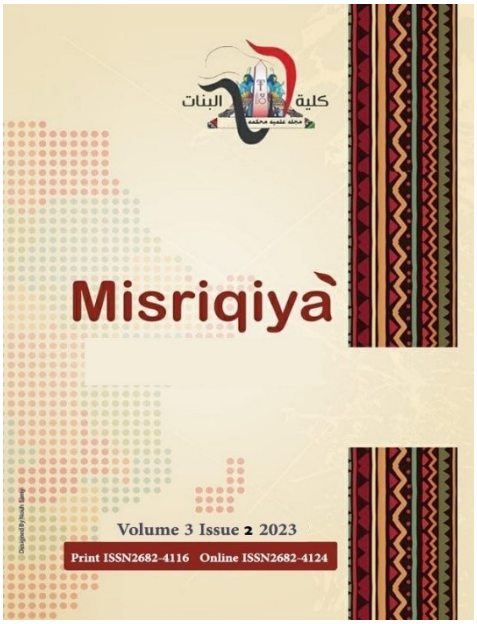

Isolation and identification of lactic acid bacteria from Egyptian pickle as bioactive molecules producing bacteria

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Received: 5-5-2023 Revised: 5-6-2023
Accepted: 11-6-2023 Published: 1-10-2023

DOI: 10.21608/MISJ.2024.209153.1045

Abstract
Gamma-aminobutyric acid (GABA) have attracted growing interest in recent decades due to its multiple health benefits with numerous and important physiological functions, such as anti-cancer, anti-hypertension, anti-diabetes, antioxidant and anti-inflammation. GABA is produced by lactic acid bacteria, which can produce a high amount of this amino acid. The food industry has leveraged this capacity to develop functional foods enriched with GABA. For this reason, the current study reports the isolation of lactic acid bacteria (LAB) and their screening for GABA production from monosodium glutamate (MSG) using pre-stained thin-layer chromatography (TLC). The isolate MGOLIV.25 was selected as a high GABA producing bacterial strain. The GABA content of MGOLIV.25 was detected by Gas chromatography–mass spectrometry (GC-MS) to be 9.02 mg/ml. The isolate MGOLIV.25 was identified biochemically and molecularly as *Lacticaseibacillus casei.* The findings of this study suggest that the isolate *Lacticaseibacillus casei* could be used for developing of healthy food products rich with GABA.
Keywords*: GABA, anti-cancer, lactic acid bacteria, probiotic, monosodium glutamate.*

1. Introduction

Probiotics are living microorganisms that when ingested in adequate quantities help maintaining a healthy gut environment, these include mainly *Lactobacillus* and *Bifidobacterium* strains, as well as some *Enterococcus* and *Streptococcus* strains (**Hill *et al*., 2014**). Beneficial impacts associated with probiotics include enhancement of the immune response, improvement of intestinal health, inhibition of lactose intolerance, prevention of cancer and a positive impact on mental health (**Chudzik *et al*., 2021**).

The use of probiotics as a more sustainable route for postbiotic (GABA) production is gaining interest among the scientific community and industrial sector, specifically from LAB **(Diez-Gutierrez *et al*., 2020).**

Gamma-aminobutyric acid (GABA) consist of four carbon with a non-protein structure amino acid. GABA, which has attracted increasing interest in recent years due to its multiple health benefits on human such as a hypotensive, diuretic, blood pressure control, stimulant of immune cells, anti-diabetic, regulation of the cardiovascular system as well as management of several psychiatric disorders (anti-stress and anti-depressive), therefore, GABA may be considered as potential alternative therapeutics for prevention and treatment of various diseases **(Ngo and Vo, 2019**).

The previous studies of GABA production focused on seeking highly productive GABA strains and optimizing the growth conditions for these bacteria (**Diana *et al*., 2014**). The food industry is mainly interested in GABA production because it is considered a bioactive compound that promotes health and is useful for the Development of Foods for Specified Health Use (FOSHU) **(Martirosyan and Singh, 2015).** Moreover, given their action on the immune system, lactic acid bacteria (LAB) have the potential to protect against damage related to the immune system, prevent intestinal infections and act as immunomodulators (**Miller *et al*., 2019).**

Pursuant to **Hill *et al*., 2018** the *Lactobacillus casei group (LCG)*, comprised mainly of the closely related *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* species that are considered the most studied species due to their industrial, commercial and applied health potential. Commercially, they are used to ferment dairy products, often producing foods with improved flavor and texture. They were also found to produce many bioactive metabolites which can confer host benefits when consumed (**Dietrich *et al*., 2014**).

