Ceylon Journal of Science 54 (4) 2025: 1115-1128

RESEARCH ARTICLE

Functional Characterization and Safety Evaluation of GABA-Enriched *Prosopis africana* (Okpeye) Fermented with *Bacillus subtilis* AQU under Optimized Conditions

U. K. Akpi, N. A. Mohd Zaini*, W. A. A. Q. I. Wan-Mohtar, W. S. A. W. M. Noor

Highlights

- First report on optimizing γ-aminobutyric acid (GABA) production in *Prosopis africana* (Okpeye) using *Bacillus subtilis* AQU.
- *Bacillus subtilis* AQU efficiently produced high levels of GABA utilizing Okpeye as a fermentation substrate.
- Response surface methodology (RSM) model showed excellent fit ($R^2 = 95.56\%$) with high predictive reliability.
- The strain showed probiotic traits such as acid and bile tolerance, hydrophobicity, and antimicrobial activity.
- Optimized Okpeye fermented with *B. subtilis* AQU was non-toxic, safe, and maintained >50% viability in L-929 cells.
- Optimized Okpeye with *B. subtilis* AQU was non-toxic, safe, and kept L-929 cell viability >50%

Ceylon Journal of Science 54 (4) 2025: 1115-1128
DOI: http://doi.org/10.4038/cjs.v54i4.8599

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Functional Characterization and Safety Evaluation of GABA-Enriched *Prosopis africana* (Okpeye) Fermented with *Bacillus subtilis* AQU under Optimized Conditions

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Received: 14.11.2024; Accepted: 29.08.2025

Abstract: Gamma-aminobutyric acid (GABA) is a non-protein amino acid synthesized via the α-decarboxylation of L-glutamate. This study explored the production of GABA using *Bacillus subtilis* AQU isolated from Okpeye and its cytotoxicity effect. *Prosopis africana* seeds sourced from the Ogege market in Nsukka Local Government Area, Enugu State, Nigeria, were used to produce Okpeye. The fermentation process for Okpeye production was conducted under optimized conditions using Response Surface Methodology (RSM). The isolated *B. subtilis* AQU were tested for probiotic properties, including low pH tolerance, bile salt tolerance, hydrophobicity, auto aggregation, antimicrobial activity, and cytotoxicity effects. The optimization of GABA production using Central Composite Design (CCD) in Minitab 21.0 software identified the optimal conditions for GABA synthesis: temperature at 40 °C, fermentation time of 177 hours, 5% MSG concentration, and 3% inoculum concentration. Under the optimized conditions, the GABA yield was experimentally validated at 310.03 mg/100 g dry Okpeye, closely matching the predicted value of 313.53 mg/100 g with 83% desirability. The probiotic potential of *Bacillus subtilis* AQU demonstrated strong tolerance to low pH and bile salts, moderate auto-aggregation capacity, and high cell surface hydrophobicity, collectively suggesting strong colonization potential in the gastrointestinal tract. Cytotoxicity assays performed on L-929 cells indicated that the optimized Okpeye contains *B. subtilis* AQU, is non-toxic, and maintains above 50% cell viability even at the highest tested concentrations. Findings of the study suggest the potential of utilizing *B. subtilis* AQU for efficient GABA production, enhancing the nutritional and functional properties of fermented Okpeye.

***Keywords*:** Cytotoxicity**;** GABA; Okpeye; Probiotic potential; *Prosopis africana.*

INTRODUCTION

Fermented foods are integral to many traditional cuisines, contributing to flavour and health benefits through the bioactive compounds produced during fermentation (Akpi et al., 2020). One such compound, gamma-aminobutyric acid (GABA), a non-protein amino acid, plays a significant role as a neurotransmitter in the central nervous system. GABA is widely recognized for its health-promoting properties, including its ability to reduce hypertension, improve mood, and induce relaxation, making it a highly valued component in functional foods (Lim et al., 2016).

Prosopis africana (African mesquite), commonly called Okpeye, is a staple in West African cuisine, particularly in Nigeria, where it is used as a fermented condiment. The seeds of *P. africana* are fermented to produce Okpeye, which is rich in protein, vitamins, and essential nutrients (Oguntoyinbo et al., 2014; Ugwuanyi, 2019). Traditionally, fermentation is spontaneous, involving diverse microorganisms that enhance the flavour and boost the product's nutritional value (Oyewole & Isah, 2012). Among these microbes, *Bacillus* spp. has gained attention for its ability to optimize fermentation and significantly enhance GABA production (Lim et al., 2016; Wan et al., 2020).

While fermentation improves the nutritional profile of Okpeye, ensuring the safety and efficacy of the product remains crucial, especially when optimized strains like *Bacillus subtilis* are involved. Cytotoxicity assays are essential for determining whether the compounds generated during fermentation, such as GABA, adversely affect human cells (Devi et al., 2023). These assays ensure that optimized fermentation processes enhance beneficial properties without compromising safety, thus validating the potential of GABA-enriched Okpeye for human consumption (Torino et al., 2013).

This study aimed to evaluate the cytotoxicity of optimized fermented *P. africana* (Okpeye) produced by *Bacillus subtilis* and its effectiveness in GABA production. By analyzing both cytotoxicity and functional properties, this research offers valuable insights into the development of safe and effective GABA-enriched functional foods, contributing to the growing field of health-oriented culinary innovation (Akpi et al., 2023; Ab Kadir et al., 2016).

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MATERIALS AND METHODS

Sources of raw materials

The Okpeye used in this study was produced at the University of Kebangsaan Malaysia, Malaysia. The seeds for the production were carefully selected from the Ogege market, located in Nsukka Local Government Area, Enugu State, Nigeria.

Microorganism

B. subtilis AQU, previously isolated, identified, and characterized from fermented Okpeye, was obtained from the Department of Food Sciences, Faculty of Science and Technology, University of Kebangsaan Malaysia, Malaysia. American Type Culture Collection L-929 mouse subcutaneous connective fibroblast cells (Mus musculus, NCTC clone 929, CLL-1TM), at passage 9, were utilized for the cytotoxicity assay.

Production of Okpeye

The production of Okpeye was done according to the Akpi et al. (2020) method, with a few slight adjustments, as shown in Figure 1. First, *P. africana* seeds were boiled for 6 hours or until they became soft. The cotyledons were then gently removed by pressing the seeds between the thumb and fingers. The cotyledons were then thoroughly rinsed, drained, and dried in an oven at 100°C for 10 minutes, using a pot lined with banana leaves. Dried cotyledons were layered in a basket, forming a 1 to 2 cm thick layer. Additional banana leaves were placed on top, facing downward, and pebbles weighed the stack down. The fermentation lasted four days at 37°C, during which the cotyledons darkened and developed a strong ammonia smell. Fermented cotyledons were ground into a fine paste using a mortar and shaped into different sizes. The balls were gently dried outdoors over several days, allowing them to harden.

Optimization of GABA production using RSM

The GABA production by the *B. subtilis* AQU was optimized using RSM with central composite design (CCD) in Minitab 21.0 software. Table 1 displays the independent factors important for the optimization of GABA-rich Okpeye. The temperature (20 – 40 °C), MSG concentration (1–5 % w/w), inoculum volume (5 – 10 % w/v), and incubation time (48 - 196 h) were selected as independent factors to optimize the rate of GABA production. The fermentation for optimization was conducted in a petri dish containing 30 g of *P. africana*. With the α-value set to 2.0, CCD's experimental design produced 31 runs listed in Table 2.

