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Importance and Evaluation of Glutamic Acid Production from Mostly Common Probiotic

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Abstract

THIS study aimed to estimate glutamic acid (Glu) production by probiotic bacteria and evaluate the impact of stimulants.70 samples, including raw milk, cottage cheese, yogurts were used. *Bacillus subtilis* and *Enterococcus faecium* were identified as the main probiotic bacteria. Out of 70 samples, 7 strains from *E. faecium* and only one strain from *B. subtilis* were obtained by cultural methods and confirmed by PCR using specific primers. LC-MS/MS was used to estimate Glu production qualitatively, with three *E. faecium* and one *B. subtilis* strain showing the highest peak areas, which were then analyzed by DNA sequencing for molecular identification. Extracellular cellular glutamic acid (ECG) and Intracellular glutamic acid (ICG) production from the untreated selected strains was performed quantitatively using the LC-MS/MS technique. Stimulation of Glu-responsible genes was performed using sucrose-supplemented medium and UV light exposure. Results showed that stimulation by UV light showed the most effective approach for both ECG and ICG in the case of *B. subtilis* and for ICG in the case of *E. faecium*, as indicated by the increase in the produced quantity if compared to the standard formulation (using glucose) or sucrose supplementation, while sucrose supplementation showed effectiveness on ECG production in the case of *E. faecium*. From this study, it can be concluded that UV light exposure to *B. subtilis* and sucrose supplementation medium for *E. faecium* have optimum effect on the safe and economic production of Glu.

Keywords: Glutamic acid, *E. faecium, B. subtilis,* LC-MS/MS, Sucrose, UV exposure.

Introduction

Glutamic acid (Glu) is an important amino acid for animal nutrition due to its various functions in the body as it is involved in many metabolic processes as a neurotransmitter. Moreover, it plays an important role in protein synthesis. Glu is also involved in the regulation of blood sugar levels, maintenance of the immune system, and regulation of nitrogen balance in the body [1]

In terms of animal nutrition, Glu is a crucial component of feed ingredients as it is important for the growth and maintenance of tissues, particularly in young animals. Glu is also essential for the maintenance of the digestive system, as it helps to produce digestive enzymes and maintain the integrity of the gut lining. Additionally, Glu has been shown to have a positive impact on animal performance and production as it improves feed conversion efficiency, increases weight gain, and improves the overall health and well-being of animals. Furthermore, Glu is a flavor enhancer, which can improve the palatability of animal feed, making it more appealing to the animals and helping to improve their consumption [2].

Commercial usage of bacteria that produce amino acids dates back to the 1950s, and regulatory mutants have since enhanced strains. The creation of amino acids like L-glutamic, L-valine, L-alanine, and L-proline using wild type bacteria depends on either inherent metabolic regulation or external stimuli that stimulate secretion. Many bacterial genera have the ability to produce amino acids e.g. Corynebacterium,

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Brevibacterium, Bacillus, Enterobacter, Mycobacterium and Eschericia[3].

Due to the high demand for this amino acid and the increasing demand for natural and sustainable food ingredients, there is a growing interest in the production of Glu using microorganisms such as bacteria. *B. subtilis* and *E. faecium* are two bacteria that have been studied for their ability to produce Glu and have shown great potential for large-scale production [4].

B. subtilis and *E. faecium* are well-known bacteria that have a long history of safe use in food and feed applications. Both of these bacteria have been investigated for their ability to produce Glu and have demonstrated positive results. This makes them attractive options for the production of Glu in large quantities. The ability to use these bacteria for the production of Glu is especially relevant given the growing demand for natural and sustainable food ingredients [5].

The production of Glu is typically limited by the activity of the gene(s) responsible for its synthesis. This gene(s) encodes enzyme(s) that catalyze the conversion of the substrate into Glu [6].To increase the production of Glu, it is necessary to stimulate the expression of the responsible gene(s) by various means, such as UV light exposure and growth in a sugar-rich medium[7,8]. UV light exposure has been shown to increase the expression of various genes in bacteria, and it has the potential to enhance the production of Glu. Additionally, growth in a sugar-rich medium can increase the production of Glu by providing the bacteria with a large amount of energy and nutrients [9,6].

