
Enterococcus faecium p187	5	42°C aerobic
Bifidobacterium longuium	5	37°C Anaerobic
Lactobacillus brevis	5	30.0°C aerobic
Lactobacillus buchneri	5	37°C aerobic
Streptococcus thermophilus	5	37°C aerobic

(1) Cairo (MIRCEN), Faculty of Agriculture, Ain Shams University
(2) Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.
(3) American Type Culture Collection, Manassas, VA.
(4) Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ, Germany).
(5) Personal Communication with Associate Prof. Dr. Hoda Mahrous Abbas, Sadat City University, Institute of Genetic engineeing and biotechnology, Industrial Biotechnology Department, Food and Dairy Biotechnology Branch.

Table 2. Oligonucleotide primer pairs sequence and their target elements.

Primer	Sequence	Fragment length	Target element	References
hdcAf/ hdcAr	5'-ATGAAGCCAGGACAAGTTGG 3' 5'-AATTGAGCCACCTGGAATTG 3'	84 bp	Histidine decarboxylase gene	Coton and Coton (2005)
Tdcf/ tdcr	5'-CAAATGGAAGAAGAAGTTGG 3' 5'-GAACCATCAGCA ACAATGTG 3'	213 bp	*tdc* gene Tyrosine decarboxylase gene	Laucas et al. (2008).
HDC3 /HDC4	5'-GATGGTATTGTTTCKTATGA 3' 5'-CCAAACACCAGCATCTTC 3'	435 bp	Histidine decarboxylase gene hdc	Coton and Coton (2005)
Odcf/ Odcf	5'-TGCA CTTCCATATCCTCCAG-3' 5'-GAATTTCTGGAGCAAATC CA-3'	127 bp	Ornithine decarboxylase gene	Granchi et al. (2006) Nannelli et al. (2008)
AGD1f/AGDIR	5'-GAACGACTAGCAGCTAGTTAT-3' 5'-CCAATAGCCGATACTACCTTG-3'	90 bp	Agmatine Dihydrolase (Deiminase) gene	Laucas et al. (2007)
ODF/ODR	5' CATCAAGGTGGACAATATTTCCG 3' 5' CCGTTCAACAACTTGTTTGGCA 3'	500 bp	detection of putrescine gene	Granchi et al. (2006)

Table 3. Time/temperature profiles for qualitative PCR with DNA extracted from microorganisms using the primer pairs described in Table 2

Primer pair	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Reference
HDC3 / HDC4	95 °C, 30 s	95°C, 30 s	48°C, 45 s	72°C, 2 min	35	Coton and Coton (2005)
hdcAf/hdcAr	95°C, 5 min	95°C, 30 s	55°C, 30 s,	72°C, 30 s	40	Coton and Coton (2005)
Tdcf/tdcr	95 °C, 30 s	95 °C, 30 s	52°C, 30s	72°C, 2 min	30	Marcobal et al. (2005)
P1rev/p0303	95°C, 1 min	95°C, 30 s	50°C, 1 min	72°C, 1min	35	Landete et al. (2007)
Agdif/agdif	95 ºC, 5 min	95ºC, 30s	55 ºC, 30s	72 ºC, 2 min	35	Lucas et al. (2007)
ODCF/OCDR	95 °C, 5 min	95°C, 30 s	55°C, 30 s	72°C, 30 s	40	Granchi et al. (2006) Nannelli et al. (2008)

2.4. BA extraction and determination

2.4.1. BA extraction

To extract and determine histamine, putrescine, cadaverine, spermidine, spermine and tyramine in all homogenised tested samples, the method described by **Mietz and Karmas (1978) and Maijala and Eerola (1993**) was applied. Briefly, samples (100mL) of microbial culture) were centrifuged and supernatants were blended with 125 ml of trichloroacetic acid (TCA, 5%) for 3 min using a Warning blender. The homogenised sample was filtered using Watman No. (1). The filtrate (10 mL) was transferred into a glass tube with 4 g NaCl and 1 mL of NaOH (50 %), then shacked and extracted with (3 x 5.0 ml) n-butanol: chloroform (1:1 v/v), the upper layers were transferred to separating funnel (100 ml) with 15 ml n-heptane and extracted with (3 x1.0 ml) HCl (0.2 N). The HCl layers were collected in a glass tube and evaporated to dryness at 95°C aided by a gentle current of air, submitted to dansylchloride derivatization (1 h at 55°C) for formation of dansylamines**.** Saturated $NaHCO_3$ solution (0.5 ml) was added to the residue of both samples and working standards and carefully mixed, then 1.0 ml dansyl chloride solution was added and thoroughly mixed using vortex mixer. The mixture was incubated at 55 °C for 45 min**.** Distilled water (10 ml) was added to the dansylated mixture and vigilantly mixed. The mixture was extracted with (3 x 5.0 ml) diethyl ether. The ether layers were carefully evaporated at 35 °C in a dry bath with aid of current air. The dry film was kept under – 20 ºC for TLC analysis of BA.

