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# Isolation and Evaluation of Egyptian Gamma-Aminobutyric Acid (GABA) Producing Psychobiotic Bacteria

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**Abstract**

Psychobiotics are unique class of probiotics that potentially improve mental health via the production of neuroactive compounds such as gamma-aminobutyric acid (GABA). This study aimed to evaluate and improve GABA-producing lactic acid bacterial strains from fermented food origin to be used as potential psychobiotics. Hundred and ten presumptive lactic acid bacteria (LAB) were isolated from rumi cheese, mish cheese and pickled green olive and screened for GABA production using thin-layer chromatography (TLC). The survival rate of isolates to tolerate acidic conditions at pH 2.5 for 3 hours and bile salt resistance at 0.3% was evaluated. Three selected isolates were identified by 16S rRNA as *Lacticaseibacillus rhamnosus* MROMI.22, *Lacticaseibacillus casei* MGOLIV.25 and *Lacticaseibacillus chiayiensis* MGOLIV.28, and their GABA contents were determined by gas chromatography–mass spectrometry (GC–MS) to be 8.61 mg/ml, 9.02 mg/ml and 10.16 mg/ml, respectively. The safety prerequisite was confirmed by the absence of hemolytic activity. The proteolytic activity, cell surface hydrophobicity and auto-aggregation of selected isolates were also evaluated. The selected isolates showed also, variable antibacterial activity degree against nine pathogenic strains and different antibiotic sensitivity to nine common antibiotics. Response Surface Methodology (RSM) was used for culture media conditions optimization for GABA production from *L. chiayiensis* MGOLIV.28 that was found to produce 65.70 mg/ml increase of GABA production more than 6.46 fold, with a 72 h fermentation time, 33.5°C temperature, 4.5 pH and at 3% mono-sodium glutamate concentration. The free radical scavenging activity of *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 was 69.7ug/ml, 23.67ug/ml and 15.78ug/ml respectively. Also, they showed various cytotoxic activities degree.

**Keywords:** Psychobiotic**;** *Lactobacillus* sp.; GABA.

## 1. Introduction

One of the most important factors influencing life satisfaction and quality is mental health. Psychiatric and psychological research has proven that the general population has a higher prevalence of stress, anxiety, and depression (SAD). Probiotics have drawn a lot of attention from the scientific community as well as the general public. The notion of the microbiome's synergistic effects on the human body, especially the bidirectional signaling that takes place between the brain and the gut, has changed since the identification of the gut-brain axis [1]. Numerous investigations on the possible advantages of psychobiotics for depression and anxiety have been conducted recently in humans. Research interest has also been focused on the possible mechanisms behind their therapeutic effects, such as enhanced synthesis of GABA or other neurotransmitters [2]. Lactic acid bacteria (LAB) are among the GABA-producing microorganisms that are frequently employed in the manufacturing of numerous fermented foods [3]. Gamma-aminobutyric acid (GABA), commonly known as 4-aminobutyric acid, is a non-protein amino acid with four carbons. It is produced by the α-decarboxylation of L-glutamate and functions differently, one of which is nutrition acquisition molecule. Furthermore, GABA is essential for the mechanisms underlying the stress response [4].

Recently, GABA has been shown different biological capability including decreasing blood pressure and stress, improving memory and sleeping in addition to anticancer abilities and food additives [6,7,8,9,10,11]. Most lactic acid bacteria (LABs) are classified as “generally recognized as safe” (GRAS) [12,13] and have been used to commercially generate GABA [14,15].

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When it comes to the production of high-yield GABA, Lactobacillus is more active in acidic environments than other bacteria. The creation of enhanced microbial cells for the synthesis of commercially valuable metabolitesis a major goal of biotechnological research [16]. This study's primary goal is to assess and enhance lactic acid bacteria that produce γ-aminobutyric acid (GABA) from fermented food sources so they can be employed as possible psychobiotics.

## 1. Materials and methods

### 2.1. Fermented food sources

Rumi cheese, mish cheese and pickled green olive were used as sources for GABA-producing LAB isolation. They were purchased from local markets in Cairo, Egypt.

### 2.2. Isolation of LAB from fermented food

De Man, Rogosa and Sharpe (MRS) (Lab M, Heywood, UK) broth and agar were used as selective media throughout this study [17]. The culture broth was streaked on MRS 1.5% (w/v) agar plates with 2% (w/v) $CaCO_3$ and cultivated at 37°C for 72 hours under anaerobic conditions in 5% $CO_2$ container. After 72 hours of incubation, bacterial isolates that displayed a clear zone in the medium were considered putative LAB and selected for further investigation [18-19]. The putative LAB isolates were confirmed by Gram staining and catalase [20].

### 2.3. Screening of γ-Aminobutyric Acid GABA)-producing Lactic Acid Bacteria (LAB)

Isolated gram-positive and catalase-negative bacteria were screened for GABA production [21, 22]. GABA (Sigma-Aldrich, St. Louis, USA) production was preliminarily evaluated by thin-layer chromatography (TLC) analysis (Silica gel 60 TLC F254 (Merck, Germany) [23]. One microliter (1 µl) of supernatant from each isolate was spotted on a silica TLC plate with dimensions of 20 × 20 cm. Then the thin layer chromatography was developed at 30°C with the developing solvent system N-butanol– acetic acid–water (5:3:2, v/v/v) solvent (ascending technique). The TLC chamber was filled to 1/4 with the solvent system, the lid was closed.

### 2.4. Screening of probiotic activities of selected isolates

#### 2.4.1. Tolerance against low pH and resistance to bile salts

The acid resistance test was carried out using spectrophotometer to measure the optical density (OD) of each sample at 650 nm every hour in triplicate [24]. The bile salt tolerance test was also performed [25]. One (1 ml) bacterial cultures were inoculated into 9 ml MRS broth pH 2.5 and incubated at 37°C for 3 h. After the incubation time, spectrophotometer was used to measure the optical density (OD) of each sample at 650 nm. The level of absorbance ($A_{650}$) has been adjusted to a value of 0.08 ± 0.05 to standardize the number of bacteria. Each acid-tolerant isolate was screened for bile tolerance. One hundred microliters of overnight grown bacterial culture was inoculated in freshly prepared MRS broth containing 0.3% bile salts (Sigma-Aldrich, St. Louis, USA). The viability of bacteria in 0.3% bile was evaluated by spreading 100 µl of the bacterial sample onto the MRS agar plate at the time (0, 1, 2, 3, 4) h. Plates with no bacterial colony were considered negative and the ones with colonies were considered positive.The acid tolerance and the survival rate were calculated using the following equation:

$$\text{Survival Rate (\%)} = \frac{\text{OD (After treatment)}}{\text{OD (Before treatment)}} x\ 100\ \%$$

### 2.5. Molecular identification of GABA-producing LAB isolates

Selected LAB isolates were identified using 16S rRNA gene sequencing using Thermo Scientific GeneJET Genomic DNA Purification Kit (Cat. No K0721) (Thermo Fisher Scientific, Massachusetts, USA). Universal primers 27F and 1492R were used [26]. The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST: http://www.ncbi.nlm.nih.gov/BLAST) and then submitted to GenBank to obtain the accession number.

### 2.6.Probiotic characteristics

#### 2.6.1. Hemolytic and proteolytic activity

For hemolytic activity, MRS agar plates with 5% human blood were streaked with 18 h old *Lactobacillus* cultures and incubated anaerobically at 37°C for 48-72 h. The plates were checked for a clearance zone indicating a hemolytic reaction [27]. The proteolytic activity of selected isolates was evaluated by the well diffusion method [28]. De Man, Rogosa and Sharpe (MRS) agar plates supplemented with 5% human blood were streaked with 18 h old *Lactobacillus* broth cultures and incubated at 37°C for 48 h in triplicate. A clear zone diameter around the well indicates positive proteolytic activity [29].