This study aimed to isolate *E. faecium* and *B. subtilis* from milk and dairy products, identify the isolated strains at the molecular level, investigate their ability to produce glutamate (Glu), and evaluate the effects of UV light exposure and a sugar-rich medium on the expression of Glu-responsible gene(s), as each strain has unique characteristics and efficiency in metabolite production. Therefore, it was crucial to estimate the capability of the strains under study to produce Glu under different conditions. The environment in which each strain grows significantly affects its behavior, so it was insufficient to rely on the results of other strains to predict the behavior of the strains under study.

Materials and Methods

Samples

A total of 70 samples (Table 1) were collected from the Egyptian market and were analyzed at both Food Safety Department, Regional Center for Food and Feed - Agricultural Research Center and the Microbiology Department - Faculty of Veterinary Medicine - Cairo University.

Isolation of B. subtilis and E. faecium producing Glu [6,10]

Five grams of each sample were mixed with 45 ml of peptone buffer Biolife (7 g peptone, 1 g sodium chloride at pH 7, autoclaved at 121^{o}C for 15 min) and homogenized for 2 minutes. Ten-fold serial dilutions were performed down to 10^{-8}. Two pre-sterilized Petri dishes were inoculated with 1 ml of each dilution, then 10-15 ml of warmed Man Rogosa and Sharpe (MRS) agar, Lab M (8 gm beef extract, 10 gm peptone, 4 gm yeast extract, 20 gm Dextrose, 2 gm dipotassium phosphate, 5 gm sodium acetate, 10 gm Tween 80, 0.2 gm magnesium sulfate, 0.05 gm manganese sulfate, 20 gm agar for solidification at pH 6.4 ± 0.2 at 25^{o}C) was added for *B. subtilis* and Slanetz and Bartley Agar (SBA) agar LAB M (20 g tryptose, 5 g of yeast extract, 2 g of glucose, 4 g of dipotassium hydrogen phosphate, 0.4 g of sodium azide, 0.1 g of 2,3,4 tetrazolium chloride, and 12 g of agar at pH 7.2 ± 0.2 at 25^{o}C)for *E. faecium*. After solidification, a second layer of molten MRS agar was poured, and plates were incubated at 37°C for 24 hours. Colonies were counted and reported by multiplying the count by the dilution factor. Single colonies were cultured in MRS broth and incubated for 24 hours at 37°C. All tubes were stored at -20°C until further analysis.

Morphological and biochemical identification of isolated bacteria

A single colony from each isolate was identified using a light microscope (Optika, Italy). Bacilli (rod-shaped, aerobic, Gram-positive bacteria) were identified as *B. subtilis* [11], and Cocci (arranged in pairs) were confirmed as *E. faecium*[12].Gram stain and biochemical tests (Catalase, Indole, Methyl Red, Voges-Proskâuer, and Citrate utilization) were performed on all strains[13]. Typical reactions confirmed the suspected isolates as *B. subtilis* and *E. faecium*."

Molecular identification

Identification confirmation of the suspected strains was performed using PCR technique as follows:

DNA extraction

A loopful from every isolated strain was inoculated in 10 ml of nutrient broth Difco (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, 5 g of NaCl, and 1 l of distilled water at pH 6.8 ± 0.2 at

25°C) The inoculated tubes were incubated at 37°C for 24 hr. One ml of each culture medium was centrifuged at 13000 rpm in an Eppendorf tube to obtain a pellet from the bacteria under study, and the supernatant containing the medium was discarded. DNA extraction was performed using Prepman Ultra reagent (Thermofisher, United Kingdom) according to the manufacturer's instructions [14].