Table 1: Independent factors for optimizing the cultivation conditions of gamma-butyric acid

Independent variables	Low	High
Temperature (°C)	20	40
Inoculum (%, w/v)	1	5
Time (hours)	48	196
MSG (%, w/w)	1	5

Preparation of the sample

The sample preparation followed the method outlined by Torino et al. (2013) with a few minor modifications. Two kilograms (2 kg) of *P. africana* seeds were weighed and boiled until they became soft. Once cooked, the seeds were manually dehulled to release the cotyledons, which were

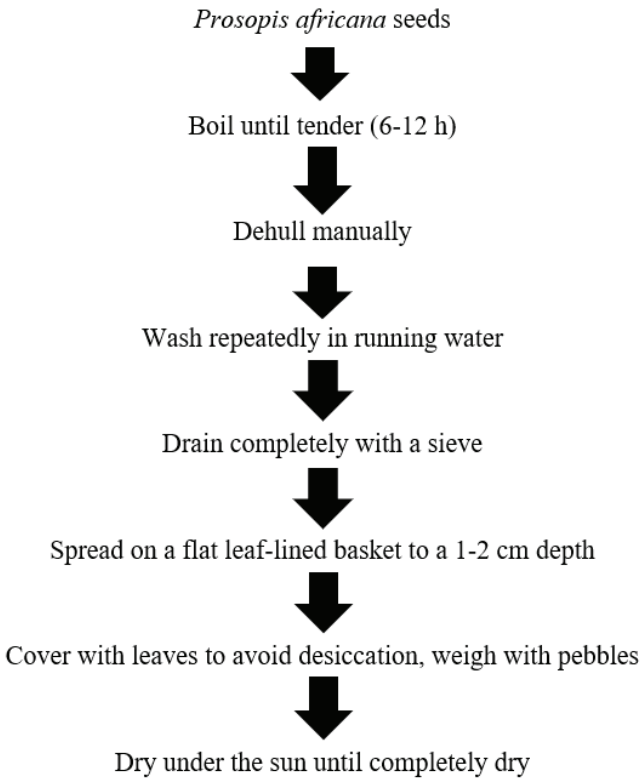


Figure 1: Steps in the traditional production of Okpeye seasoning.

Table 2: Central composite design (CCD) with tested variables and responses for the Gamma-aminobutyric acid (GABA) production (mg/100 g dry fermented Okpeye) by *Bacillus subtilis* AQU

Variables					Response		
					GABA (mg/100 g dry fermented)		
Run	Temp. (°C)	Time (hours)	MSG (%, w/w)	Inoculum (%, w/v)	Experimental	Predicted	Residual
1	30	122	1	3	202.11	212.16	-10.05
2	30	122	3	3	225.23	202.65	22.58
3	30	122	3	1	116.78	110.82	5.96
4	30	122	3	3	222.20	202.65	19.56
5	30	48	3	3	160.36	159.36	1.00
6	30	122	3	3	196.22	202.65	-6.43
7	30	122	3	3	180.63	202.65	-22.02
8	30	196	3	3	104.90	97.69	7.21
9	30	122	3	5	180.29	178.01	2.30
10	30	122	3	3	180.50	202.65	-22.15
11	30	122	3	3	200.14	202.65	-2.51
12	30	122	3	3	213.61	202.65	10.96
13	30	122	5	3	115.87	97.61	18.30
14	**35**	**85**	**2**	**3**	**360.01**	**347.00**	**13.01**
15	35	85	2	4	320.17	302.36	17.81
16	35	159	2	2	146.82	174.09	-27.27
17	35	159	2	2	302.89	294.13	8.76
18	35	85	4	4	170.66	172.67	-2.01
19	35	85	4	2	140.27	167.81	-27.54
20	35	159	4	4	196.63	190.84	5.81
21	35	159	4	4	128.06	120.29	7.77
22	25	159	2	2	80.25	56.57	23.68
23	25	85	2	2	126.31	137.95	-11.64
24	25	159	2	4	126.31	128.65	-2.34
25	25	85	2	4	131.13	141.27	-10.14
26	25	159	4	2	103.84	121.20	-17.36
27	25	85	4	2	117.38	130.01	-12.63
28	25	85	4	2	100.12	77.20	22.92
29	25	159	4	4	122.12	143.80	-21.68
30	40	122	3	3	350.41	352.68	-2.27
31	20	122	3	3	155.03	144.55	10.50

washed thoroughly several times with water. The cleaned cotyledons were placed in a sieve to drain completely. After draining, the cotyledons were evenly spread out on a flat tray and left to air dry for 1 hour at room temperature to reduce moisture and ensure a consistent wetness level across the samples. About 30 grams of the cotyledons were carefully measured and placed into sterile petri dishes.

Sample inoculation and fermentation

Each petri dish containing 30g of sterile cotyledons of *P. africana* was aseptically inoculated with a suspension of appropriate standardized inoculums (10^6 cells/mL) of *B. subtilis* AQU and MSG, according to the RSM. The contents of the petri dishes were thoroughly mixed using a sterile glass rod. Fermentation was allowed to proceed in the incubator. The various parameters to be optimized were varied as specified by RSM. Two control experiments were set up: one dish contained a sterile sample with no pre-inoculation, and the other had an unsterile sample that was accidentally inoculated. Once the fermentation process was complete, the fermented mash was ground in a sterile mortar and pestle, and the paste was shaped into balls or any preferred forms. The finished products were then dried in an oven at 45°C for 168 hours.

Determination of GABA content in Okpeye samples

Standard solution

The stock solution of GABA and 1-fluoro-2,4-

dinitrobenzene (FDNB) was purchased from Sigma (USA). The stock solution (1.0, 1.5, 2.0, 2.5, and 5.0 mg/L) of GABA was prepared with de-ionized water and stored at 4 °C before use. A stock solution of FDNB (1 %, w/v) was prepared by dissolving 1 g of FDNB in 100 mL of acetonitrile and stored at 4 °C before use.

Derivatization

1 g of Okpeye sample was added to 10 mL of water and vortexed for 5 mins, after which 1 mL of the Okpeye sample, 1 mL of 0.5 mol/L $NaHCO_3$ (pH 9.0), and 1 mL of FDNB (1%, w/v) reagent were added to a volumetric flask of the volume of 10 mL. The mixture was vortexed for 5 s and kept in the dark at 60 °C for 1 h. After cooling to room temperature, phosphate buffer (0.02mol/L, pH 7.0) was added to the scale line of the flask. After mixing, the solution was filtered through a 0.22mm membrane filter (Whatman, USA). A similar approach was carried out for the working standard of GABA.

GABA analysis

A modified version of the Kalu et al. (2025) method was employed to assess the GABA content in fermented Okpeye samples and by an isolated strain. The derivative determination from the samples was done using Shimadzu high-performance liquid chromatography (HPLC) (Japan) equipped with a UV spectrophotometric detector set at 360 nm. InertSustainSwift C18, a reversed-phase column paired with a C18 cartridge was used. The temperature of the column was maintained at 35 °C. The mobile phase comprised solvent A (50% acetonitrile) and solvent B (Phosphate buffer, 0.02 mol/L, pH 7.0). Before use, the column was conditioned and equilibrated with 16% solvent A and 84% solvent B. The gradient elution profile was 0 to 20 minutes: linear gradient from 16% to 100% solvent A, 20 to 26 minutes: linear gradient from 100% back to 16% solvent A, 26 to 30 minutes: maintained at 16% solvent A. A membrane filter of 0.22 µm was used to filter the mobile phase and degassed before use. Each sample (5 microliters) was injected into the chromatograph column. The chromatographic peak of the fermented Okpeye extract was compared against a calibration curve to determine the GABA content in the samples.