### 2.7. Adhesion activity detection

#### 2.7.1. Assessment of cell surface hydrophobicity (CSH)

The hydrophobicity of selected strains was checked [30]. It determined the attachment of bacteria to a polar solvent xylene. The hydrophobicity determines the attachment of bacteria to a polar solvent xylene. Overnight grown cultures were centrifuged at 7000 rpm for 10 min at 4°C. Supernatants were discarded and pellets were washed twice with phosphate buffer saline (PBS) (pH 7.2). Absorbance at 650 nm was adjusted to 0.08 ± 0.05 (Ao). In test tubes, 3 ml of the above suspensions was taken and 0.6 ml of xylene was added followed by homogenization using vortex for 1 min. Test tubes were incubated at 37°C for 2 h to allow the separation of two phases. After incubation, aqueous phase was taken and absorbance at 650 nm was measured (A).

$$\text{Cell surface hydrophobicity (H\%)} = \frac{A0 - A}{A0} \; x100$$

Where H% = cell surface hydrophobicity (%), Ao = initial absorbance at 600 nm and A = final absorbance of the aqueous phase.

**2.7.2. Cellular autoaggregation assay (AA)**

The autoaggregation assay was performed [31]. Auto-aggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells. Bacterial isolates were grown for 20 h in MRS broth in anaerobic condition at 37°C. The bacteria were centrifuged at 5000 rpm for 20 min, washed twice with PBS and then re-suspended in PBS. The level of absorbance ($A_{650}$) was adjusted to a value of 0.08 ± 0.05 to standardize the number of bacteria. The suspension (4 ml) was vortexing ($A_{initial}$) and then incubated for 2 h at 37°C ($A_{2h}$). Autoaggregation was expressed with the following equation:

$$\text{Cellular auto-aggregation (AA \%)} = 1 - \frac{A2h}{Ainitial} \; x \; 100$$

where AA% = cellular autoaggregation (AA) (%), Ainitial = initial absorbance at 600 nm and A2h = final absorbance after two hours.

**2.8. Antibiotic susceptibility test**

The LAB strain antibiotic susceptibility was assessed on MRS agar plates by the antibiotic disc diffusion method using nine antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and Vancomycin) suggested by the European Food Safety Authority (EFSA) guidelines [32]. Antibiotic susceptibility patterns (Himedia, Maharashtra, India) were also examined [33]. The MRS agar medium was poured and allowed to solidify at room temperature. The overnight LAB cultures (100 µl) were spread on MRS agar plates and allowed to dry. The antibiotic discs were placed onto the inoculated plates and incubated at 37°C for 24 h. The level of absorbance ($A_{650}$) was adjusted to a value of 0.08 ± 0.05 to standardize the number of bacteria. The results were compared with the interpretative zone diameters as described in the Performance Standards for Antimicrobial Disc Susceptibility Tests [34].

**2.9. Evaluation of antioxidant activity by the DPPH radical scavenging method**

The free radical scavenging activity of bacterial extracts was measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) [35]. In brief, (0.1 mM) solution of DPPH in ethanol was prepared. This solution (1 ml) was added to (3 ml) of different extracts in ethanol at different concentration (3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, 1000 µg/ml). Only those extracts were used which were solubilized in ethanol and their various concentrations were prepared by dilution method. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min in dark place. Absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Milton roy). Ascorbic acid was chosen as the reference antioxidant (Sigma-Aldrich, St. Louis, USA).

**2.10. Antibacterial activity of selected strains**

The antibacterial activity was investigated [36, 37]. Pathogenic strains used in this test were obtained from the Botany Department, Faculty of Women for Art, Science and Education, Ain Shams University (Egypt, Cairo), which included *Bacillus subtilis* (MW391723), *Frendinandcohnia humi* (MW391719), *Sternotrophomonas maltophilia* (MW390826), *Bacillus* anthracis (MW390831), *Bacillus stratosphericus* (MW391714), *Enterobacter cancerogenus* (MW390637), *Staphylococcus aureus* (MW390870), *Pseudomonas aeruginosa* (MW390638), *Klebsiella pneumoniae* (MW390509) and *E.coli* aerobically grown in Luria–Bertani broth (LB) at 37ºC for 24 hours. Briefly, the cultural broths of the isolates were obtained from overnight cultures grown in MRS broth at 37ºC. Cell free supernatants were obtained by centrifuging the culture broth at 8000 rpm for 15 min. The supernatants were filter-sterilized by Millex-GP syringe filter 0.22 µm (Merck, Darmstadt, Germany). Then, the supernatants were lyophilized with Labconco freeze dryer (Kansas City, Missouri, USA). The dried sample was dissolved in 4 ml of sterilized distilled water and its antimicrobial properties were investigated by agar-well diffusion [37].

**2.11. Cytotoxicity activity determination using MTT assay**

The cytotoxicity of the fermented extract of bacterial strains against Caco-2 cancer cells was investigated using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [38]. The analysis was performed at the Center for Drug Discovery Research and Development, Faculty Pharmacy, Ain Shams University. The ninety-six well tissue culture plate was inoculated with (100 ul / well) and incubated at 37°C for 24 h to develop a complete monolayer sheet then the growth medium was decanted from 96 well micro titer plates after confluent the sheet of cells were formed, cell monolayer was washed twice

with phosphate buffer saline. Two-fold dilutions of the tested sample was made in Roswell Park Memorial Institute (RPMI) medium with 2% serum (maintenance medium), (0.1 ml) of each dilution was evaluated in different wells leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37°C and examined.

**2.12. Gas chromatography–mass spectrometry (GC–MS) analysis**

Gas chromatography–mass spectrometry (GC–MS) was used to detect GABA production [39]. A TSQ Quantum triple quadrupole GC-MS/MS instrument coupled with a Thermo Scientific™ TRACE™ 1300 GC (Thermo Scientific, Austin, TX, USA) was used. Sample introduction was performed a Thermo Scientific™ AS3000 autosampler, and chromatographic separation using a Thermo Scientific™ Trace GOLD TG-5MS 30 m × 0.25 mm I.D. × 0.25 μm film capillary column. Additional details of instrument parameters are: Oven temperature program: initial temperature 50 C for 1 min, increased by 4 C/min to 250 C and held there for 5 min, ion source and transfer line were 250, and 270 C, respectively. Helium was used as carrier gas at a flow rate of 0.7 mL/min. Split Injection mode was used. The mass spectrometer (MS) was operated in electron impact ionization/selective ion monitoring (EI/SIM) mode (70 eV). The identification mass fragment of 4-Aminobutanoic acid, 3TMS derivative(GABA) used in this method were: m/z 304 and 174. Based on the mass spectral analysis, GABA is confirmed by determining the mass of the compound. The elemental composition of GABA is (C4H9NO2), which has a molecular weight of 103.

**2.13. Optimization of γ-aminobutyric acid (GABA) for selected LAB strains using response surface methodology (RSM)**

Response surface methodology is a systematic analytical method for investigating the effects of an independent variable (fermentation temperature, time, pH and MSG). Additionally, it is an optimization tool widely used in production processing, as shown in Table (1) [40]. A set of 27 experiments was planned, with each variable at four levels, as shown in Table (2). The relations between the coded values and actual values, independent variables and responses were calculated according to the quadratic model. The relative effects of two variables on the response were examined from three-dimensional (3D) contour plots. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The GABA production was optimized using RSM with Box–Behnken Design (BBD) in Design Expert 7.0 software (Version 7, Godward St NE, Suit 6400, Minneapolis, MN, USA) [41].