2.4.2. Determination of dansylamines by TLC densitometer

One-dimensional silica gel G 60 TLC plates (20 x 20 cm) (Merck, Darmstadt, Germany) were used for the chromatographic separation of the studied dansylamines. The dry film of standards and samples were dissolved in 500 µl methanol. The dissolved standards and samples (10 µl) were spotted. The plate was developed using chloroform: benzene: triethyl amine (6: 4.5: 1 v/v/v). The plate was dried at room temperature and visualized using UV lamp (365 nm). The resulting spots were then marked and the marked areas were determined in Microanalysis Centre, Faculty of science, Cairo University using CS- 9000 Dual wavelength flying spot scanning densitometer (SHIMADZU) at wavelength 254 nm. Standard curve of each dansylamine was used to calculate the concentrations of BA in the tested samples.

3. Results and Discussion

3.1 DNA isolation

The detection of genes that gave microorganisms the ability for BA production involved three steps. The first step involves genomic DNA extraction and amplification of specific microorganisms sequence (16s r DNA) from microorganisms DNA. This procedure is necessary to discriminate between negative and positive results due to inhibited amplification (**Gezginc et al., 2013)**.The second step entails amplification of specific sequences, represented by each histidine decarboxylase gene, tyrosine decarboxylase gene, ornithine decarboxylase gene, agmatine dihydrolase (Deiminase) gene and detection of putrescine gene. The quality of the extracted DNA from the strains using CTAB method was examined by electrophoresis through a 0.8% agarose gel (Fluka). DNA bands of high intensity appeared in the lanes (**Figs. 1 A**), showing high yield of genomic DNA. The results presented in this study confirmed that the CTAB protocol can be used for DNA extraction and purification from the strains as an initial step of detection of different decarboxylase genes. After agarose gel electrophoresis, the DNA was present as a high molecular weight band from all of the strains (**Figs. 1 B**).

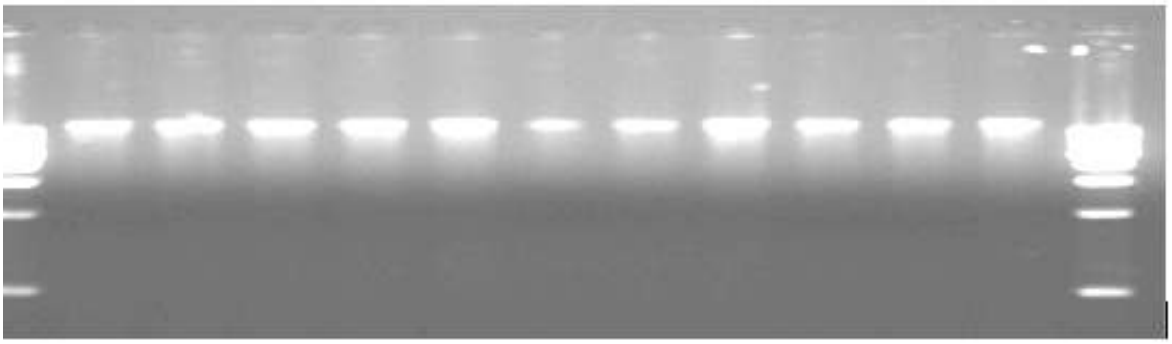

Fig. 1A. Example of DNA electrophoresis on 0.5 agarose gel of DNA extracted from the strains

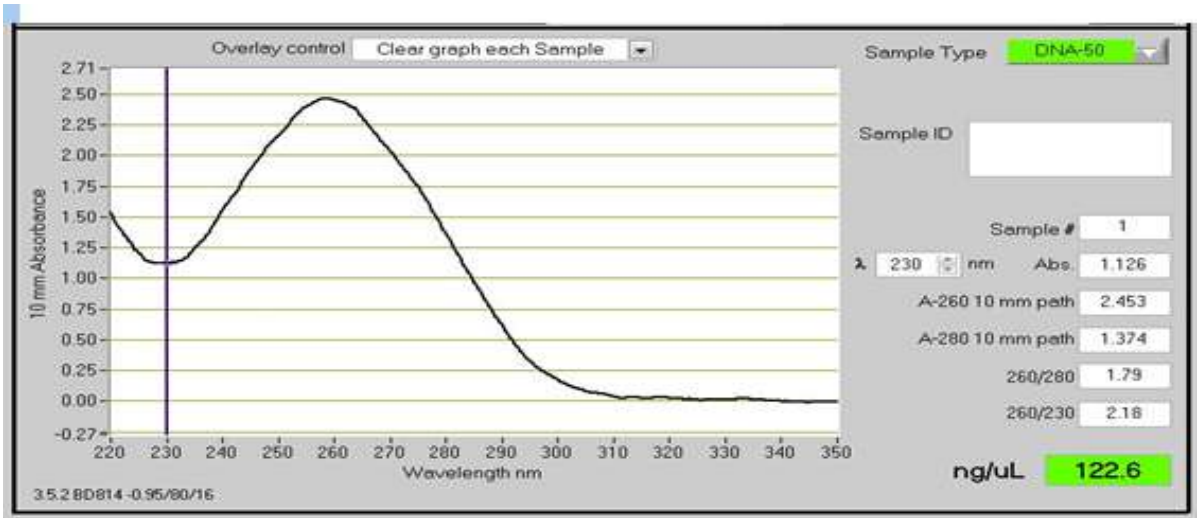

Fig.1.B. Examples of the results obtained by NANODROP 2000 spectrophotometer for DNA amount of extracted DNA from some strains using CTAB method.