Evaluation of probiotic attributes of *B. subtilis* AQU

Tolerance to low pH

The isolate's low pH tolerance was assessed following the procedure of Ahire et al. (2021). *B. subtilis* AQU was grown overnight in nutrient broth at 37 °C and centrifuged at 8000 × g for 5 minutes at 4 °C. The cells were washed twice with sterile phosphate-buffered saline (pH 7.3) before being suspended in approximately 1 mL of phosphate-buffered saline. The strain was diluted in PBS (1:100) at different pH values (1-4) and incubated at 37 °C for various time intervals (0-3 hours). The bacterial cell viability was measured in CFU/mL on nutrient agar plates, and the percentage survival at various pH levels after 3 hours of incubation was recorded.

Tolerance to bile salt

Bile salt tolerance of the *B. subtilis* AQU was determined using a slight modification of the method of Unban et al. (2020). An aliquot (1 mL) of the *B. subtilis* AQU culture grown overnight in nutrient broth was incubated in 10 mL of freshly prepared nutrient broth containing 0.3% (w/v) bile salts at 37 °C for 3 h under 150 rpm agitation. Samples were collected before (T_0=0 h) and after (T_1=3 h) incubation and spread onto nutrient agar plates using a glass rod, then incubated at 37 °C for 24 h, after which viable cells were counted. The viability of the organisms in the nutrient broth containing 0.3% bile salts was calculated as survivability (%) = $T_0/T_1 \times 100$

Hydrophobicity assay

B. subtilis AQU was tested for cell surface hydrophobicity according to Thapa et al. (2004). Ethyl acetate and chloroform were used to determine surface hydrophobicity. The cells were grown overnight, centrifuged, washed several times with PBS, and suspended in Hartmann's solution (10 mL). The control's optical density (OD) (A_0) was determined at 600 nm. In the test sample, an equal volume of solvent was mixed with the cell suspension, vortexed for 2 minutes, and left at room temperature for 30 minutes. Following removing the aqueous phase, the absorbance was measured at 600 nm (A_1). This was determined by formula $(1 - A_1/A_0) \times 100$.

Autoaggregation assay

The autoaggregation assay for *B. subtilis* AQU was done as Patel et al. (2009) described. Centrifugation was used to harvest the overnight-grown cells, which were washed several times with PBS. The cell pellets were suspended again to achieve an OD_{595} of 0.5. Four milliliters of this cell suspension were gently vortexed for 10 seconds before incubating at 37 ºC for two hours. After incubation, the supernatant was removed, and the absorbance was read at 595 nm using a spectrophotometer. The autoaggregation percentage was calculated using the formula: $1 - (A_t / A_0) \times 100$, where A_t is the absorbance of the cell suspension after 2 hours and A_0 is the absorbance at the start.

Antimicrobial activity assay

The agar well diffusion method described by Akpi et al. (2022) was used for the antimicrobial activity and antibiotic susceptibility assay with minor modification. The pathogens tested for antimicrobial activity included *V. cholerae*, *L. monocytogenes, Salmonella* sp., *E. coli, K. pneumonia*, *S. aureus,* and *P. aeruginosa*. Overnight cultures of these pathogens were swabbed onto nutrient agar plates with wells. The supernatant from 24 h cultures of *B. subtilis* AQU grown in nutrient broth was added to the wells at 0.5% (v/v) final concentration and incubated for 24 hours at 37 °C. Negative control wells were filled with distilled water, and a ciprofloxacin (10 mcg) antibiotic disc was used as a positive control. The inhibition zones were measured to determine the resistance and sensitivity patterns of the strains.

Antibiogram profile

For the antibiotic susceptibility test, *B. subtilis* AQU was inoculated in nutrient broth and incubated for 24 h at 37 °C. The sample culture of about 100 µl was spread on Mueller-

Hinton agar, and the antibiotic discs were gently applied to the plates and incubated at 37 °C for 24 hours. The antibiotics used were ciprofloxacin (10 mcg), gentamicin (10 mcg), tetracycline (30 mcg), cephalothin (30 mcg), penicillin (10 mcg), nalidixic (30 mcg), vancomycin (30 mcg), trimethoprim (25 mcg), and Erythromycin (15 mcg).

Cytotoxicity assay

The cytotoxicity potential of the optimized Okpeye was evaluated using the MTT assay, following the protocol established by Mosmann (1983), which measures cell viability through optical density readings. L-929 fibroblast cells were cultured in a tissue culture flask using Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, non-essential amino acids, 1 mM sodium pyruvate, and 1% antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). These cells were maintained at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% carbon dioxide and 95% relative humidity, with daily observations to ensure healthy growth. Once the cells reached confluence, they were detached via trypsinization, and the number of viable cells was determined. The cells were then seeded into a 96-well plate at a density of 10,000 cells per well and incubated under the same conditions for at least 12 hours to reach 70–80% confluency.

To prepare the optimized Okpeye for testing, it was first dissolved in the complete growth medium at a concentration of 2 mg/mL, then sterilized through a 0.2 µm membrane filter. This stock solution was subsequently serially diluted with the growth medium to achieve final concentrations of 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.13 mg/mL, and 0.06 mg/mL. The growth medium in each well of the 96-well plate was then replaced with 200 µL of Okpeye solution at the specified concentrations or with control media. The cells were exposed to these treatments for 24 hours ± 30 minutes at 37 ± 1 °C under the same humidified and CO_2-enriched conditions.

Following the exposure period, 5 mg/mL MTT solution was added to each well, and the plate was incubated for another 4 hours ± 30 minutes to allow for formazan crystal formation, indicating metabolically active cells. Dimethyl sulfoxide (DMSO) was then added to dissolve the formazan crystals, and optical density (OD) readings were taken at 570 nm using a spectrophotometer. These OD values provided quantitative data on cell viability, thus allowing the cytotoxicity of the Okpeye preparation to be assessed across the tested concentrations.

Statistical analysis

All data represented the average of the three replications (n=3), and the values were recorded as mean ± standard deviation. GABA production by *B. subtilis* AQU was optimized via RSM. Analysis of variance (ANOVA) with Tukey's pairwise comparisons and 95% confidence intervals was used. A $p < 0.05$ indicates a significant difference in the experimental responses performed using Minitab 21.0 software.

GABA analysis of fermented Okpeye samples

The GABA content of Okpeye produced through conventional and spontaneous fermentation methods was analyzed. The GABA content in the Okpeye seasoning showed 22.86 ± 5.70 mg/100 g dry fermented Okpeye. GABA is said to be synthesized in plants by glutamate decarboxylase, activated by low temperature or low atmospheric oxygen. GABA is typically metabolized to succinic acid in plants; however, stress inhibits GABA metabolism to succinic acids, and as a result, GABA accumulates in stressed plants. The presence of GABA-producing microorganisms could explain the existence of GABA in Okpeye. The GABA content of the Okpeye product may be attributed to differences in fermentation conditions, including time and microorganisms.