The optimal fermentation conditions for GABA production were modeled using response surface methodology (RSM). The independent variables of culture media optimization are shown in Table (1).

**Table 1:** Independent variables levels to optimize the culture condition to produce GABA

| Independent variables | **Model parameter** | **+1** | **0** | **-1** |
|---|---|---|---|---|
| Temperature (ºC) | **A** | **30** | **33.5** | **37** |
| Time (h) | **B** | **24** | **72** | **120** |
| pH | **C** | **3.5** | **4.5** | **5.5** |
| Monosodium glutamate (MSG) (%) | **D** | **1** | **3** | **5** |

**2.14. Statistical analysis**

The data obtained in this research was expressed as mean ± SD (standard deviation) of triplicates and were analyzed using one-way analysis of variance (ANOVA). The mean and (SD) were calculated by subjecting the values to Statistical Package for the Social Sciences (SPSS) 16.020 statistical analyses when required using Microsoft Excel 2020.

**Table 2:** Box-Behnken design (BBD) with studied variables for the GABA production of selected strains

| Run | Temperature (ºC) | Time (h) | pH | MSG (%) |
|---|---|---|---|---|
| **1** | **33.5** | **24** | **5.5** | 3 |
| **2** | **37** | **72** | **5.5** | 3 |
| **3** | **30** | **24** | **4.5** | 3 |
| **4** | **33.5** | **120** | **4.5** | 5 |
| **5** | **33.5** | **24** | **4.5** | 5 |
| **6** | **33.5** | **72** | **4.5** | 3 |
| **7** | **33.5** | **72** | **4.5** | 3 |
| **8** | **33.5** | **24** | **4.5** | 1 |
| **9** | **37** | **72** | **4.5** | 1 |
| **10** | **33.5** | **72** | **3.5** | 5 |
| **11** | **33.5** | **72** | **4.5** | 3 |
| **12** | **30** | **72** | **4.5** | 5 |
| **13** | **37** | **72** | **4.5** | 5 |
| **14** | **30** | **72** | **4.5** | 1 |
| **15** | **30** | **120** | **4.5** | 3 |
| **16** | **30** | **72** | **3.5** | 3 |
| **17** | **33.5** | **72** | **5.5** | 1 |
| **18** | **30** | **72** | **5.5** | 3 |
| **19** | **37** | **72** | **3.5** | 3 |
| **20** | **33.5** | **24** | **3.5** | 3 |
| **21** | **33.5** | **120** | **4.5** | 1 |
| **22** | **33.5** | **72** | **5.5** | 5 |
| **23** | **33.5** | **120** | **5.5** | 3 |
| **24** | **33.5** | **120** | **3.5** | 3 |
| **25** | **37** | **120** | **4.5** | 3 |
| **26** | **37** | **24** | **4.5** | 3 |
| **27** | **33.5** | **72** | **3.5** | 1 |

## 3. Results

### 3.1. Isolation, purification and evaluation of lactic acid bacteria (LAB) for their selection as probiotics

In the present study, 110 lactic acid bacteria were isolated from Rumi cheese (45 isolates), Mish cheese (30 isolates) and pickled green olive (35 isolates) plated on MRS agar supplemented with 2% $CaCO_3$. The primary characteristics of the bacterial isolates revealed that all isolates were gram-positive and were catalase negative.

### 3.2. Screening of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB)

GABA production was confirmed by observing red spots on TLC plates. The red spot mobility from the culture supernatant was consistent with the GABA standard, as a retention factor (Rf) value (0.75 cm) of the sample was matched with the GABA standard at detectable concentrations. Only strains with Rf value corresponding to that of the GABA standard (0.75 cm) were selected for GABA quantification and accordingly, only 42 selected isolates were considered GABA producers.

### 3.3. Screening of probiotic activities of isolated bacteria in vitro

#### 3.3.1. Tolerance against low pH

Selected LAB 42 isolates (15 isolates from Rumi cheese, 11 isolates from Mish cheese and 16 isolates from pickled green olive) were screened for their ability to tolerate acidic conditions at pH 2.5. The results indicated that the isolates (MROMI.22, MGOLIV.25 and MGOLIV.28) were able to withstand pH 2.5 for 3 hours, as indicated by the absorbance with survival rates of 96%, 94% and 99%, respectively, as illustrated in Figure (1).

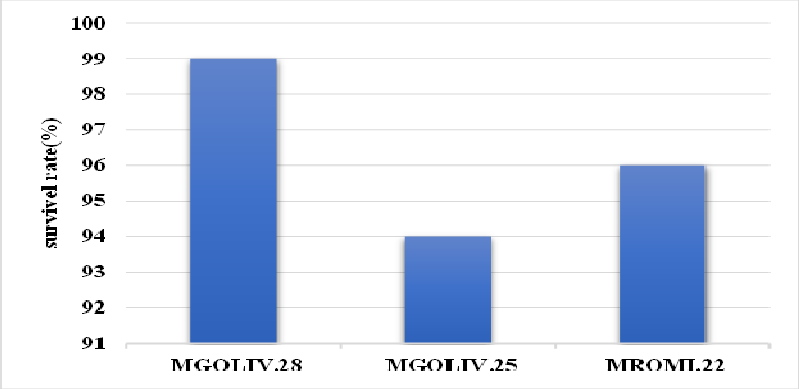



**Figure 1:** Lactic acid bacteria (LAB) probiotic isolates survival rate (%) after 3 hours incubation at pH (2.5)

### 3.3.2. Resistance to Bile Salts

The strains that showed acid resistance at pH 2.5 for 3 hours were selected to be screened for bile salt resistance. The isolates (MROMI.22, MGOLIV.25 and MGOLIV.28) showed survival rates of 91%, 87% and 93%, respectively, at 0.3% concentration of bile salt, as presented in Figure (2). Therefore, the results suggest that the three strains possess probiotic characteristics with high survival rates at low pH (2.5) and bile concentrations (0.3%).

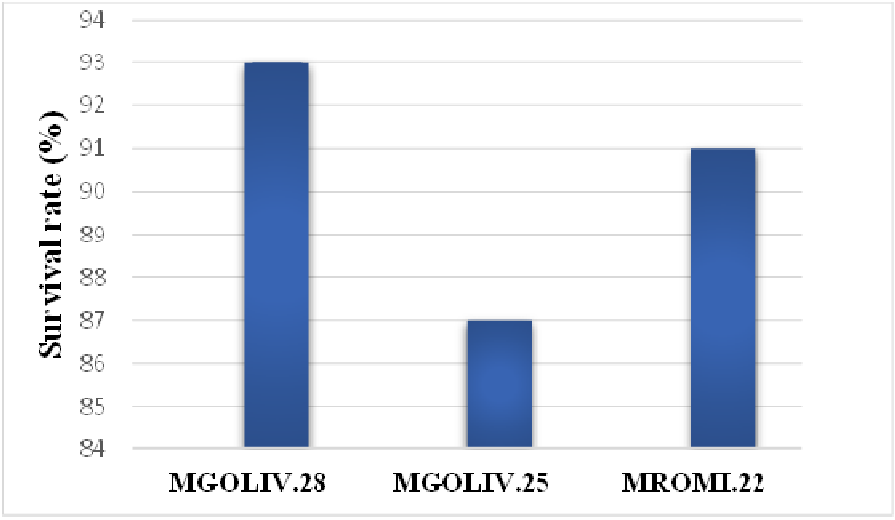



**Figure 2:** Lactic acid bacteria (LAB) probiotic isolates survival rate (%) after 4 h incubation in bile salt (0.3)

### 3.4. Molecular identification of the selected GABA-producing LAB isolates

Partial sequence data of 16S rRNA genes of isolates *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 were deposited in the database of the National Center for Biotechnology Information (NCBI) with accession no. ON796543, ON796544 and ON796545, respectively.