PCR reaction

PCR analysis was performed using the extracted DNA, specific primers (Table2)[15,16], and PCR premix (iNtRON Biotechnology, Korea). The PCR setup was performed according to the manufacturer's instructions. DNA amplification was performed using a thermal cycler (T100™ Thermal Cycler Singapore) for *B. subtilis*and *E. faecium*, respectively. PCR products were visualized on a 2% agarose gel using gel electrophoresis (Cleaver Scientific Ltd., Rugby, Warwickshire, United Kingdom)[17].

Estimation of Glu production from tested strains using LC-MS/MS

Preparation of bacterial suspensions

The colony-forming unit densities of all tested strains were adjusted to give 10^8cfu/ml in MRS broth, which was then incubated at 30 °C for 96 hours[6].

Extraction of produced Glu[6]

Extracellular glutamic acid (ECG) and Intracellular glutamic acid (ICG) were estimated**.** Cells were collected from the culture medium by centrifugation at 10000 rpm for 15 min at 4°C. The extracellular production was estimated using the supernatant that was diluted 50-fold with 7% (v/v) glacial acetic acid and kept at 4°C for 15 minutes, after which the diluted sample was centrifuged at 10,000 rpm and stored for further investigation after being filtered using a Nylon membrane with a 0.22 mm pore size. Estimation of intracellular production was conducted by the collected cells that were washed three times with 0.9% NaCl solution and were re-suspended in 1 ml of phosphate buffered saline (PBS, pH 7.0). One milliliter of 75% (v/v) ethanol was used to suspend the cells. The homogenate was centrifuged at 10000 rpm for 15 minutes at 4 °C, and the supernatant was then collected for additional analysis after being filtered through a Nylon 0.22 mm pore size filter.

Calibration, Tuning, Optimization, Method Development, and Batch Submission of Glutamic Acid

Instrument calibration followed the Applied Biosystems Sciex 4000Q Trap user manual for both ion modes. Parameters were set for a mass range of 100-200 Da, targeting 148.1 m/z using Analyst® software. Compound optimization was done in positive ion mode with a molecular weight of 147.1. The acquisition method for LC and MS was developed per the manual, setting parent and daughter masses, declustered potential (DP), collision energy (CE), and collision exit potential (CXP)values for Glu, using 70% acetonitrile:30% H2O with 0.2% formic acid [18]. Glu analysis by LC-MS/MS showed the parent ion at 148.1 m/z and daughter ions at 84, 102, and 130 m/z, with 84 m/z used for quantitative detection[19].

Establishing Calibration Curves for Glutamic Acid

A series of glutamic acid standard solutions (Acros, China) with concentrations of 10 ng/ml, 20 ng/ml, 40 ng/ml, and 80 ng/ml were injected into the HPLC(Agilent 1200 series) by separating column for building the standard curves of glutamic acid, from which the linearity and accuracy were evaluated [18].

Measuring ECG and ICG produced by tested strains

Aliquots from the extracellular and intracellular the extracts of the tested strains were injected in LC-MS/MS (Liquid chromatography mass spectrometry mass spectrometry) instrument after validation of test method. Qualitative estimation of its Glu was performed by detecting of its intensities and comparing the height of the obtained peaks related to every strain which corresponded to ECG and ICG intensities.

DNA sequencing

DNA sequencing was performed on 4 strains that showed the highest Glu production as follows:

Specific bands of tested bacteria were eluted from agarose gel using a specialized Qiagen DNA gel purification kit "QIAquick Gel Extraction kit" (Qiagen, German).PCR products were sequenced in Biotechnology lab, Regional Center for Food and Feed - Agricultural Research Center using Sanger technology with an eight capillary Applied Biosystems GA-3500 sequencer(genetic analyzer device) from Hitachi High-Tech Corporation, Tokyo, Japan[20].The sequences of the bacterial isolates were submitted to the NCBI GenBank database(National Center for Biotechnology for Biotechnology Information) the NCBI BLAST program was used to compare the data to the sequences published in the same database (http://www.ncbi.nlm.nih.gov/BLAST/).Neighbor joining phylogenetic tree reconstruction was done using MEGA 11. software using the maximum

likelihood method to elucidate the phylogenetic position of the selected isolates.