3.2. Detection of BA genes

3.2.1. Detection of histidine, tyrosine decarboxylase hdc and *tdc* genes

Sensitive detection of BA-producing bacteria in the first stages is important for preventing BA production and accumulation in food. The application of conventional culture technique for detecting BA-producing bacteria is often tedious and unreliable. On the other hand, it is more important to find a good correlation between histamine production and the presence of hdcA gene. To detect the histidine decarboxylase gene, hdc in the strains under investigation, the primer pair HDC3 /HDC4 was used and the positive samples gave PCR products at 435 bp (**Coton and Coton 2005**). **Figure 2 A** presented the positive results obtained using the primer pair HDC3 /HDC4 lines 2, 3, 4, 5, 6, 7. To confirm these results the primer pair hdcAf/ hdcAr was used as it is more sensitive and the PCR reveal at 123 bp (**Coton and cotton, 2005**) as shown in **Figure 2.B**. Based on the outcomes exhibited in figure 2.B, it can be concluded that only six strains (*S. thermophilus, L. lactis, L. brevis, L. pentosus p160, Enterococcus faeciumand and L., rhamnosus TISTR 541)* from the strains under investigation have given positive results for the presence of histidine decarboxylase gene hdc. Data in **Figure 3** indicate the positive results acquired by the primer pair Tdcf/ tdcr by PCR to detect the tyrosine decarboxylase gene tyc. The PCR amplicon appears at 213 bp. Notably, the same strains that gave positive results for the existence of histidine decarboxylase hdc, have also demonstrated positive results for the presence of tyrosine decarboxylase gene tdc. These findings are in agreement with the results of **Gezginc et al. (2013)** who showed that *S. thermophilus* isolates have the ability to form BA, especially histamine, and tyramine. A similar evidence is provided by **Lorencová et al. (2012)** who found that some probiotic strains (*Bifidobacterium* and *L. rhamnosus*) are detected for BA production. The orientation of these genes polycistronic hdc cluster (hdcAPB) was identified in *S. thermophilus*. The cluster began with the hdcA gene, followed by a transporter hdcP gene and ended with hdcB gene, which catalyses the maturation of the pyruvoyl-dependent hdcA (**Calles-Enriquez et al. 2010**). The gene order of hdcAPB oper and is similar to *S. capitis* and *C. perfringens*, which, however, lack hdcB (**De las Rivas et al., 2008**). HdcB is a functional enzyme and substoichiometric amounts of hdcB were required to cleave hdcA (**Trip et al., 2011**). The hdc cluster is located either on the plasmid or on the chromosome. In *L. hilgardii* and *T. halophilus* strains hdc cluster is located on the plasmid (**Satomi et al., 2008**), whereas in *S. thermophilus* strain the cluster is located on the chromosome (**Calles-Enriquez et al., 2010).** The sequence of the hdc cluster has homology with a phase resistance pER35 plasmid from *S. thermophilus*. In addition, tyrosine decarboxylase gene (tdcA) was identified in *S. thermophilus* strains *and L. brevis* (**La Gioia et al., 2011**, **coton and coton 2009**). These similarities point to a horizontal transfer of hdc cluster and tdcA in *S. thermophilus.* Moreover, the strains that gave positive results were similar with gene sequence have showed similarity with tdcA and hdc genes. The hdcA enzyme was synthesized in milk even in the absence of histidine. **Rossi et al. (2011)** stated that the enzyme activity in cell-free extract obtained from cultures grown with or without histamine was not significantly different.