In the present study, the focus is on the isolation of high GABA producer of lactic acid bacteria (LAB), because LAB show possibilities for commercial use as starters of production in functional fermented foods sector.

2. Materials and methods

2.1. Isolation of lactic acid bacteria

One gram of green olive pickle samples was serially diluted and inoculated on De Man, Rogosa and Sharpe (MRS) agar supplemented with 1% calcium carbonate ($CaCO_3$) then incubated at 37° C for 24-48 hours under anaerobic conditions in anaerobic Jar. The bacterial isolates that formed clear zone were considered as putative LAB. Different single colonies were streaked onto MRS agar to obtain pure cultures (**Yogeswara *et al*., 2018**).

2.2. Screening of GABA-producing LAB by thin layer chromatography

To select LAB with high GABA producing ability, bacterial were grown on MRS (**Himedia, India**) broth supplemented with monosodium glutamate (MSG) 3% and incubated at 37° C for one day and then the cultured broth was centrifuged at 1000 rpm for 10 min at 4° C. To select LAB with high GABA producing ability, bacterial were grown on MRS (**Himedia, India**) broth supplemented with monosodium glutamate (MSG) 3% and incubated at 37° C for 48 hours and then the cultured broth was centrifuged at 1000 rpm for 10 min at 4° C. The

supernatants (0.5 µl) and (0.5 µl) GABA solution 1% were spotted on TLC silica plates (**Merck, Germany)** using solvent solution consisted of 1% ninhydrin with a combination of butanol-acetic acid and water (5:3:2, v/v/v) as the mobile phase according to **Wu *et al*., 2018**. After development, the plate was directly dried at 105° C.

The GABA-producing strain were identified based on Gram staining, according to the **Coico, 2006** protocol and were observe under microscope with 100x achro oil objective (**CX31 Binocular Microscope, Olympus Co., Japan**).

Catalase test was conducted by adding two drops of hydrogen peroxide (H_2O_2) 3% to 24 hours cultures on an object glass slide (**Reiner, 2010**), followed by 16S rDNA sequence amplification.

2.3. Molecular identification of GABA-producing isolates

2.3.1. Amplification and Sequencing of 16S rDNA

For molecular identification of potential GABA-producing LAB isolates 16S rDNA gene sequencing was used. Deoxyribonucleic acid (DNA) from LAB was extracted and purified using (**Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721**, **#K0722, USA**) for Gram-positive bacteria according to the manufacturer's instructions. Universal primers 27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492R: 5′-GGT TAC CTT GTT ACG ACT T-3′ were used to amplify a target sequence of the 16 S rDNA gene in the purified DNA template. Polymerase chain reaction (PCR) was performed in (T100 Bio-Rad, USA) PCR thermal cycler with initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification (94°C, 30s-50 °C, 30s-72°C, 2 min and 72°C, 10 min). Then, the single purified bands were sent to (**Macrogen, South Korea**) company for sequencing. The obtained sequences were analyzed and compared with those in GenBank DNA database (NCBI) using the Basic Local Alignment Search Tool (BLAST: https ://www.ncbi.nlm.nih.gov/BLAST) and the highest identity score was considered for species identification (**Karimian *et al*., 2020**).

2.4. Measurement of GABA Content

2.4.1. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Gas chromatography-mass spectrometry (GC-MS) was used to verify the GABA production (**Kim *et al*., 2013**). According to **Lim *et al*., 2017**, the freeze-dried samples (10 mg) from culture supernatants were extracted by a 1 ml solvent mixture of methanol, chloroform and water (2.5:1:1 (v/v/v)). After two stage derivatizations of oximation followed by trimethylsilyl etherification, the derivative (1 µl) was separated by using the GC-MSQP2010 Ultra system with an AOC-20i autosampler (Shimadzu, Japan) and a DB-5 capillary column (30 m × 0.25 mm, 1.0 µm, J&W Scientific, USA). The temperature program was 100°C for 4 min, followed by a temperature gradient of 100- 320°C at 10°C/min and a hold at 320°C for 11 min.