### 3.4.1. Construction of a Circos plot for 16S rRNA

To illustrate the differences and similarities between the isolated strains *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 with the public database in GenBank, bioinformatics tool Circos plot analyses were constructed at the genus level as illustrated in Figure (3). A spectrum of colors, ranging from a distinct color tone for low identity to a different tone for high identity, was employed to accentuate the variation in conservation levels. The ribbons transition from one color tone, denoting lower sequence identity, to another tone signifying higher similarity. The color gradient was calibrated based on the

calculated percentage identity values, with a minimum value of 76.47% representing the least conserved regions and a maximum of 100.00% denoting perfect sequence conservation.

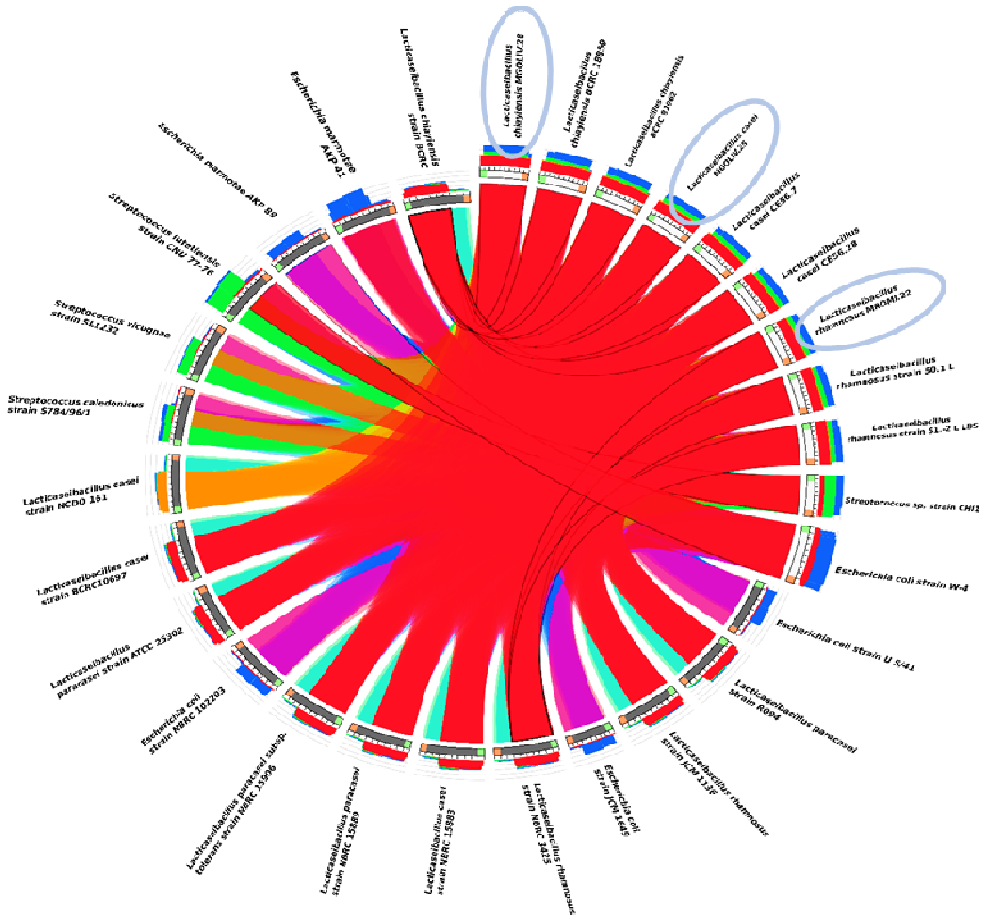


**Figure 3:** Circos plot illustrated the difference and the similarity between LAB strains

### 3.5. Gas Chromatography–Mass Spectrometry (GC–MS) analysis

GABA production for *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 bacterial strains were confirmed by GC–MS analysis. The three strains could convert MSG to GABA. The identity of the putative GABA peak was confirmed by GC with (MS) detection. *L. chiayiensis* MGOLIV.28 was the highest GABA producer at 10.16 mg/ml and was selected for further experiments, followed by *L. casei* MGOLIV.25 (9.02 mg/ml) and *L. rhamnosus* MROMI.22 (8.61 mg/ml).

### 3.6. Probiotic characteristics

#### 3.6.1. Hemolytic activity

Absence of hemolytic activity is considered as a safety prerequisite for the selection of a probiotic strain. None of the three GABA-producing *Lacticaseibacillus* (*L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28) strains showed α and β hemolytic activity when grown on blood agar.

#### 3.6.2. Proteolytic activity

The proteolytic activity of the three selected strains (*L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28) was determined by measuring diameters of halo zone on the skimmed milk agar, *L. chiayiensis* MGOLIV.28 (28 mm), *L. casei* MGOLIV.25 (25mm) and *L. rhamnosus* MROMI.22 (23 mm).

### 3.7. Adhesion activity detection

#### 3.7.1. Assessment of cell surface hydrophobicity (CSH)

The hydrophobicity of the three selected strains *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 showed different hydrophobicity. *L. chiayiensis* MGOLIV.28 showed the highest hydrophobicity at (73.8%) followed by *L. rhamnosus* MROMI.22 (71.3%) and *L. casei* MGOLIV.25 (59.9%) as illustrated in Figure (4).

**Table 3:** Concentration of γ-aminobutyric acid (GABA) of *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 strains analyzed by GC-MS to determine the GABA concentration

| LAB strains | Source | Concentration (mg/ml) |
|---|---|---|
| *L. rhamnosus* MROMI.22 | Rumi cheese | **8.61** |
| *L. casei* MGOLIV.25 | Pickled green olive | **9.02** |
| *L. chiayiensis* MGOLIV.28 | Pickled green olive | **10.16** |

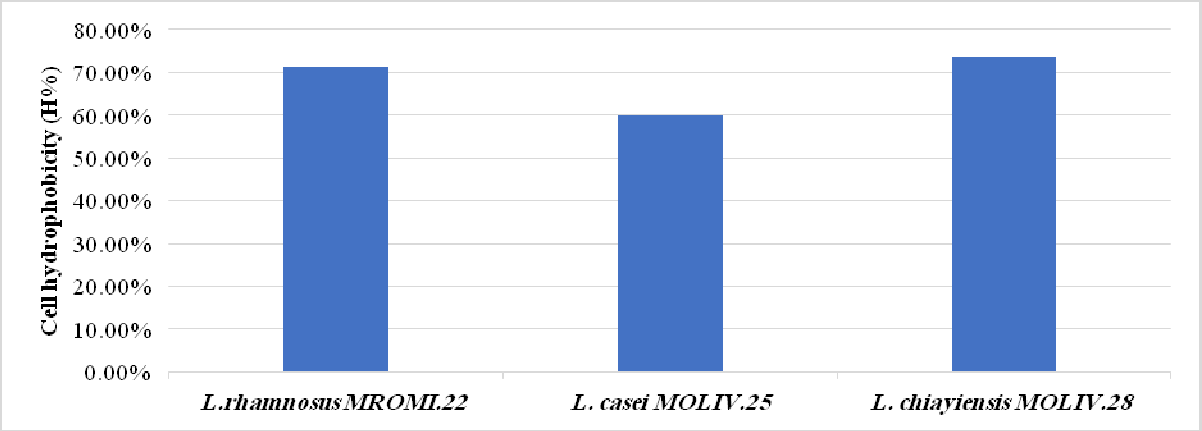



**Figure 4:** Hydrophobicity potential of the three selected strains. Each bar represents the mean ± standard deviation (SD)

### 3.7.2. Cellular auto-aggregation assay

*L. chiayiensis* MGOLIV.28 strain showed the highest auto aggregation at (54.03%) followed by *L. casei* MGOLIV.25 (39.78%) and *L. rhamnosus* MROMI.22 (20.93%) Figure (5).