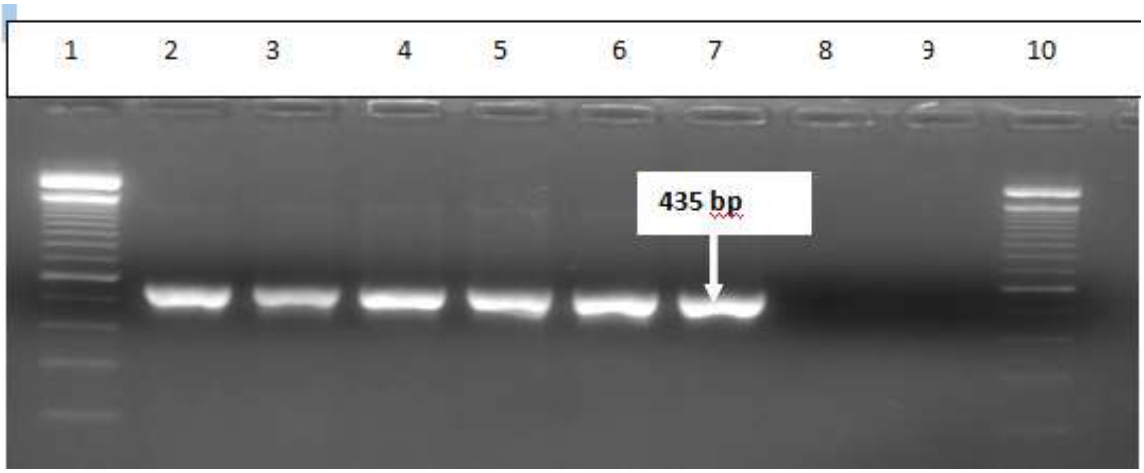

Figure 2A. Presented the positive results that obtained by using the primer pair HDC3 /HDC4, in the strains *S. thermophilus*, *L. lactis*, L. brevis, *Lactobacillus pentosus p160*, *Enterococcus faecium and L. rhamnosus* TISTR 541 respectively lines 2, 3, 4, 5, 6 and 7**.**

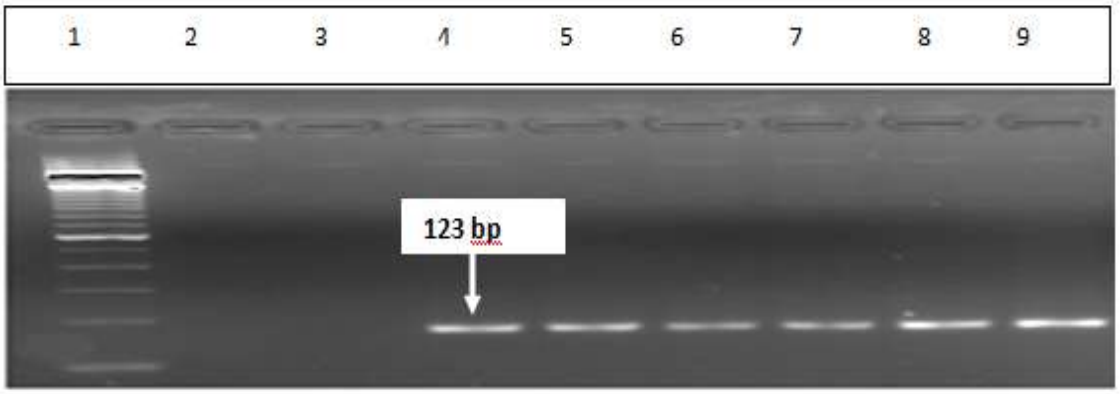

Figure 2B. Presented the positive results that obtained by using the primer pair hdcAf/ hdcAr, *S. thermophilus*, *L. lactis*, *L. brevis*, *Lactobacillus pentosus p160*, *Enterococcus faeciumand Lactobacillus*

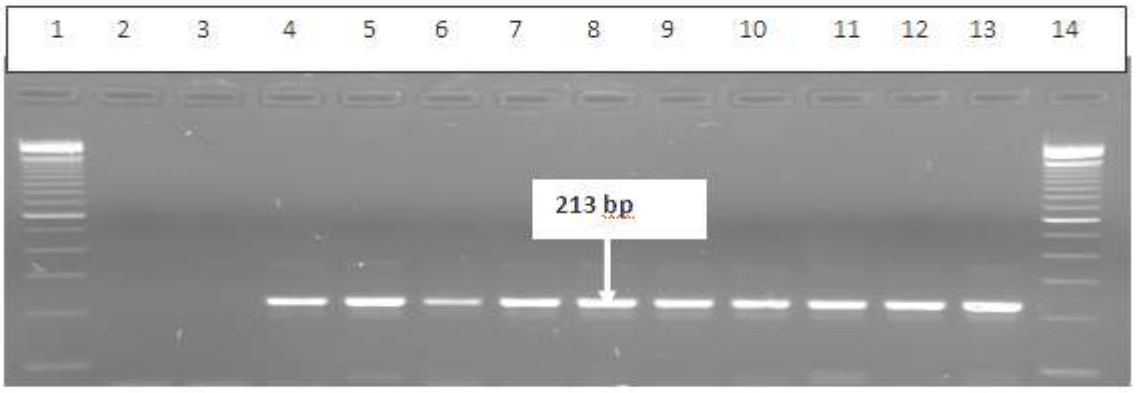

Figure 3. Presented the positive results that obtained by using the primer pair hdcAf/hdcAr for identification of Tyrosine decarboxylase gene lanes 1 and 14: DNA ladder 100 bp, Lane 2 and 3 PCR control negative, lanes 4 and 5: PCR products by DNA from *S. thermophilus,* lanes 6 and 7, PCR products by DNA from *L. brevis p102 and L. brevis,* lanes 8 and 9:PCR products by DNA from *Lactobacillus pentosus p160* and *Enterococcus faecium,* lanes 10 and 11 PCR products by DNA from *L. rhamnosus TISTR 541* and *L. casei DSMZ* 20011, lanes 11 and 12: PCR products by DNA from *L. paracasei* TISTR 453 and *Lb. delbrueckii* subsp. *bulgaricus* respectively**.**