Optimization of GABA production by *B. subtilis* AQU

Since Okpeye contains gamma-aminobutyric acid (GABA), it demonstrates promising potential as a functional food ingredient. An optimization study was conducted to maximize GABA production and enhance its GABA content. Specifically, optimization of the culture conditions for GABA synthesis in Okpeye was examined using Bacillus subtilis AQU as the fermenting agent. Key variables were selected for analysis using response surface methodology (RSM) to simulate the fermentation process. These variables, including temperature, fermentation time, inoculum concentration, and monosodium glutamate (MSG) concentration, were initially identified through single-variable optimization (Table 2). A central composite design (CCD) was employed within the RSM framework to explore the interactions and effects of these variables on GABA production. This led to a quadratic multiple regression analysis, establishing a predictive quadratic regression model. The resulting quadratic regression equation is as follows:

GABA (mg/100 g)

= -319 + 0.6 TEMPERATURE (°C) + 1.59 TIME (H) + 195.2 MSG (%, w/w) + 7.2 INOCULUM (%, w/v) + 0.460 TEMPERATURE (°C)*TEMPERATURE (°C) - 0.01354 TIME (H)*TIME (H) - 11.94 MSG (%, w/w)*MSG (%, w/w) - 14.55 INOCULUM (%, w/v)*INOCULUM (%, w/v) - 0.0589 TEMPERATURE (°C)*TIME (H) - 5.92 TEMPERATURE (°C)*MSG (%, w/w) + 2.40 TEMPERATURE (°C)*INOCULUM (%, w/v) + 0.513 TIME (H)*MSG (%, w/w) + 0.509 TIME (H)*INOCULUM (%, w/v) - 12.37 MSG (%, w/w)*INOCULUM (%, w/v)

ANOVA was conducted to validate the regression coefficient for GABA production (Table 3). The ANOVA results showed a very small "model p-value" (<0.0001), indicating high significance. The "lack of fit" was not significant, with a p-value of 0.323. The model also had a suitable coefficient of determination (R^2 = 95.56%) and an adjusted coefficient of determination (R^2 adjusted = 91.68%). Hence, the quadratic polynomial model was significant and effectively captured the true relationship between the response and the key variables.

Table 3: Analysis of Variance for gamma-aminobutyric acid GABA production by *B. subtilis* AQU.

Sources	Df	Adj SS	Adj MS	F - value	P -value
Model	14	152920	10922.80	24.61	<0.00*
Linear	4	97147	24286.90	54.71	<0.00*
TEMPERATURE	1	64980	64980.30	146.38	<0.00*
TIME (H)	1	5706	570.70	12.85	<0.00*
MSG (%)	1	19682	19682.00	44.34	<0.00*
INOCULUM (%)	1	6779	6779.50	15.27	0.01*
Square	4	23653	5913.40	13.32	<0.00*
TEMPERATURE*TEMPERATURE	1	3776	3776.40	8.51	0.01*
TIME (H)*TIME (H)	1	9819	9819.30	22.12	<0.00*
MSG (%)*MSG (%)	1	4077	4077.10	9.18	0.01*
INOCULMN (%)*INOCULUM (%)	1	6057	6057.40	13.65	<0.00*
2-Way Interaction	6	32119	5353.20	12.06	<0.00*
TEMPERATURE*TIME (H	1	1899	1898.60	4.28	0.06
TEMPERATURE*MSG (%)	1	14026	14026.30	31.60	<0.00*
TEMPERATURE*INOCULUM (%)	1	2300	2299.90	5.18	0.04*
TIME (H)*MSG (%)	1	5760	5759.70	12.97	<0.00*
TIME (H)*INOCULUM (%)	1	5686	5685.50	12.81	<0.00*
MSG (%)*INOCULUM (%)	1	2449	2449.00	5.52	0.03*
Error	16	7103	443.00		
Lack-of-Fit	10	5067	506.70	1.49	0.32
Pure Error	6	2035	339.20		
Total	30	160022			
Sum = 21.07	R-sq = 95.56%			R-sq (adj) = 91.68% R-sq (pred) = 80.03%	

Assessment of parameter interaction on GABA production

With the MSG concentration and inoculum size fixed at 3%, this study investigates the effect of temperature and fermentation time on GABA production (Figure 2a). The surface plot shows that the highest GABA production, reaching approximately 400 mg/100 g dry fermented Okpeye, occurs at temperatures around 40 °C and fermentation times between 100 and 150 hours. This demonstrates that elevated temperatures and extended fermentation durations contribute significantly to GABA synthesis. Conversely, GABA production is markedly reduced at lower temperatures (20 - 25 °C) and shorter fermentation times (below 75 hours). These findings suggest that optimal GABA yields in fermented *P. africana* (Okpeye) are closely tied to maintaining higher temperatures and more extended fermentation periods, highlighting the critical role of these variables in enhancing the fermentation process.

With the temperature fixed at 30 °C and inoculum size fixed at 3%, this study investigates the effect of MSG concentration and fermentation time on GABA production (Figure 2b). The surface plot reveals that the highest GABA production, exceeding 200.0 mg/100 g dry fermented Okpeye, is achieved at MSG concentrations of approximately 1-3% and fermentation times around 50-150 hours. This indicates that lower MSG concentrations and fermentation times maximize GABA synthesis. At higher MSG concentrations (4 – 5%) and higher fermentation times (>175) hours), GABA production is notably reduced. This implies that sufficient MSG concentration and adequate fermentation time are crucial for optimal GABA production. Therefore, optimizing MSG concentration and fermentation duration is vital to boost GABA yields, highlighting the importance of these parameters in the fermentation process.

With the fermentation time held constant at 122 hours and the inoculum size fixed at 3%, this study evaluates the impact of MSG concentration and temperature on GABA production (Figure 2c). The surface plot illustrates that the highest GABA production, exceeding 450.0 mg/100 g dry fermented Okpeye, occurs at temperatures around 30 - 40 °C and MSG concentrations between 2 – 3%. This suggests that higher temperatures and moderate MSG concentrations favour maximizing GABA synthesis. GABA production is significantly lower at lower temperatures (20 – 25 °C) and MSG concentrations (1 – 2%). This implies that adequate temperature and appropriate MSG concentration are essential for optimal GABA production. Therefore, optimizing both temperature and MSG concentration is critical to enhancing GABA yields, highlighting the importance of these parameters in the fermentation process.

Figure 2d shows the effect of inoculum and MSG concentration on GABA production, with fermentation time and temperature constant at 122 hours and 30 °C, respectively. The surface plot reveals that GABA production is highly sensitive to variations in both MSG and inoculum percentages. GABA concentrations exceeding 200.00 mg/100 g dry fermented Okpeye are achieved with an MSG concentration between 2 and 4% and an inoculum size around 3%. The GABA production decreases significantly when the MSG concentration drops below 2 or exceeds 4 % and the inoculum size is below 2%. This analysis suggests that maintaining an MSG concentration of 2 to 4 % and an inoculum size of around 3 to 5% is optimal for maximizing GABA production under the given conditions.