3. Results and Discussion

A total of (7) lactic acid bacterial isolates were isolated from pickled green olive by appropriate dilutions with saline and was plated on MRS agar supplemented with 1% $CaCO_3$ to recognize

the acid-producing bacteria from other bacteria. Colonies of acid-producing bacteria was identified by a clear zone around each colony Figure (1), were randomly selected from MRS agar plates and purified by replating on MRS agar which is similar with results obtained in the study of **Goa *et al*., 2022.**

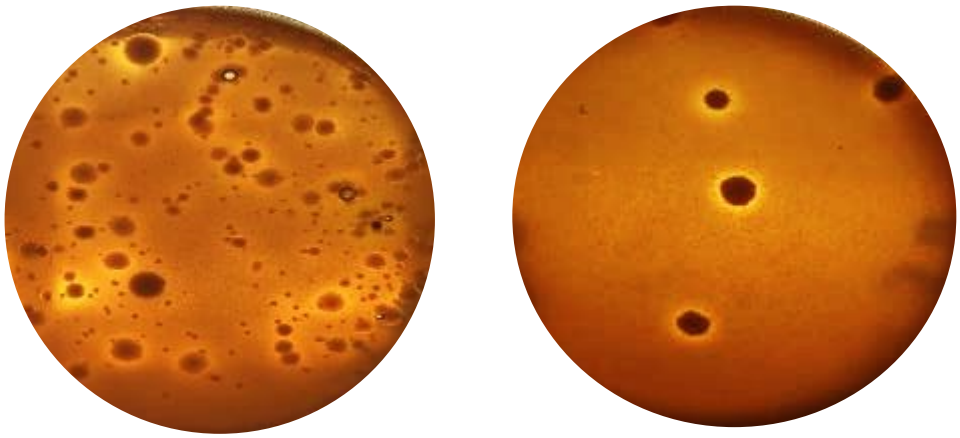


Figure (1). Lactic acid bacteria colonies grown on MRS agar media.

Gram staining results showed that the (7) isolates were Gram-positive, bacilli shaped and have different arrangements. These results are supported by research **Gong *et al*., 2021** which states that the lactic acid bacteria (LAB) are in the group of Gram-positive with the shape of cell bacilli as represented in Table (1) and Figure (2).

Table (1). Biochemical characteristics of LAB isolated from pickled green olive.

LAB isolate code	LAB genus detected	Gram stain	Cell morphology Cellular arrangement	Catalase
MGOLIV.7	Lactobacillus	+	Diplobacillus	Negative
MGOLIV.10	Lactobacillus	+	Monobacillus	Negative
MGOLIV.13	Lactobacillus	+	Streptobacilli	Negative
MGOLIV.19	Lactobacillus	+	Monobacillus	Negative
MGOLIV.21	Lactobacillus	+	Diplobacillus	Negative
MGOLIV.23	Lactobacillus	+	Diplobacillus	Negative
MGOLIV.25	Lactobacillus	+	Streptobacillus	Negative

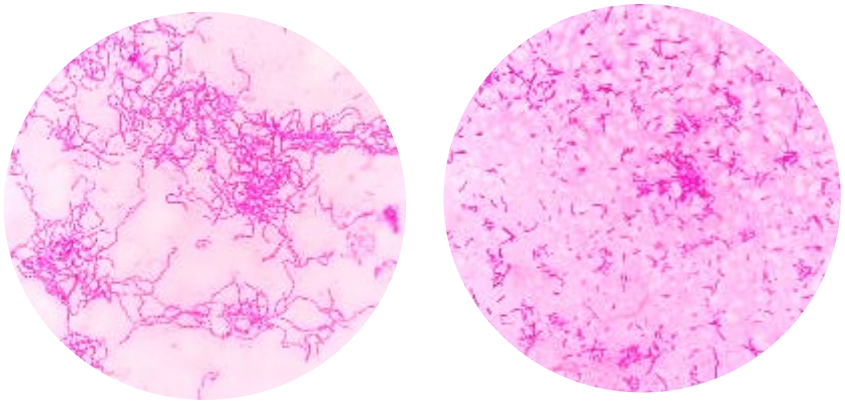


Figure (2). Microscopic appearance of Gram-stained selective *lactobacillus spp*.