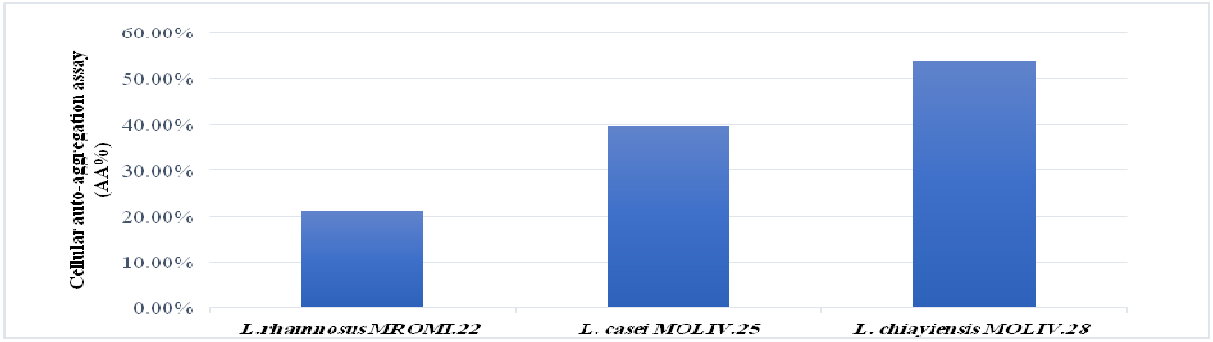



**Figure 5:** Auto-aggregation potential of the three strains. Each bar represents the mean ± standard deviation (SD)

### 3.8. Antibiotic susceptibility test

Antibiotic susceptibility is a key factor associated with the safety of using probiotic bacteria. Therefore, the antibiotic susceptibility of the three GABA-producing LAB strains (*L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28) to nine commonly used antibiotic was evaluated based on the diameter of the inhibition zone (mm) in the disc diffusion test. A variable antibiotic sensitivity was observed in all the selected strains and is reported accordingly in Table (4).

**Table 4:** Inhibition zone (mm) of the selected LAB strains

| Antibiotics | Concentration (µg/disc) | LAB strains | | |
|---|---|---|---|---|
| | | *L.rhamnosus* MROMI.22 | *L. casei* MGOLIV.25 | *L. chiayiensis* MGOLIV.28 |
| Ampicillin | 10 µg | **0 R** | **0 R** | **0 R** |
| Chloramphenicol | 30 µg | **13 MS** | **25 S** | **20 S** |
| Clindamycin | 2 µg | **20 MS** | **30 S** | **15 MS** |
| Erythromycin | 15 µg | **30 S** | **30 S** | **30 S** |
| Gentamicin | 10 µg | **15 S** | **17 S** | **17 S** |
| Kanamycin | 30 µg | **0 R** | **0 R** | **0 R** |
| Streptomycin | 10 µg | **0 R** | **10 R** | **0 R** |
| Tetracycline | 15 µg | **10 R** | **10 R** | **15 MS** |
| Vancomycin | 30 µg | **0 R** | **0 R** | **0 R** |

S sensitive, MS medium-sensitive, R resistant, µg - Microgram

### 3.9. Antibacterial activity

Based on the zone of inhibition (mm), significant antimicrobial activity was observed in the three selected strains. Strains *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 showed various degrees of antibacterial activity against the pathogenic strains including: Gram positive (*Bacillus anthracis* (MW390831), *Bacillus subtilis* (MW391723), *Fredinandcohnia humi* (MW391719), *Bacillus stratosphericus* (MW391714), *Staphylococcus aureus* (MW390870)) and Gram negative (*Stenotrophomonas maltophilia* (MW390827), *Enterobacter cancerogenus* (MW390637), *Pseudomonas aeruginosa* (MW390638), *Klebsiella pneumoniae* (MW390509) and *E.coli* ). The results indicated that *L. chiayiensis* MGOLIV.28 cell free supernatant has strong antimicrobial activity against all tested pathogens and the diameter of inhibition zones ranged from 23 to 40 mm.

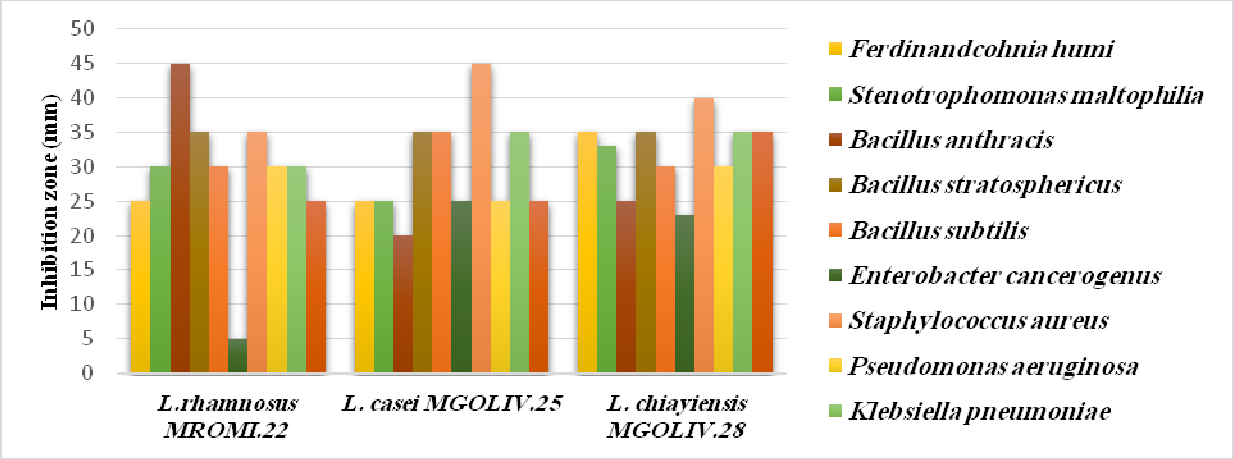



**Figure 6:** Evaluation of antimicrobial activity of the three selected strains supernatants against clinically relevant pathogens

The highest antimicrobial activity for *L. rhamnosus* MROMI.22 was recorded against Gram-positive bacteria *Bacillus anthracis* (45mm) and the lowest activity was recorded against Gram-negative bacteria *Enterobacter cancerogenus* (5 mm). While the highest antimicrobial activity *L. casei* MGOLIV.25 was recorded against Gram-positive bacteria *Staphylococcus aureus* (45 mm) and the lowest activity was recorded against Gram-positive bacteria *Bacillus anthracis* (20 mm). The highest antimicrobial activity for *L. chiayiensis* MGOLIV.28 was recorded against Gram-positive bacteria *Staphylococcus aureus* (40mm) and the lowest activity was recorded against Gram-negative bacteria *Enterobacter cancerogenus* (23 mm) as illustrated in Figure (6).

### 3.10. Antioxidant DPPH scavenging activity

*In vitro* scavenging activity of strains fermented extract, *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 were evaluated for their antioxidant activity using DPPH assay test. Ascorbic acid was chosen as the reference antioxidant in this test.