Comparison of Glu production under different conditions

Production of Glu using traditional cultural technique (standard)

All investigated strains' colony forming unit densities were adjusted to produce 10^8cfu/ml in MRS broth without any modifications, and they were then cultured for 48 hours at 37°C[6].

Production of Glu using sucrose-supplemented medium (Treatment 1)

The colony-forming units' densities of all tested strains were adjusted to give 10^8cfu/ml in MRS broth -supplementation with sucrose instead of glucose (20 g/l), which then were incubated at 37°C for 48 hours [6].

UV stimulation of Glu-responsible gene(s) expression (Treatment 2)

The method was performed [9] as follows:

Five ml of every bacterial suspension were inserted in centrifugation tubes and were centrifuged at 6000 rpm for 15 minutes. The pellets were washed with PBS, Merck(137 mM sodium chloride, 2.7 mM potassium chloride, 1.8 mM potassium dihydrogen phosphate, and 10 mM disodium hydrogen phosphate)at pH 7 twice and were suspended in the same amount of buffer. Three ml were transferred to sterile Petri dishes, which were then introduced to an irradiation unit (dimensions 15 × 25 cm) to be exposed to ultraviolet rays directly through a UV lamp (Model x-30 G/F Spectro line, 230 V, 0.45 Amps) at a wavelength of 254 nm. The distance between radiation source and bacteria was 30 cm for 60 seconds at a dose of 69000 J/m2.

Quantitative analysis of Glu by LC-MS/MS after stimulation

Quantitative measurement of the Glu was performed by running the LC-MS/MS technique [19] using Agilent 1200 infinite HPLC (High-performance liquid chromatography) coupled with a 4000 Q TRAP LC/MS/MS system equipped with a Zobrax C-8 column (2.1 x 150 mm, 5um partical size; Agilent). Liquid chromatography was carried out at a column temperature of 25°C. 50% (A) water and 50% (B) ACN with 0.2% formic acid each made up the mobile phase. The gradient conditions were 70% (A), 30% (B) at 0 minutes, and 70% (A), 30% (B) for 5 minutes. The positive ion mode was used for MRM. Gas temperature at 300°C, CE at 18 volts, DP at 51 volts, and CXP at 14 volts were additional MS parameters. The MRM sitting according to Analyst® 1.6.3 with Hotfix used were for Glu: 148.1 > 84.1.

Statistical analysis

All obtained results were analyzed using the General Linear Model (GLM) by SAS 9.4 software (2013). Means are compared using Tukey's range tests. The mean differences are considered significant at the p-value ($P < 0.05$) [21].

Results

Data illustrated in table 3 showed the prevalence of strains under study in different food categories related to milk and its products. It was clear that, both *B. subtilis* and *E. faecium* could not be isolated from raw milk. *B. subtilis* was detected in one commercial yogurt sample only in percentage of 6.7% while *E. faecium* could be isolated from 20% and 20% of tested sampled of both cottage cheese and locally enriched yogurt, respectively. Data obtained from this table also revealed that, *B. subtilis* was isolated from examined milk and milk product samples in a percentage of 1.4% while the percent of isolated *E. faecium* was 10%, As according to the most recent research, these two bacteria play a significant role in Glutamic acid production and are commonly found in milk and dairy products.

The isolated strains were identified using morphological appearance of *B. subtilis* as bacilli and the shape of *E. faecium* as diplococcic.

The confirmed *B. subtilis* and *E. faecium* strains have been verified using PCR technique to detect the specific typical gene for each micro-organism.

Figure1A showed the result of DNA visualization of *E. faecium* which was confirmed by the appearance of its specific band at the relevant molecular weight 550bp while figure 1B showed the specific band of *B. subtilis* which was at 1311bp level.

Figure2A showed the characteristics peak of Glu at positive ion mode which appeared at 148.1 m/zDa while Figure 2B showed the result of fragmentation of the obtained parent ion using specific MS parameters as illustrated previously. The parent ion 148.1 was clear to be fragmented to its daughter ions 84, 102 and 130 m/z,Da showing successful Ms/Ms interference.