Lactic acid bacteria do not produce catalase enzyme that converts hydrogen peroxide into water and oxygen because it includes in groups of anaerobic bacteria which is similar with results obtained in the study of **Ismail *et al*., 2018**.

Qualitatively, the GABA production by bacterial isolates was determined by observing red-colored spots on thin-layer chromatography (TLC) plate. Among the seven LAB isolate only one isolates (MGOLIV.25) showed high GABA production according to TLC and R_f value (0.75) of the sample that was matched with standard GABA as represented in Figure (3). The amounts of GABA in supernatant were further analyzed GC-Mass.

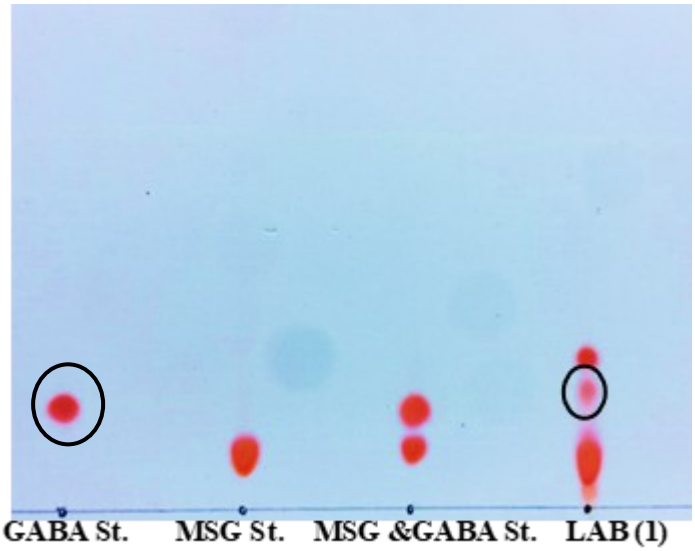


Figure (3). Thin-layer chromatography (TLC) analysis of GABA producing-LAB. Lane 1: GABA standard (G); lane 2: monosodium glutamate (MSG); lane 3: standard GABA and MSG; lane 4: GABA-positive strains.

Yogeswara *et al*., 2020 reported that the bacterial isolates were able to convert MSG during one day of incubation and showed the same Rf as that of GABA standard ($R_f = 0.61$) which is close to the R_f in the present study.

Based on the mass spectral analysis, GABA is confirmed by determining the mass of the compound. The concentration of GABA in the fermented extract was found to be 9.02 mg/ml.

The report by **Siragusa *et al*., 2007** is the first to show the synthesis of GABA by *L. casei*. In a research paper conducted by **Sharafi *et al*., 2020** the potential of GABA production by probiotic bacteria *L. casei* in the culture medium of MRS broth was evaluated and was found to be 53.82 mg/ml.

The highest GABA producer was (MGOLIV.25) were Gram positive, streptobacilli cell type. The identification of the bacterial isolate was further confirmed by molecular identification through 16S rRNA gene by PCR as represented in Figure (4), sequencing analysis and BLAST software of the NCBI was used to carry out sequence homology search. The isolate (MGOLIV.25) was identified as *Lacticaseibacillus casei* with similarity percentage of 99 % and the sequence was submitted to the GenBank under the accession number ON796544 as showed in Figure (5).