This surface plot evaluates the impact of temperature and inoculum size on GABA production while holding the fermentation time and MSG concentration constant at 122 hours and 3%, respectively (Figure 2e). The plot indicates that the highest GABA production, exceeding 400 mg/100 g dry fermented Okpeye, is achieved at temperatures around 30 – 40 °C and an inoculum size between 2.5 and 4%. Conversely, lower GABA production is observed at lower temperatures (20 – 25 °C) and lower inoculum sizes (1 – 2%), as indicated by the blue areas in the plot. This analysis further highlights that optimal GABA production occurs at higher temperatures and larger inoculum sizes within the studied range, emphasizing the importance of these parameters in maximizing GABA yields in the fermentation process.

This surface plot explores the effect of fermentation time and inoculum size on GABA production, with temperature and MSG concentration held constant at 30 °C and 3%, respectively (Figure 2f). The plot demonstrates that the highest GABA production, exceeding 200.0 mg/100g dry fermented Okpeye, is achieved with a fermentation time between 100 and 150 hours and an inoculum size of around 3 to 4%. Lower GABA concentrations are observed with shorter fermentation times (50 - 75 hours) and smaller inoculum sizes (1 – 2%), as indicated by the lighter shades of green and blue areas. This analysis highlights that extended fermentation time and a moderate inoculum size are critical for achieving high GABA production under the given conditions.

Validation of the optimized conditions

Figure 3 illustrates the optimization of culture conditions using Okpeye condiment to achieve maximum GABA production by the *B. subtilis* AQU. According to results obtained with Minitab software 21.0, the optimal conditions, temperature of 40 °C, fermentation time of 173.20 hours, MSG concentration of 3.28%, and inoculum of 4.95%, predicted a GABA production of 313.53 mg/100 g dry fermented Okpeye with an 83% desirability. In the experiment, the conditions were set at a temperature of 40 °C, a fermentation time of 173 hours, an inoculum concentration of 3% (w/v), and MSG at 5% (w/w). This resulted in a GABA yield of 310.03 mg/100 g dry fermented Okpeye. The less than 5% difference between the predicted and experimental values confirms that the model is highly accurate and reliable.

Probiotic potential of the GABA-producing *B. amyloliquefaciens* AUK

Tolerance to low pH

For probiotic strains to be effective in the gut, they must survive the ingestion process and the harsh conditions of the gastrointestinal tract, particularly the stomach's acidic environment. The survival of *B. subtilis* AQU in gastric juice hinges on its ability to tolerate low pH, a crucial trait for probiotics (Kavitha et al., 2018; Nithya et al., 2013). In this study, *B. subtilis* AQU demonstrated resilience under acidic conditions (Table 5). Similar variations in acid tolerance have been observed across different *Bacillus* species. The strain survived after exposure to low pH levels (pH 2.0 and 4.0) for 3 hours. *B. subtilis* AQU demonstrated survival rates of 97.7% and 100%, respectively. These values are comparable to probiotic *Bacillus* strains like MKSKE1, MKSK-J1, and MKSK-M1, which showed survival rates of 93.1, 91.9, and 96.0% (Lee et al. 2017).

The ability to remain viable after 3 hours in an acidic environment suggests that the strain can effectively adapt to acid stress and resume growth. This resilience may be attributed to genetic and physiological mechanisms typically found in acidophilic microorganisms (Dabire et al., 2022). Additionally, acid-tolerant strains may gain extra protection from high-protein and high-fat diets, which could further enhance their ability to provide health benefits to consumers.

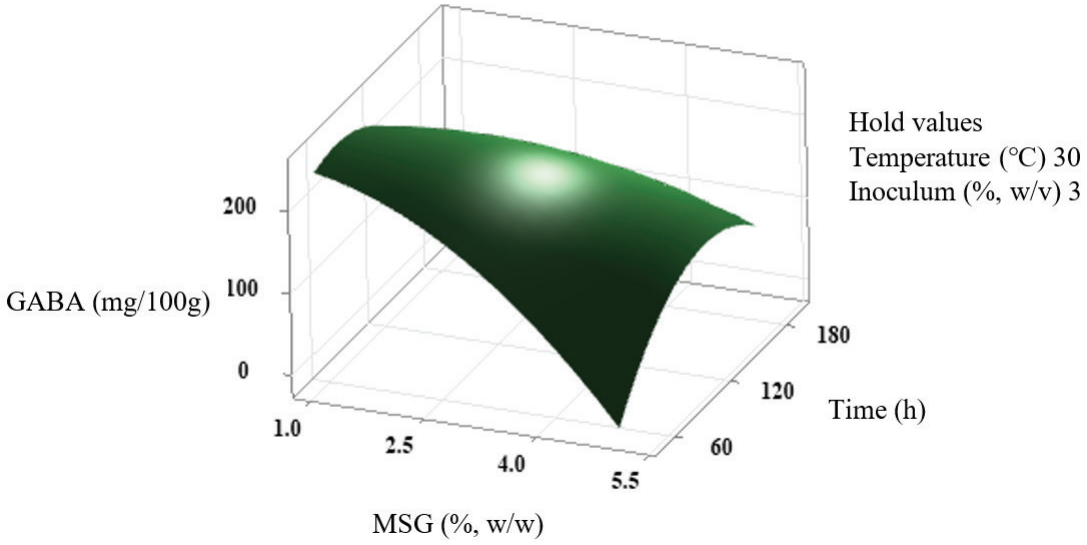
Tolerance to bile salt

The results demonstrated that *B. subtilis* AQU exhibited strong bile tolerance, maintaining 70.37% viability after 3 hours of incubation in a nutrient broth containing 0.3% bile salts (Figure 4). Bile, which enters the small intestine through the duodenum, typically inhibits bacterial growth because the cell membranes of bacteria consist of lipids and fatty acids that are susceptible to bile salts (Nwagu et al., 2020). However, resistance to bile salts is crucial for bacteria to survive and thrive in the gut environment, making it an essential criterion for selecting probiotic strains (Nithya et al., 2013; Jena et al., 2013). Bile tolerance tests are usually performed using a 0.3% bile solution, which closely resembles human bile and is considered a benchmark concentration for assessing the bile tolerance of potential probiotics (Brashears et al., 2003; Gilliland et al., 1984). The study observed that *B. subtilis* AQU could tolerate the 0.3% bile concentration.