3.2.2. Detection of Ornithine, Decarboxylase, Agmatine Dihydrolase genes.

The primer Odcf/ Odcr was used to detect the Ornithine decarboxylase gene and PCR products were appeared at 127 bp (**Granchi et al., 2006**), whereas, the primer AGD1f/AGDIR was used to detect the agmatine dihydrolase gene. The PCR products were revealed at 90 bp (**Laucas et al., 2007**). Data in Figure 4 A (lanes 4 and 5) *L. Paracasei* TISTR 453, lanes 6 and 7 for *L. casei DSMZ* 20011, lanes 8 and 9 *L. brevis*, lanes 10 and 11 *S. thermophilus* presented the positive results obtained from tested stains. While the remaining strains did not given any positive results. The results reported herein show that only 4 strains have the gene for Ornithine decarboxylase. Similar results were observed by **Landete *et al.* (2007)** who found that some LAB species *such as L. casei* and *L. paracasei and Oenococcus oeni* have the genetic capability of BA production **(Moreno-Arribas and Polo, 2003)**. The primer pair AGD1f/AGDIR was used to discover agmatine Dihydrolase (Deiminase) gene. Figure 4 B presented the positive results revealed from PCR. The same strains that gave positive results for the presence of Ornithine decarboxylase gene, have also given positive results for the existence of agmatine dihyrolase (Deiminase) gene (Figure 4 lanes 2 to 9). Such findings accord with the results reported by **Landete *et al*. (2007)** who discovered the genes for BA production in *Lb. delbrueckii* subsp. *bulgaricus*. On the other hand, many other lactobacilli can produce BA, including tyramine, e.g., some strains of *Lb. brevis* or *Lb. paracasei* (**Landete et al., 2008**). Some studies have shown the existence of a strain-dependent ability to form BA. For example, *O. oeni* strains were described as being histamine- or putrescine-formers (**Coton et al., 1998; Marcobal et al., 2006**). A *Lb. hilgardii* strain, IOEB 0006, was also described as being a histamine-former; in this case the amine producing pathway was harbored by an unstable plasmid (**Lucas et al., 2005**). Concerning the ability of *Lb. brevis* to form tyramine, **Lucas et al. (2003)** showed, that this trait was encoded at the chromosomal level for the strain IOEB 9809. Additionally, **Lucas et al. (2007)** indicated that the presence of the tyrdc gene was a strain-dependent trait in *Lb. brevis* strains isolated from wines, which is confirmed in this study.

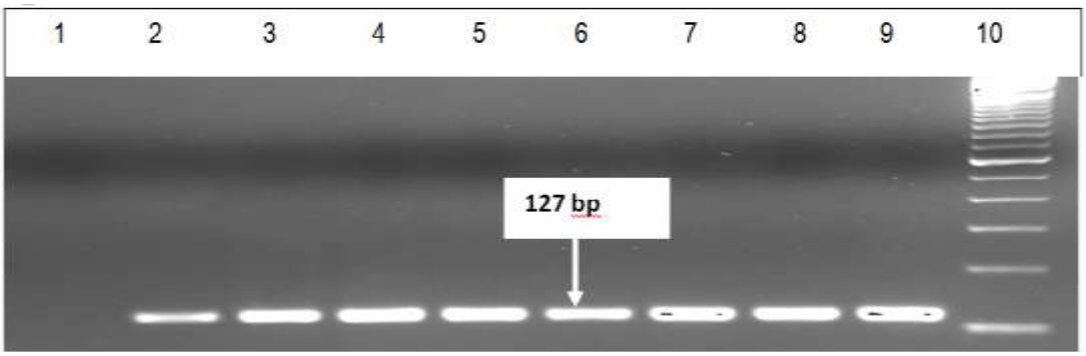

Figure 4 A. Detection of Ornithine decarboxylase gene in some strains. PCR products obtained with the primer AGD1f/AGDIR., lanes 1 and PCR control, lanes 2 and 3 PCR products of DNA from *Lactobacillus Paracasei* TISTR 453, lanes 4 and 5 PCR products of DNA from for *Lactobacillus Casei DSMZ* 20011, lanes 6 and 7 PCR products of DNA from *Lactobacillus brevis*, lanes 8 and 9 PCR products of DNA from *S. thermophilus,* lane 10 DNA ladder 100 bp.