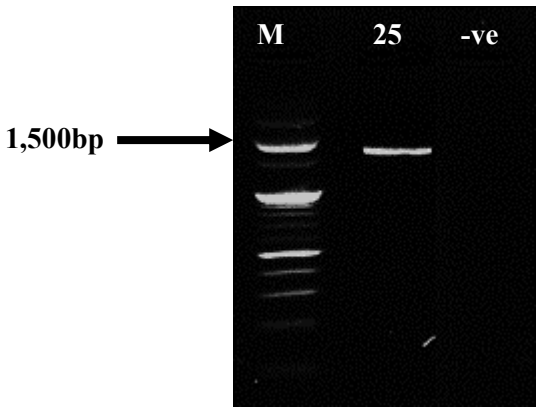
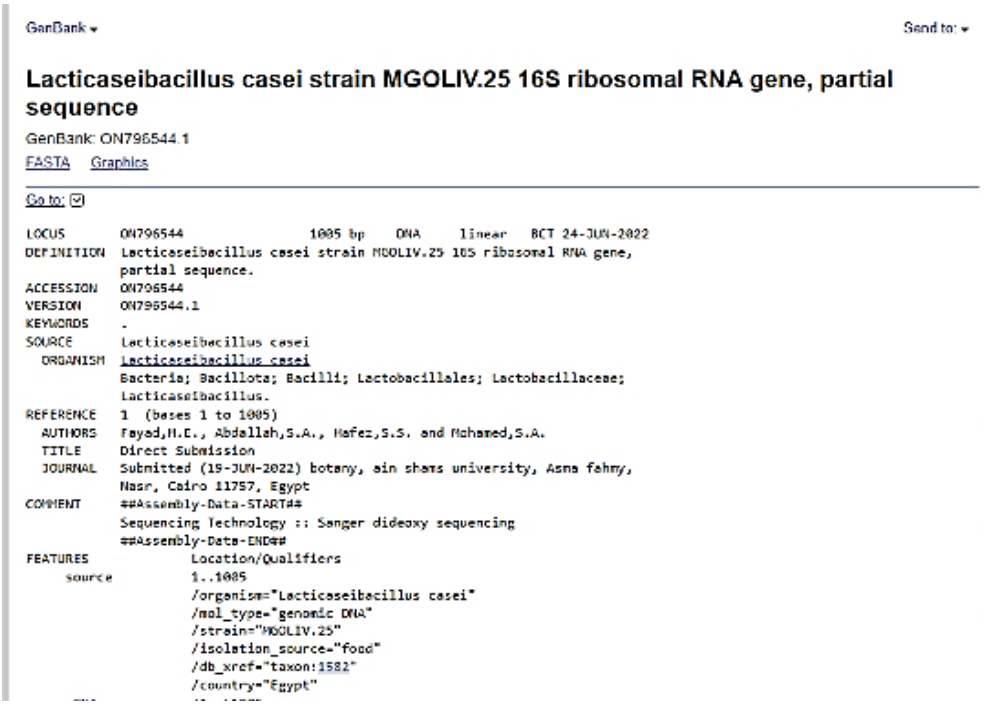


Figure (4). Agarose gel showing PCR amplification of 16S rDNA. Lane 1: 100 kb DNA ladder. Lanes from 2: represented MGOLIV.25 LAB isolates. Lane 3: represented negative control.



GenBank ▾ Send to: ▾

Lacticaseibacillus casei strain MGOLIV.25 16S ribosomal RNA gene, partial sequence

GenBank: ON796544.1

FASTA Graphics

Go to:

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VERSION     ON796544.1
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  ORGANISM  Lacticaseibacillus casei
            Bacteria; Bacillota; Bacilli; Lactobacillales; Lactobacillaceae;
            Lacticaseibacillus.
REFERENCE   1  (bases 1 to 1005)
  AUTHORS   Fayad,H.E., Abdallah,S.A., Hafez,S.S. and Mohamed,S.A.
  TITLE     Direct Submission
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Figure (5).16S rDNA partial submission of *Lacticaseibacillus casei MGOLIV.25*.

4. Conclusions

The fact that consumers have paid much approach toward different functional foods derived from natural products have been developed along with the tendency of consumers. GABA has been evidenced as a potent bioactive compound with numerous health beneficial effects. Therefore, the functional foods produced from GABA are thought to be able to prevent and/or treat different diseases, particularly diabetes, hypertension and neurological disorders. That's why GABA-producing strains will be remarkedly increased in food industry.

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