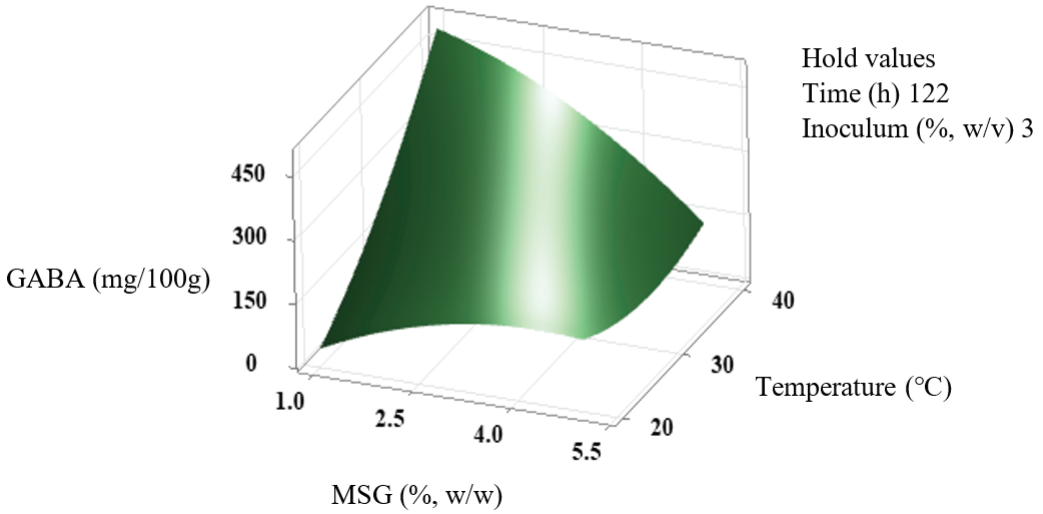
Cell hydrophobicity assay

The findings revealed that *B. subtilis* AQU exhibited 42% adhesion to ethyl acetate and 60% adhesion to chloroform (Figure 5). Hydrophobicity is a critical characteristic that supports the attachment of probiotic microorganisms to the intestinal lining, aiding their establishment and interaction with the host (Lee et al., 2019). Through their ability to adhere, probiotic strains can block pathogenic access by creating a physical barrier or explicitly interacting with cell receptors (Compaore et al., 2013). The *B. subtilis* AQU strains tested in this study demonstrated varying levels of hydrophobicity and self-aggregation, which suggests that they have strong adhesion and colonization capabilities,

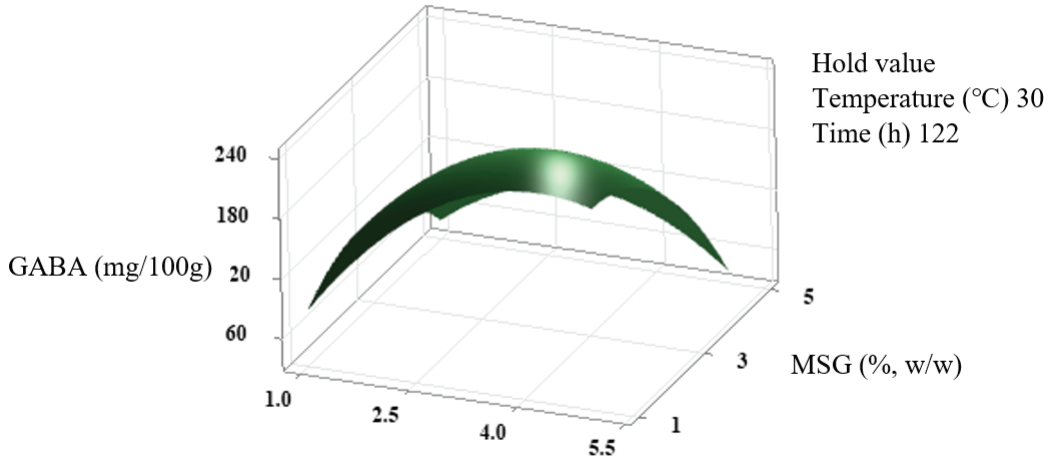
a Surface Plot of GABA (mg/100g) vs TIME (H), MSG (%, w/w)



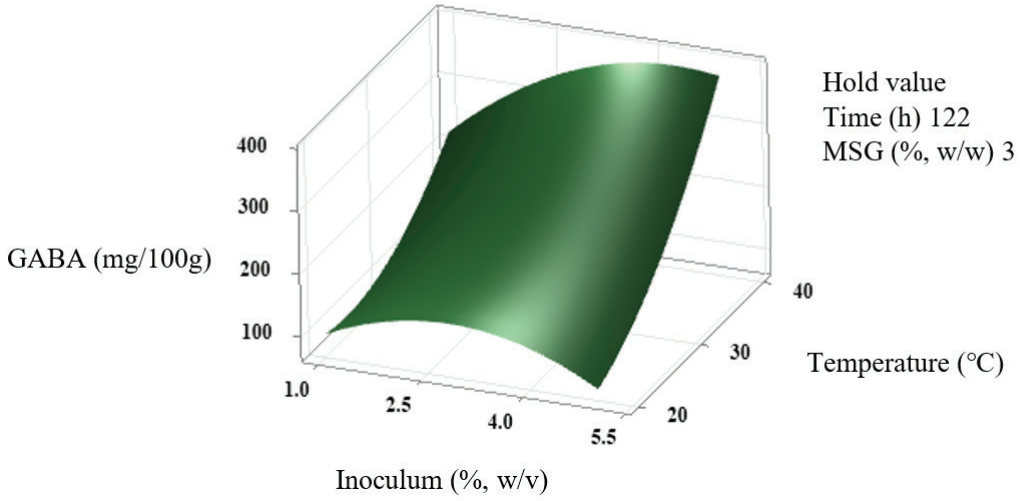
b Surface Plot of GABA (mg/100g) vs TEMPERATURE (°C), MSG (%, w/w)



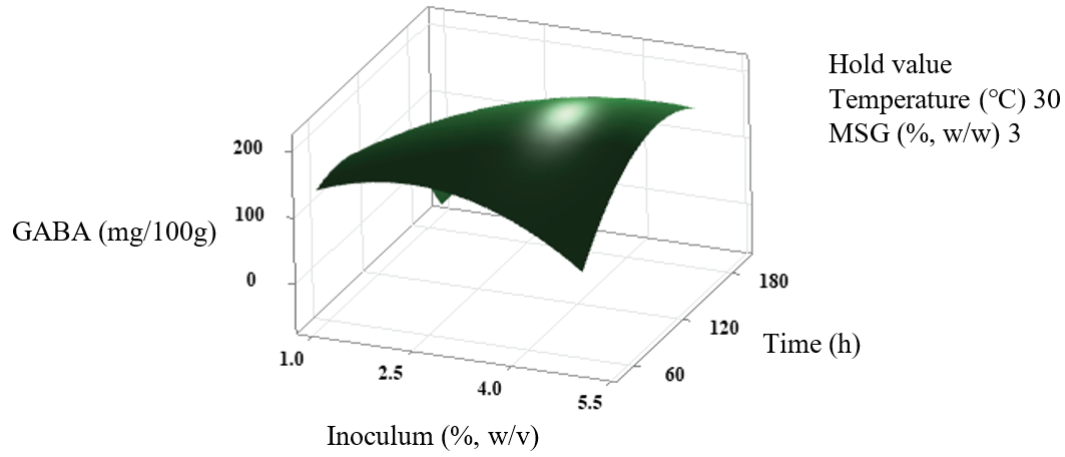
c Surface Plot of GABA (mg/100g) vs MSG (%, w/w), INOCULUM (%, w/v)



d Surface Plot of GABA (mg/100g) vs TEMPERATURE (°C), INOCULUM (%, w/v)



e Surface Plot of GABA (mg/100g) vs TIME (H), INOCULUM (%, w/v)



f Surface Plot of GABA (mg/100g) vs TIME (H), TEMPERATURE (°C)