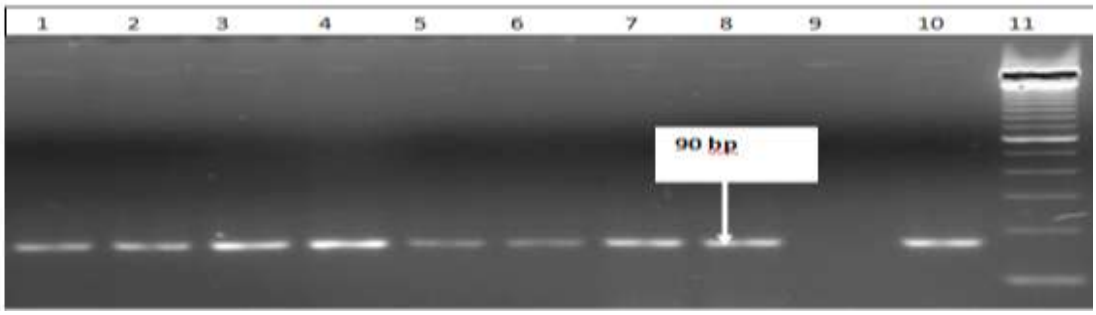

Figure 4 B. Detection of agmatine Dihydrolase (Deiminase) gene in some strains. PCR products obtained with the primer AGD1f/AGDIR. lane 1: PCR products by DNA from *Lactobacillus paracasei* TISTR 453, lanes 2: PCR products by DNA from *Lactobacillus casei DSMZ* 20011, lane: 3 PCR products by DNA from *Lactobacillus brevis*, lanes 4: PCR products by DNA from *S. thermophilus,* lane 5: PCR products by DNA from *Lactobacillus brevis* p102, lane 6: PCR products by DNA from *Lactobacillus buchneri, lane 7:* PCR products by DNA from *Lactobacillus sanfrasiscensis DSM20451, lane 8:* PCR products by DNA from *Lactobacillus pentosus p160*, lane 9: PCR products by water, lane 10 PCR control positive , lane 11: DNA ladder 50 bp.

3.2.3. Detection of putrescine gene

The primer ODF/ODR was used to detect the ornithine decarboxylase gene and PCR products appeared at 500 bp (**Granchi et al., 2006).** Data in Figure **5** demonstrated the positive results produced by PCR technique. From data shown in figure 5 we could conclude that the putrescine gene is detected in *L. paracasei* TISTR 453, *L. casei DSMZ* 20011, *L. brevis, Streptococcus. Thermophilus, Enterococcus faecium p187, Lb. delbrueckii* subsp. *bulgaricus.* These findings agree with the results reported by **Costantini et al. (2013)** who found that the majority of the analyzed *L. brevis* harbour both AgDI and tdc genes and are tyramine and putrescine-producers. Conversely, among the other LAB tested, only one *L. hilgardii* and one *Pediococcus pentosaceus* produced putrescine. The AgDI gene was also discovered in two other LAB (*Lactobacillus mali* and *pediococcus parvulus*), but no putrescine production was observed. The hdc gene and histamine production were found in strains. Such results agree with a previous report by **Costantini et al. (2006)** wherein the presence of hdc gene *in L. hilgardii* 5211 strain isolated from wine was confirmed. The hdc gene was also present in *L. casei* 18 isolated from cider. These two strains were not putrescine-producers. Tyramine and histamine are the most represented BA in wine and cider. Significantly, tyramine has toxicological properties and is presumed to play an active role in the development of migraine, hypertension, psychological depression, schizophrenia, and Parkinson disorder (**Branchek and Blackburn, 2003**). Histamine has been associated with headache, allergies, and hypotension **(Maintz and Novak, 2007**). In the present investigation, most tyramine production observed is ascribable to *L. brevis*. Furthermore, results indicate that two strains belonging to different species of *L. hilgardii 5211*, isolated from wine, and *L. casei* 18, isolated from cider, also biosynthesize histamine are in agreement with their genetic pattern. Histamine production by *L. casei* has been previously demonstrated (**Garai et al., 2007**).

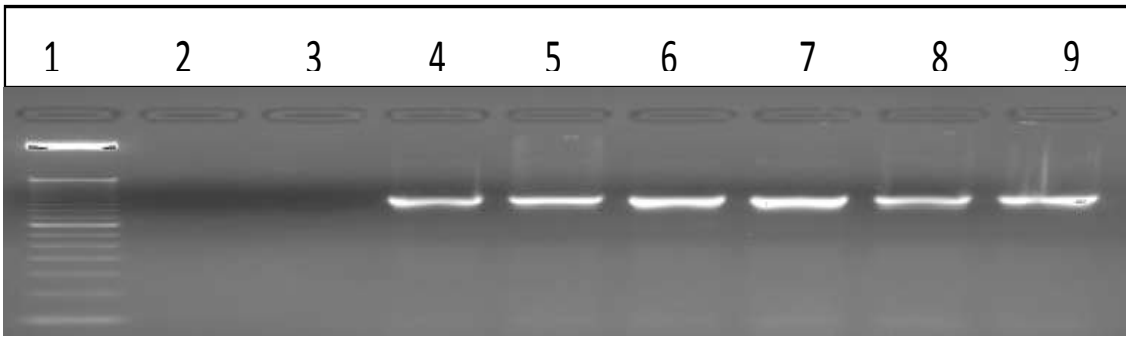

Figure 5: Detection of putrescine gene in some strains. PCR products obtained with the primer ODF/ODR. lanes 1 DNA ladder 50 bp, lanes: 2 and 3 PCR control without DNA, lane 4: PCR products by DNA from *Lactobacillus paracasei* TISTR 453, lane 5 PCR products by DNA from for *Lactobacillus casei DSMZ* 20011, lane 6: PCR products by DNA *Enterococcus faecium p187*, lane 7: PCR products by DNA from *Lactobacillus brevis*, lane 8: PCR products by DNA from *S. thermophilus, lane 9:* PCR products by DNA from *Lb. delbrueckii* sub*sp bulgaricus*