The $IC_{50}$ of the fermented extract of *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 were 69.7 ug/ml, 23.67 ug/ml and 15.78 ug/ml, respectively. The $IC_{50}$ of ascorbic acid (standard) was 7.43 µg/ml Figure (7)**.** The free radical scavenging activity of different extracts and ascorbic acid was in the following order: ascorbic acid > *L. chiayiensis* MGOLIV.28 > *L. casei* MGOLIV.25 > *L. rhamnosus* MROMI.22 as presented in Figure (7)**.**

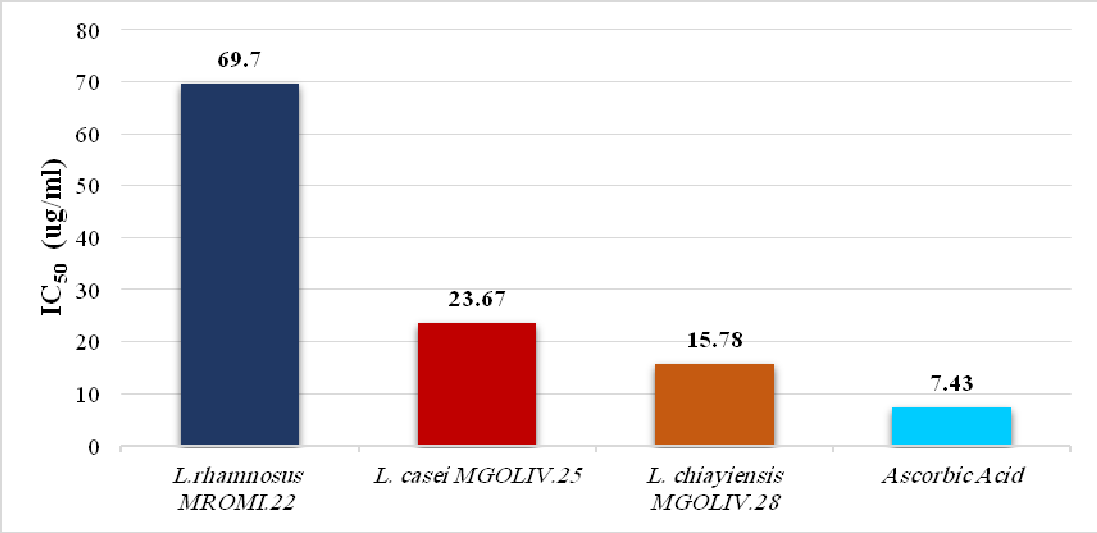



**Figure 7:** $IC_{50}$ of DPPH radical scavenging activity for ascorbic acid. *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28

### 3.11. Cytotoxicity assay on Caco-2 cell line

The current study showed various cytotoxic activities degrees of different fermented extracts against Caco-2 cells lines using MTT. The results confirm that the $IC_{50}$ value of fermented extract of *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25 and *L. chiayiensis* MGOLIV.28 against Caco-2 cells line were 293.07 ± 3.86 µg/ml, 245.27 ± 4.42 µg/ml and 155.19 ± 1.14 µg/ml respectively as compared with Vero cells $IC_{50}$ value (833.43 ± 5.22 ug/ml).

### 3.12. Optimization of gamma-aminobutyric acid (GABA) production by *L. chiayiensis* MGOLIV.28 using Response Surface Methodology (RSM) experimental design

To increase the amount of produced GABA from *L. chiayiensis* MGOLIV.28 strain (that gave the best GABA producer strain), culture media conditions were optimized by Response Surface Methodology (RSM). The predicted results were confirmed by the experiments and no difference was observed between the predicted and experimental data as showed in Table (5).

The interaction effect of these factors on GABA concentration produced by *L. chiayiensis* MGOLIV.28 is described by the below equation: where R is the concentration of GABA (mg/ml); A, B, C and D are coded values of the independent variables (A: temperature, B: Time, C: pH and D: MSG) respectively. The equation demonstrates the relationship between the determined GABA yield and the four fermentation factors:

$$R = +64.37-6.22A-5.92B+1.27C-9.48D-1.98AB\ 2.67AC+3.70AD+0.6000BC+2.27BD-1.43CD-24.66A^2-11.31B^2-18.56C^2-20.65D^2$$

Table (6) showed the statistical analysis of variance ANOVA used to determine the model's significance. The p-value ($p \leq 0.05$) indicates that the experimental data obtained fit well with the model. The coefficient of determination ($R^2$) was used to evaluate the quality of the model. The values of $R^2$ and the adjusted $R^2$ were 0.9772 and 0.9507, respectively which indicates that the model could explain 97.72% of GABA variation while a minor remainder (2.28%) could not be predicted by the model. The lack of fit was insignificant and its p-value was 0.1051 ($p \leq 0.05$). All these values indicate that the reliability and general quality of the model were highly acceptable in predicting the GABA yield.

**Table 5:** Various combination sets produced and the corresponding GABA yield (actual and predicted values)

| Run order | **Actual value** | **Predicted value** |
|---|---|---|
| 1 | 42.20 | 41.08 |
| 2 | 11.70 | 13.52 |
| 3 | 35.70 | 38.56 |
| 4 | 14.40 | 19.37 |
| 5 | 23.30 | 26.66 |
| 6 | 64.30 | 64.37 |
| 7 | 63.10 | 64.37 |
| 8 | 55.40 | 50.18 |
| 9 | 18.50 | 18.72 |
| 10 | 17.00 | 15.93 |
| 11 | **65.70** | **64.37** |
| 12 | 13.60 | 12.18 |
| 13 | 10.40 | 7.15 |
| 14 | 36.50 | 38.55 |
| 15 | 31.00 | 30.67 |
| 16 | 24.50 | 23.43 |
| 17 | 34.90 | 37.43 |
| 18 | 32.40 | 31.31 |
| 19 | 15.50 | 16.34 |
| 20 | 41.40 | 39.75 |
| 21 | 37.40 | 33.79 |
| 22 | 18.20 | 15.61 |
| 23 | 30.00 | 30.45 |
| 24 | 26.80 | 26.72 |
| 25 | 15.70 | 14.29 |
| 26 | 28.30 | 30.08 |
| 27 | 28.00 | 32.04 |

**Table 6:** Analysis of variance (ANOVA) from obtained results of GABA production by *L. chiayiensis*MGOLIV.28 strain

| Source | **Sum of Squares** | **df** | **Mean Square** | **F-value** | **p-value** | |
|---|---|---|---|---|---|---|
| Model | 6618.63 | 14 | 472.76 | 36.78 | < 0.0001 | **significant** |
| A-Temperature | 463.76 | 1 | 463.76 | 36.08 | < 0.0001 | |
| B-Time | 420.08 | 1 | 420.08 | 32.68 | < 0.0001 | |
| C-pH | 19.25 | 1 | 19.25 | 1.50 | 0.2445 | |
| D-MSG Conc. | 1079.20 | 1 | 1079.20 | 83.95 | < 0.0001 | |
| AB | 15.60 | 1 | 15.60 | 1.21 | 0.2922 | |
| AC | 28.62 | 1 | 28.62 | 2.23 | 0.1615 | |
| AD | 54.76 | 1 | 54.76 | 4.26 | 0.0613 | |
| BC | 1.44 | 1 | 1.44 | 0.1120 | 0.7436 | |
| BD | 20.70 | 1 | 20.70 | 1.61 | 0.2285 | |
| CD | 8.12 | 1 | 8.12 | 0.6318 | 0.4421 | |
| $A^2$ | 3242.84 | 1 | 3242.84 | 252.26 | < 0.0001 | |
| $B^2$ | 682.02 | 1 | 682.02 | 53.05 | < 0.0001 | |
| $C^2$ | 1836.86 | 1 | 1836.86 | 142.89 | < 0.0001 | |
| $D^2$ | 2254.11 | 1 | 2254.11 | 175.34 | < 0.0001 | |
| Residual | 154.26 | 12 | 12.86 | | | |
| Lack of Fit | 150.88 | 10 | 15.09 | 8.91 | 0.1051 | **not significant** |
| Pure Error | 3.39 | 2 | 1.69 | | | |
| Cor Total | 6772.89 | 26 | | | | |

Figure (8) shows the merged effect of pH, MSG concentration, temperature and time on GABA production displayed as the response surface profiles. Figure (**a**) shows the effect of temperature and MSG Figure (**b**) shows the effect of MSG and pH, Figure (**c**) shows the effect of temperature and pH, Figure (**d**) shows the effect of temperature and time, Figure (**e**) shows the effect of time and pH on GABA production of *L. chiayiensis* MGOLIV.28.