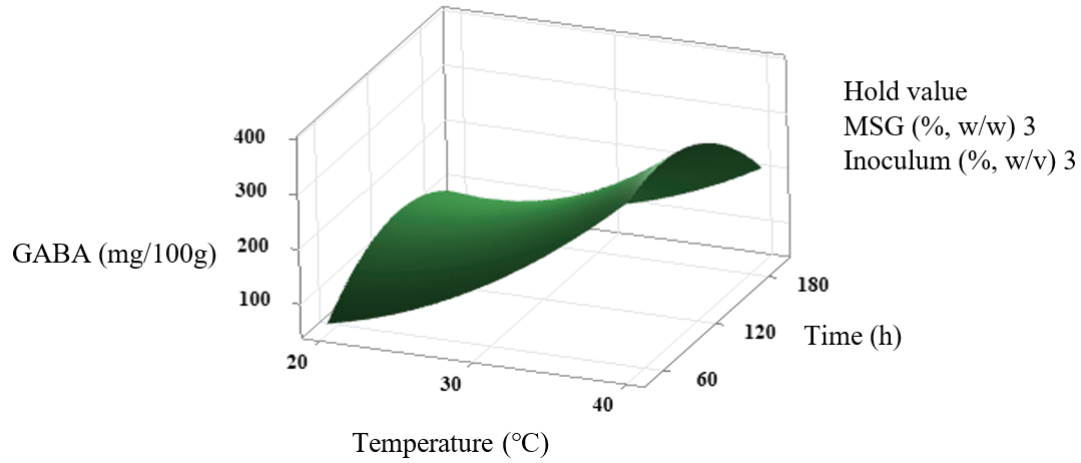


Figure 2: Effects of parameter interactions on gamma-aminobutyric acid (GABA) production: a) time vs MSG, b) temperature vs MSG, c) MSG vs inoculum, d) temperature vs inoculum, e) time vs inoculum, f) time vs temperature.

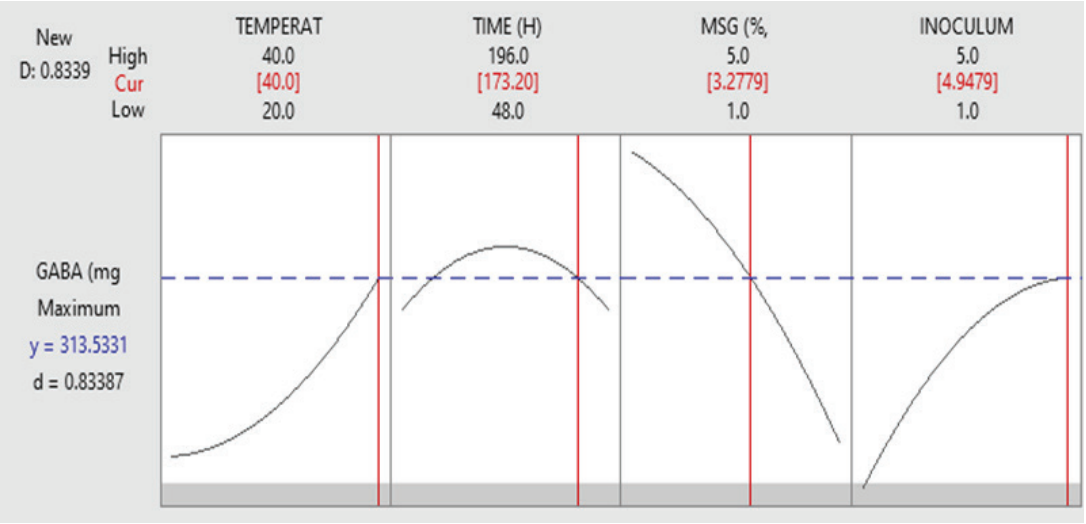


Figure 3: Optimization of the extracted gamma-aminobutyric acid in Okpeye condiment produced by *Bacillus subtilis* AQU

Table 5: pH tolerance of *Bacillus subtilis* AQU isolated from Okpeye

pH	Microbial count (Log cfu/ml)				% survival (after 3 h)
	0 h	1 h	2 h	3 h	
1	4.51 ± 0.03	0	0	0	0
2	4.71 ± 0.03	4.68 ± 0.01	4.63 ± 0.03	4.60 ± 0.02	97.7
3	4.93 ± 0.02	4.91 ± 0.00	4.90 ± 0.01	4.90 ± 0.02	99.4
4	5.25 ± 0.01	5.25 ± 0.02	5.25 ± 0.01	5.25 ± 0.02	100

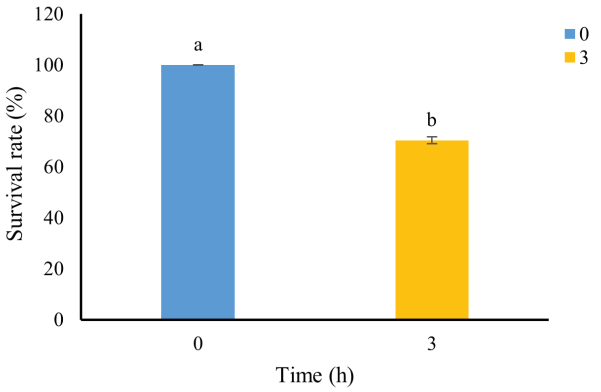


Figure 4: Tolerance of *Bacillus subtilis* AQU to 0.3% bile salt.

thereby potentially preventing pathogens from attaching to the gut by either steric interference or receptor blockage (Claudie et al., 2004). Differences in the adhesion levels observed among the tested strains may be influenced by factors such as surface charge and hydrophobic interactions (Nithya et al., 2013). These findings are consistent with the observations made by Talebi et al. (2018), who reported hydrophobicity levels ranging from 57.4% to 98.0% for various *Bacillus* strains, including 437F, 1630F, and 1020F.

Autoaggregation assay

Auto-aggregation refers to a microorganism's capacity to stick to gut epithelial cells (Patel et al., 2009). This trait is essential for successful colonization and long-term survival in the host's gastrointestinal system. This characteristic allows beneficial bacteria to establish themselves within the gut and potentially exert antimicrobial effects against harmful pathogens. In this study, *B. subtilis* AQU demonstrated an auto-aggregation rate of 26.5%. The observed auto-aggregation levels were lower compared to those reported by Nwagu et al. (2020) for probiotic *B. cereus* strains BC1 (53.7%) and BC2 (48.69%), but slightly higher than those found for *Bacillus* strains 437F (23.2%) and 1630F (20.5%) after 3 hours of incubation, as reported by Talebi et al. (2018).

Antimicrobial activity

B. subtilis AQU showed significant antimicrobial effectiveness against *S. aureus*, but *S.* typhimurium, *E. coli, P. aeruginosa, K. pneumoniae*, and *L. monocytogenes* resisted it (Table 4). The ability to produce antimicrobial compounds is an important characteristic when assessing the probiotic potential of bacteria (Duc et al., 2004). Previous research has highlighted the antimicrobial properties of various *Bacillus* strains isolated from traditional plant-based fermented food condiments in Africa. For instance, Compaoré et al. (2013) reported a broad antimicrobial spectrum for *Bacillus* species derived from Bikalga. However, this was not observed in the current study. It is well-established that the antimicrobial activity can vary depending on the testing methods used, the type and concentration of antimicrobial compounds produced, or the nature of the target pathogens (Compaoré et al., 2013). These factors may influence the findings of this study.