3.3. Determination of BA by TLC

Data in Table 4 and Figure 6 show the levels of BA and the positive samples for different BA in microorganisms under investigation as determined by TLC. Only six strains from the strains under investigation were given positive results for producing putrescine. The strains *L.. Casei DSMZ* 20011 exhibited the greatest capacity for putrescine production (17.38 ppm), followed by *L. Paracasei* TISTR 453 (14.65 ppm) *L. brevis*13.59, *Enterococcus faecium p187* 10.53, *S. thermophilus* (3.53) and the strain *L.. delbrueckii* sub*sp bulgaricus*. demonstrated the lowest ability for producing putrescine amine. In the same table the ability of microorganisms for producing histamine was presented. The *Enterococcus faecium p187* strain was given the highest ability for histamine production then *L. brevis (12.88 ppm), L. pentosus p160* (4.46ppm), *L. casei DSMZ* 20011 (4.24 ppm), the *L. rhamnosus* TISTR 541 (2.75) while the *S. thermophilus* showed the lowest ability for producing histamine. Data reported in this study clearly show that seven strains gave positive results for tyramines production and *casei DSMZ* 20011 displayed the highest potential for tyramine production and the level was (2.85) while the lowest ability for tyramine production was for *L. brevis* p102 strain and the level was (0.76). Only eight strains were able to produce agamantine and they varied in this. The strains *L. paracasei* TISTR 453 showed the highest ability for agamantine production (174.50 ppm) while the *S. thermophilus* produced the lowest yield (1.35 ppm). Comparable conclusions were documented by **Costantini et al., (2013),** who found that the majority of the *L. brevis, L. hilgardii and Pediococcus pentosaceus* produced putrescine.

Table 4. Concentration of biogenic amines produced by lactic acid and bifidiobacteria

Strains	Concentration of biogenic amines mg/kg (ppm).			
	Putrescine	**Histamine**	**Tyramines**	**Agamantine**
Lactobacillus acidophilus ATCC 20552	-	-	-	-
Lactobacillus paracasei TISTR 453	14.65			174.50
Lactobacillus rhamnosus TISTR 541	-	2.75		-
Lactobacillus salivarius TISTR 390	-			-
Lactobacillus acidophilus TISTR 450	-			-
Lactobacillus johnsonii ATCC 33200	-			-
Lactobacillus casei DSMZ 20011	17.28	4.24		10.63
Lactobacillus acidophilus DSM 9126	-	-		
Lactobacillus acidophilus DSM20079	-	-		
Lactobacillus acidophilus DSM20242	-	-		
Lactobacillus sanfrasiscensis DSM20451	-	-		4.89
Lactobacillus plantrum	-	-		-
Bifidobacterium infantis DSMZ 20088	-	-		-
Bifidobacterium angulatum DSMZ 20098	-	-		-
Lb. delbrueckii subsp *bulgaricus*	1.96	-		-
Lactobacillus acidophilus P 2	-	-		-
Lactobacillus acidophilus P4	-	-		-
Lactobacillus acidophilus P5	-	-		-
Lactobacillus acidophilus P 6	-	-		-
Lactobacillus acidophilus P7	-	-		-
Lactobacillus acidophilus P 8	-	-		-
Lactobacillus acidophilus P 9	-	-		-
Lactobacillus acidophilus P112	-	-		-
Lactobacillus acidophilus P106	-	-		-
Lactobacillus plantarum p1	-	-		-
Lactobacillus brevis p102	-	-		3.49
Lactobacillus pentosus p160	-	4.46		6.34
Enterococcus faecium p187	10.53	31.77		-
bifidobacterium longuium	-			-
L. brevis	13.59	12.88		42.40
L. buchneri	-	-		11.76
S. thermophilus	3.53	1.74		1.35

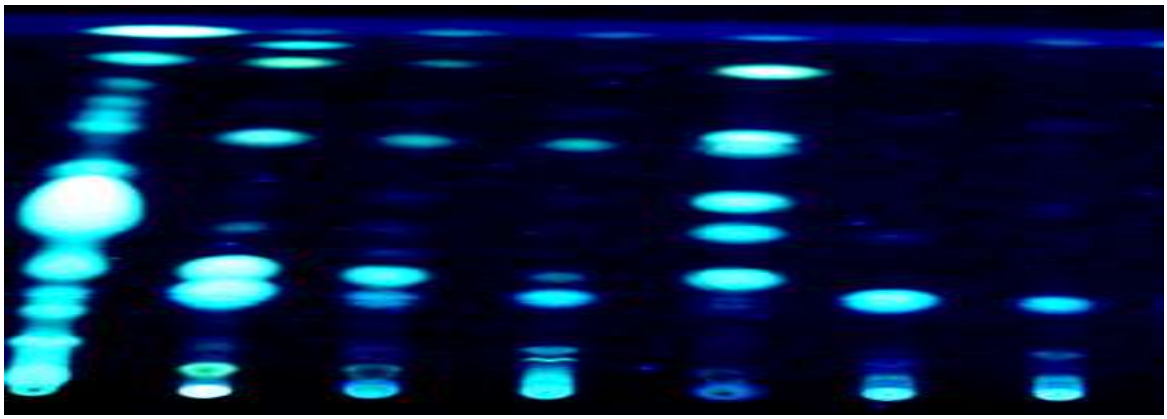

Figure 6: Examples of TLC plate for determination of different biogenic amines and the positive samples in some microorganisms under investigation.