Figure 3A showed the result of injection of Blank solution which revealed reliable starting point while Figure3B showed the resulted peak at specific retention time.Validation of the used analytical method was performed using Glu standard solution to verify that the method can be performed precisely and accurately in the lab environment.

Data in Figure4 showed the standard curve of the results obtained by injection of different concentrations of standard solution (0, 10, 20, 40 & 80ng/ml). It was clear that standard curve gave a linear mode with regression = 0.9994. Also it was clear that the accuracy % ranged from 92.9 to 103% indicating accurate dilutions, calculations and handling of the standard solutions and precise method of analysis.The results of the estimated amount of produced Glu by isolated and confirmed strains were mentioned in Table4 which showed that in case of *B. subtilis*, the amount of ECG was higher than that was found ICG.The same trend was found in the results of Glu produced by *E. faecium*as all tested strains showed higher ECG than ICG production.

From the same table and fromFigure5it was found also that some strains showed higher affinity of Glu production. The selected four strains were tested using DNA sequencer3500 GA (genetic analyzer device)for complete identification of its genome. Phylogenetic tree illustrated in figure6 confirmed that the sequenced *B. subtilis* strain belonged to strain L62 and other three *E. faecium* strains were strain HB2-2, HBUAS9-2 and SVU3. So one strain of *B. subtilis* and three strains from *E. faecium* strains were selected for applying further investigations. Also from the above mentioned figures it was clear that all tested samples (one *B. subtilis* and seven *E. faecium*) gave the typical specific chromatogram of Glu when tested by LC-MS/MS under specific parameters as mentioned previously and all obtained chromatograms showed reliable findings which were clear by obtaining the same peak at the same retention time with different intensities.

After complete identification of the strains under study, further investigations were performed to elucidate the effect of external stimulants on the expression of Glu producing gene(s).The four selected strains according to the highest of the obtained peak areas were cultivated on MRS medium under ordinary composition using glucose as the sole sugar source, another set of the four strains were cultivated on MRS medium using sucrose as the sole source of sugar while the last batch of the four selected strains set was exposed to UV light to elucidate its effect.Table5 showed the effect of the individual factor of tested strains on the ECG and ICG production. It was clear from the obtained data that, there were no significant differences between the three tested strains of *E. faecium* in ECG production while strain *E. faecium*1(E1) showed the highest amount of ICG followed by the strain *E. faecium*2(E2) then strain *E. faecium*3(E3). Concerning *B. subtilis* strain, it was clear that it had the lowest ECG amount if compared to the three *E. faecium* strains. Strain(E3) of *E. faecium* showed the lowest value of ICG production among the all three *E. faecium* strains.

Table 6 showed the comparative results of the amount of Glu production in normal conditions and as affected by sucrose and UV stimulation of the strains under study. The obtained results revealed that, in case of *B. subtilis* UV stimulation had the best effect as stimulant as it caused highly significant increase of ECG production if compared to other results. Also, sucrose had a better effect if compared to glucose as the sole source of sugar as it showed significant increase in the amount of expressed Glu. Also the same trend was obtained concerning of ICG as it was clear that UV had the best effect followed by sucrose which both gave better results if compared to the results obtained by cultivation under ordinary conditions.

From the same table it was clear that cultivation of *E. faecium* into the sucrose supplemented media gave the best results concerning ECG production as indicated by the highly significant increase of the amount of expressed Glu. Also using UV could stimulate the *E. faecium* strains coded E2 to produce more Glu if compared to the strains cultivated in glucose-contained media while the other 2 strains showed more ECG in medium contained glucose than media exposed to UV. Concerning ICG production of Glu by the three *E. faecium* strains under study, it was clear that UV stimulation had the best effect on Glu producing pathway as the obtained values of ICG in the *E. faecium* tested strains. These results were hard to be supported with findings of other scientific research work as it seemed to be not very well recognized. It was clearly demonstrated also from the same table that the highest ECG content was obtained by strain E1 of *E. faecium* followed by strain E3 of *E. faecium* then strain E2 of *E. faecium* as affected by sucrose supplementation. Also it was clear that UV had the best stimulation effect as *B. subtilis* strain for Glu production.