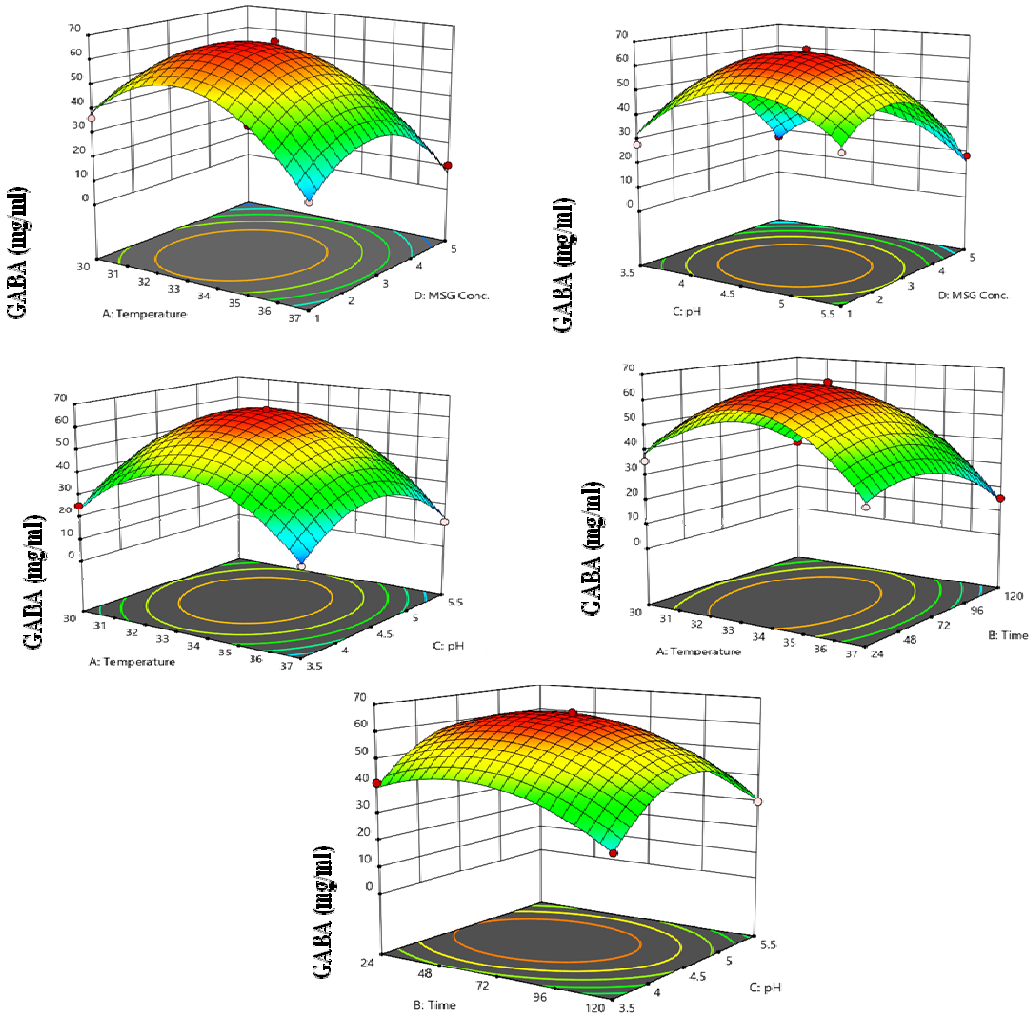
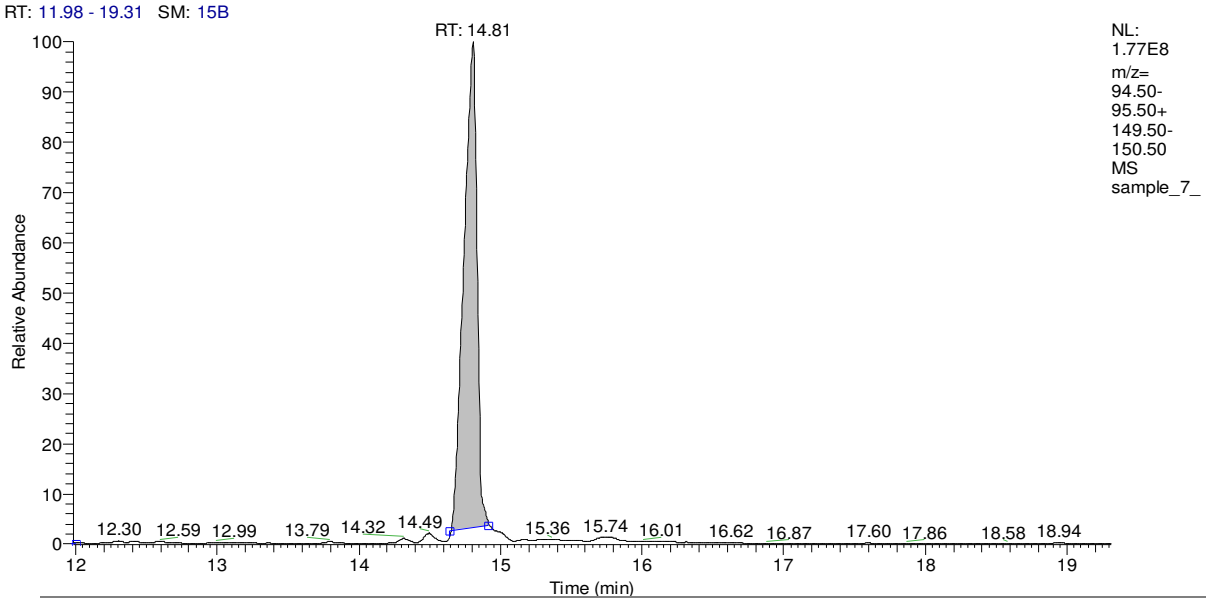



**Figure 8:** Response surface profile of GABA production from *L. chiayiensis* MGOLIV.28 indicated the effects between (a) temperature amd MSG (b) MSG and pH (c) temperature and pH (d) temperature and time and (e) time and pH

### 3.13. Selection of optimal condition and validation of model

The results of GABA production by *L. chiayiensis* MGOLIV.28 strains showed the optimal conditions for GABA production had a temperature of 33.5°C, time 72 h, pH of 4.5 and a concentration of 3% of MSG. In the above conditions, the amount of GABA production by the selected strain was 65.70 mg/ml obtained from run number 11 as showed in Figure (9).