In addition, **Al Bulushi et al. (2009)** found that most of the analyzed *L. brevis* strains were also able to produce tyramine, putrescine should be regarded not only as a molecule altering the wine organoleptic properties but also as a factor enhancing the risks of health effects exerted by tyramine on humans

(headache and hypertension). The results obtained are in agreement with the statement that the production of BA in bacteria seems to be strain-dependent rather than related to bacterial species or even genera. This research also revealed that with the elevated amount of the precursor (tyrosine), the production of the corresponding BA (tyramine in this case) is increased in decarboxylation broth, which is in accordance with other studies (**Fernández et al., 2007).**

Conclusions

In conclusion, this work indicated that the ability to form BA are not a species-dependent trait in LAB. For the first time, the molecular basis for strains to strain variation in BA-formation in this species is provided. Most important, the obtained results will allow for studying the BA formation trait in other LAB encountered in various fermented food products. Based on these findings, it is recommended to carefully select and screen the strains for amino acid decarboxylase activity before selecting LAB as appropriate starter or probiotic strains in food and dairy production because of the potentional of horizontal transfer of gene(s) from microorganisms to others. This procedure would benefit from using specific and highly sensitive methods. i.e., utilizing molecular methods such as PCR and TLC methos.

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تقييم بعض بكتريا حمض اللاكتيك والمعاونات الحيوية لقدرتها علي إنتاج الأمينات الحيوية

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تعتبر الأمينات الحيوية من المواد السامة التي لها تأثير سيء علي صحة الإنسان لذلك ينبغي أن يؤخذ في الاعتبار القدرة المحتملة للميكروبات المستخدمة في الصناعات الغذائية وذلك من حيث قدرتها علي أنتاج الأمينات الحيوية للبادئات و ذلك للحد من المخاطر الصحية والسمية التي تسببها هذه المركبات .وبالتالي، يهدف هذا العمل إلى دراسة احتمالية إنتاج الأمينات الحيوية من بكتريا حمض اللاكتيك (LAB) والمعاونات الحيوية المستخدمة في المنتجات الغذائية وغيرها المعزولة من الأطفال الرضع. اشتمل بروتوكول التحليل استخدام تقنيات تفاعل البلمرة المتسلسل PCR وكروماتوغرافيا الطبقة الرقيقة (TLC) لتحديد قدرتها علي تكوين الأمينات الحيوية .أشارت النتائج إلى أن هناك بعض الميكروبات من الميكروبات التي اختيرت لها القدرة على إنتاج الأمينات الحيوية. حيث أعطت ستة سلالات–من أصل أثنين وثلاثين سلالة—نتائج إيجابية لإنتاج putrescine وكان الحد الأقصى عند مستوى 14.65 ملجرام / كجم . ومن ناحية أخرى، أظهرت ست سلالات نتائج إيجابية لإنتاج الهستامين، وكان الحد الأقصى لإنتاجها 31.77 ملجرام / كجرام. كما أوضحت وجود الجين المسئول عن المقدرة للإنتاج كما أظهرت معطيات الدراسة نتائج إيجابية لإنتا tyramines من سبع سلالات وبلغ الحد الأقصى لذلك 2.85 مليجرام/ كجرام، كما أنها أكدت وجود جين تيروزين كربوكسيل (تي دي سي) .علي صعيد أخر قدمت ثماني سلالات من الاثنين وثلاثين المختبرة نتائج إيجابية لإنتاج أجمانتين (agamantine) وكان الحد الأقصى لقدرتها 174.50 مليجرام / كجم، كما أظهرت دلالات إيجابية علي وجود جين dihydrolase (deiminase) وهو الجين المسئول عن تكوين أجماتين. بناءا علي النتائج التي تم الحصول عليها فأننا نوصي بعمل فحص دقيق للتعرف علي قدرة الكائنات الحية الدقيقة المستخدمة في إنتاج الأغذية ومنتجات الألبان علي إنتاج الأمينات الحيوية بصفة مستمرة قبل وأثناء استخدامها في صناعات الأغذية والألبان، حيث هناك فرص محتملة لحدوث انتقال أفقي للجينات المسئولة بين الميكروبات وبعضها مما يودي إلي حدوث اختلاف في الميكروبات ويكسبها القدرة علي تكوين مركبات غير مرغوبة صحيا في الأغذية.