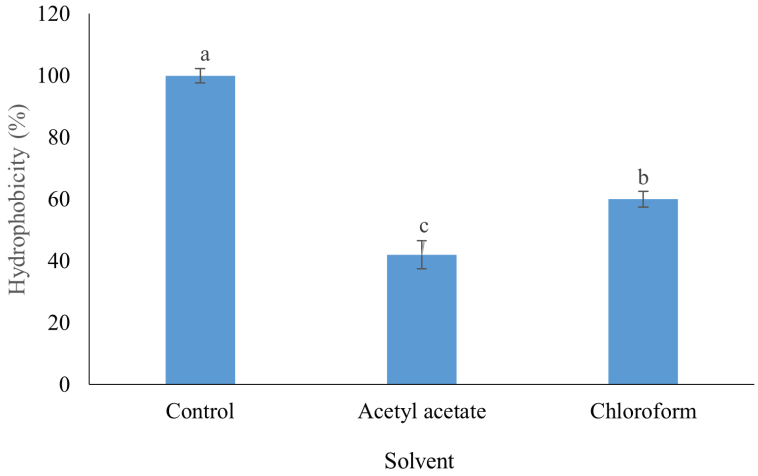


Figure 5: Cell surface hydrophobicity of *Bacillus subtilis* AQU against ethyl acetate and chloroform.

Antibiogram

Our study found that *B. subtilis* AQU was susceptible to most antibiotics tested, indicating that this strain is unlikely to harbour antibiotic-resistance genes that could be transferred to pathogenic microorganisms (Dabire et al., 2022). These results align with earlier research that also reported the susceptibility of *Bacillus* species to various antibiotics commonly used in clinical practice (Adimpong et al., 2012; Campore et al., 2013; Kavitha et al., 2018; Nwagu et al., 2020).

Cytotoxicity assay

The results of the MTT assay indicate that the optimized Okpeye formulation containing *B. subtilis* AQU is generally non-toxic to L-929 cells at the tested concentrations (Figure 6). Throughout the study, cell viability remained above 50%, even at the highest concentration of 2.00 mg/mL, demonstrating that the formulation did not exert significant cytotoxic effects. This is an important finding as it suggests that the optimized Okpeye with *B. subtilis* AQU does not compromise cell health, which is a crucial consideration when evaluating the safety of food products or supplements. The absence of a pronounced cytotoxic response indicates that the bacteria strain in this formulation is unlikely to cause harmful effects in the cells of the gastrointestinal tract.

Moreover, the dose-response relationship depicted in the graph shows a relatively stable cell viability trend across varying concentrations, with only a slight decline observed as the concentration increases. This trend suggests that the *B. subtilis* AQU strain might be well-tolerated at lower and moderate doses, providing an added safety assurance for potential consumption. The results also imply that *B. subtilis* AQU in the optimized Okpeye does not induce significant stress on the L-929 cell's metabolic activity. As a result, this could translate to minimal or no adverse effects on other cell types in the digestive system, thus supporting the strain's probiotic potential.

Table 4: Antimicrobial activity and antibiotic susceptibility pattern of *B. subtilis* AQU

		Zone of inhibition (mm)
Pathogens	*S.* Typhimurium	-
	S. aureus	15.7 ± 1.3
	E. coli	-
	Klebsiella pneumonia	-
	Pseudomonas aeruginosa	-
	Listeria monocytogenes	-
Antibiotics	Ciprofloxacin (10 mcg)	13 ± 0.8
	Gentamicin (10 mcg)	5.5 ± 0.5
	Tetracycline (30 mcg)	8.0 ± 1.0
	Cephalothin (30 mcg)	12.5 ± 0.5
	Chloramphenicol (30 mcg)	11.5 ± 1.5
	Penicillin (10 mcg)	7.0 ± 1.0
	Nalidixic (30 mcg)	9.0 ± 0.5
	Vancomycin (30 mcg)	7.5 ± 0.5
	Trimethoprim (25 mcg)	13.4 ± 1.4
	Erythromycin (15 mcg)	9.5 ± 0.5

The MTT assay confirmed that the optimized Okpeye with *B. subtilis* AQU does not exhibit cytotoxic properties at the tested concentrations. It is a promising candidate for further in vivo studies and eventual application as a probiotic food product. The results suggest that incorporating this strain in Okpeye could enhance its functional and health benefits without harming consumers. Future studies should consider the effects of long-term exposure and higher concentrations to provide a more comprehensive safety profile. However, based on this study's findings, the optimized Okpeye formulation with *B. subtilis* AQU appears to be a safe and viable option for dietary inclusion.

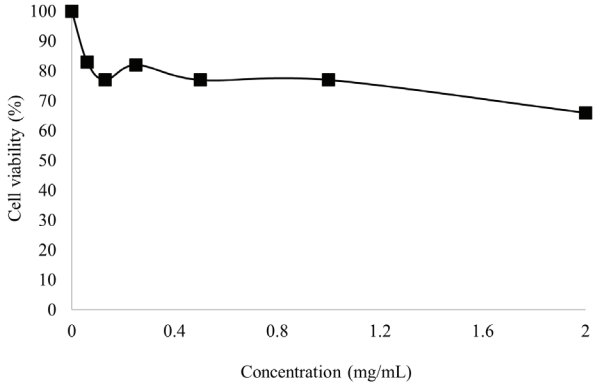


Figure 6: Viability of L-929 cells at various concentrations of the optimized Okpeye with *Bacillus subtilis* AQU

CONCLUSIONS

In conclusion, the study successfully demonstrated that the optimized fermentation of *P. africana* (Okpeye) with *B. subtilis* AQU significantly enhanced the production of gamma-aminobutyric acid (GABA), a compound known for its health benefits. The optimized conditions, including temperature, MSG concentration, inoculum size, and fermentation time, were effectively established using response surface methodology (RSM). Under ideal conditions, GABA production reached 315.03 mg/100 g of dry fermented Okpeye. The probiotic potential of *B. subtilis* AQU was further validated by its ability to survive in low pH and bile salt conditions, which mimics the gastrointestinal environment. Additionally, its cell surface hydrophobicity and autoaggregation properties suggest that the strain can adhere to the intestinal lining, enhancing its probiotic efficacy. The antimicrobial activity of the strain also highlighted its capacity to inhibit specific pathogens, although the results were variable across different bacterial species. Notably, the cytotoxicity assays revealed that the optimized Okpeye formulation was non-toxic to L-929 cells even at high concentrations, indicating its safety for potential consumption as a functional food product. The findings provide a strong foundation for developing safe, health-enhancing food products based on traditional fermentation practices.

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Miss Zainun Nurzahim in conducting the GABA analysis, as well as the support provided by the Innovation Centre for Confectionery Technology (MANIS), Faculty of Science and Technology.

DECLARATION OF COMPETING INTEREST

All the authors declare that they have no conflict of interest.

FUNDING INFORMATION

We gratefully acknowledge the support of the Tertiary Education Trust Fund (TETFUND) and Universiti Kebangsaan Malaysia for their support through the University Research Grant TAP-K020183.

AUTHOR CONTRIBUTION

Conceptualization, Funding acquisition: **U.K.A., N.A.M.Z.**; Data collection: **U.K.A.**; Statistical analysis: **U.K.A., N.A.M.Z., W.S.A.W.M.N.**; Data interpretation: **U.K.A., N.A.M.Z.**; Writing the first draft: **U.K.A.**; Manuscript editing & reviewing: **N.A.M.Z., W.A.A.Q.I.W., W.S.A.W.M.N.**; Project Supervision: **N.A.M.Z., W.A.A.Q.I.W., W.S.A.W.M.N**.

DATA AVAILABILITY

All the data generated or analyzed during this study are included in this published article.

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