**Run 11**

| **Apex RT** | Start RT | End RT | Area | %Area | **Concentration(ppm)** |
|---|---|---|---|---|---|
| **14.81** | 14.68 | 14.87 | 1015478374 | 6.57 | **65700 (65.70 mg/ml)** |

**Figure 9:** Optimal treatment GC-MS for run number 11

## 4. Discussion

The potential use of fermented foods as a probiotic delivery system in the management of mental health was addressed, noting that there is a growing evidence suggesting psychobiotics can be a nutritional supplement able to improve mental well-being in addition to physical health [42]. Franciosi *et al.* identified high-producing LAB cultures from fermented foods, such as cheese, that produce γ-aminobutyric acid (GABA) [43]. Glutamate decarboxylase (GAD) catalyse the irreversible α-decarboxylation of L-glutamate, which is the main process by which GABA is generated [44, 32]. The current study demonstrated that *L. casei* MGOLIV.25, *L. chiayiensis* MGOLIV.28 and *L. rhamnosus* MROMI.22 were displayed autoaggregation phenotypes which is in line with previous findings of [41, 45 and 46].

A lack of hemolytic activity is crucial when choosing probiotic strains for safety reasons because these strains are nonvirulent, and a lack of hemolysin guarantees that virulence won't arise among the bacterial strains [47]. A bacterial strain is classified as proteolytic when it produces a clear zone on skimmed milk agar measuring between 15 and 21 mm [48]. In the present study proteolytic activity was demonstrated by the three GABA-producing selected strains (*L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25, and *L. chiayiensis* MGOLIV.28) that showed lack of hemolytic activity. Oh and Jung found that five Lactobacillus species isolated from traditionally fermented millet-based alcoholic beverages showed γ-hemolytic or no hemolytic activity [49]. In another study *L. plantarum* cells and their supernatant showed strong protease activity on skimmed milk agar in a research previously conducted [28 , 50].

The current study's findings demonstrated that the selected strains *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25, and *L. chiayiensis* MGOLIV.28 produced different amounts of GABA and yielded GABA concentrations of 8.61 mg/ml, 9.02 mg/ml, and 10.16 mg/ml, respectively. Isolates including *L. rhamnosus* YS9, *Lb. namurensis*, *and Lb. plantarum* produce large levels of GABA [51,52]. By adding 249 mg/L of MSG, found that the GABA content of their fermented milks was 0.185 and 0.319 g/L, respectively, due to the use of two mixed starters (*Lactis and Bacillus rhamnonus*) [53].

The strains' ability to scavenge DPPH radicals was examined in order to observe the probiotic property known as the antioxidant effect. The findings demonstrated the ability of the three GABA-producing strains to scavenge DPPH. These findings were consistent with a previous study, that showed certain metabolites and extracellular enzymes might be the source of the antioxidant ability [41]. Since *L. plantarum* DM5 was the first probiotic strain to be reported to have both antioxidant and GABA-generating capabilities, it can be credited with creating a cell factory capable of producing wide range of nutraceuticals [54].

The majority of Lactobacillus species are susceptible to erythromycin and chloramphenicol [55, 56 and 46], where *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25, and *L. chiayiensis* MGOLIV.28 of the current study have sensitivities to these two antibiotics but resistant to ampicillin. *L. chiayiensis* MGOLIV.28 possesses antibacterial activity, according to an *in vitro* assessment of its

antimicrobial qualities. Li *et al.*'s stated that *L. chiayiensis* AACE3 has broad-spectrum antibacterial action against common gram-positive and gram-negative infections [56]. Antibiotics may be replaced by the inhibitors that probiotic strains produce. Another study proposed that *L. rhamnosus* YT possessed antibacterial action against bacteria that cause food spoiling and other foodborne pathogens in the food sector [57].

Extracts from the three examined strains, *L. rhamnosus* MROMI.22, *L. casei* MGOLIV.25, and *L. chiayiensis* MGOLIV.28 were found to exhibit anticancer activity. *Lactobacillus plantarum* has the potential to be a therapeutic agent by generating bioactive chemicals that may be advantageous to the host [58]. *L. plantarum*, which produces GABA, triggers anti-migration, anti-invasion, and anti-proliferative activities against HT-29 cells.

The optimization of GABA production by *L. chiayiensis* MGOLIV.28 was carried out in this study using RSM [59]. The majority of LAB are unable to produce enough L-glutamate to be used in the synthesis of GABA, however, adding MSG to the culture media is therefore essential [60]. The GAD enzyme's activity in LAB is related to the ideal MSG concentrations, which vary accordingly to the GABA-producing capacity [61]. Culture conditions have a major impact on strains' ability to produce GABA. According to our findings, 4.5 was the ideal pH for *L. chiayiensis* MGOLIV.28 GABA synthesis. Lactic and acetic acids are among the organic acids that are primarily produced by LAB metabolism in addition to bacteriocins, nitric oxide and hydrogen peroxide that are active against pathogens [62]. This pathway became active during the stationary phase and after the pH was adjusted to 4.6 [63]. The pH of the culture medium plays a critical role in the production of GABA by various bacteria that affects bacterial growth and glutamate decarboxylase activity [64].

The maximum GABA yield by fermentation is also significantly influenced by the incubation temperature. The ideal temperature range for GABA production by *L. plantarum* DSM19463 was between 30 and 35°C [65]. However, the current study's incubation temperature was 33.5°C validated the strain *L. chiayiensis* MGOLIV.28 to produce the largest amount of GABA. GAD activity responds to temperature increases by increasing to a maximum and then progressively decreasing with additional temperature rises [66]. Thus, the ideal conditions for the *L. chiayiensis* MGOLIV.28 strain to produce the highest amount of GABA were as follows: an initial concentration of 3% monosodium glutamate, a culture temperature of 33.5°C, an initial pH of 4.5, and an incubation period of 72 hours. This will cause an increase in GABA production from 10.16 mg/ml in MRS broth to 65.7 mg/ml. Meanwhile, in another study, the ideal conditions for the highest amount of GABA production by *Lb. plantarum* Taj-Apis362 were 497.97 mM, a culture temperature of 36°C, and initial pH of 5.31, and an incubation period of 60 hours [67].

## 5. Conclusions

After screening for GABA-producing *Lactobacillus* in fermented foods from Egypt, three strains were selected: *Lactobacillus chiayiensis*, *Lactobacillus casei*, and *Lactobacillus rhamnosus*. The three strains showed high GABA production ability and probiotic properties in the context of the gut (microbiota): brain axis. These strains could be used as psychobiotic starter cultures to produce GABA-enriched probiotic dairy and other novel functional foods or as capsules. In addition to supporting a healthy gut environment, this may improve mental health and even be able to treat depression. When compared to unoptimized levels, Response Surface Methodology (RSM) can help increase GABA production by more than 6.4 times, offering a reasonably priced means of increasing output. However, *in vivo* study is still necessary. It is possible to generate GABA-enriched functional meals or use the strains and growth conditions chosen to give the highest concentrations of this bioactive chemical to other biotechnological applications. Further studies are needed to assess the efficacy and stability of the *L. chiayiensis* MGOLIV.28 and the importance of possible new therapeutic targets in the field of nutritional neuropsychopharmacology.

**Competing interests:** No potential conflict of interest was reported by the authors.

**Author contributions:** Conceptualization, S.A.; methodology, M.El, S.A.H.M.; formal analysis, M.El, S.S.H., S.A.H.M.; investigation, M.El., S.S.H., S.A.H.M..; writing—original draft preparation, M.El.; writing—review and editing, S.S.H., S.A.H.M., S.A. All authors have read and agreed to the published version of the manuscript.

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