Discussion

Identifying reliable sources of glutamic acid in animal nutrition is crucial due to its significant role in protein synthesis and metabolic processes. Traditional sources, such as plant and animal-derived ingredients, often face limitations in availability and sustainability, prompting the need for alternative sources. Microbial production of glutamic acid, particularly through fermentation processes using bacteria like *B. subtilis* and *E. faecium*, offers a promising solution. This approach not only ensures a consistent supply but also enhances the nutritional profile of animal feed by improving gut health and overall growth

performance. The utilization of microbial glutamic acid can thus address the growing demand for efficient and sustainable animal nutrition [1].

Among various microorganisms, *B. subtilis* and *E. faecium* are recognized as predominant producers of glutamic acid, a key amino acid in animal nutrition. These bacteria are particularly efficient in synthesizing glutamic acid under controlled fermentation conditions. Typically, *B. subtilis* and *E. faecium* are cultured in de Man, Rogosa, and Sharpe (MRS) broth, which provides an optimal environment for their growth and metabolic activities. The standard conditions for producing glutamic acid involve incubating the cultures at 37°C for 48 hours with the MRS broth. This method not only ensures high glutamic acid output but also maintains the stability and viability of the bacterial cultures, making it a reliable approach for manufacture of fermented dairy products to increase their number and measure the percentage of glutamic acid in the products[1,22].

A percentage of 6.7% prevalence of *B. subtilis* in yogurt samples suggests that this bacterium is present but not highly common, likely due to environmental contamination or natural occurrence. This finding contrasts with other studies reporting higher prevalences of 75%, 68%, and 80% [23,24], which could be attributed to regional differences or specific production methods.

Geographic and environmental factors, along with differences in yogurt production practices likely explain these discrepancies. Also, a percentage of 10% of *E. faecium* in milk products suggests a moderate presence of this bacterium, likely due to environmental contamination or natural occurrence. This finding contrasts with other studies reporting higher prevalences of 20% and 22.3% in raw cow's milk and dairy products, respectively [25,26]which could be attributed to regional differences or specific production methods.

The appearance of rod shape of *B. subtilis* and the diplococcic shape of *E. faecium* which were typical to what was mentioned in some studies [11,12].

E. faecium and *B. subtilis*, which produced a specific bands at 550 and 1311 bp, respectively. These results corresponded with the findings of Nasiri and Hanifian, and Dutka et al., [16,27] who could visualize the specific bands of *E. faecium* and Ashe et al., and Fang et al. [15,28], who could demonstrate the specific bands of *B. subtilis*. When glutamic acid is evaluated using LC-MS/MS, the parent ion is detected at 148.1 m/z, while the fragmented daughter ions are found at 84, 102, and 130 m/z. These had been confirmed by the findings of Purwaha et al.[19] and Le et al.[29].

B. subtilis tends to produce higher levels of ECG compared to ICG levels under optimal conditions. This is due to the bacterium's robust secretion mechanisms and the role of Glu in various extracellular functions, such as acting as a signaling molecule or participating in biofilm formation. This was supported by the results of Gomaa et al.[30].Under optimal conditions, *E. faecium* appears to produce higher levels of ECG production compared to ICG levels under optimal conditions. This is due to the bacterium's efficient secretion mechanisms and the role of Glu in various extracellular functions[31].

E. faecium has the greatest affinity for Glu production if compared to other producing microorganisms. These results agreed with those reported by Mamuad and Lee [4]and Yang et al.[30].Al-Attar et al.[9], Zeng et al.[32] reported the enhancing effect of UV as a stimulant for Glu production as it causes stimulation of the gene expression of some of the responsible genes participating in its metabolic pathway.

Glucose, as the sole carbon source in MRS broth, plays a crucial role in the production of glutamic acid by *B. subtilis* and *E. faecium*. This monosaccharide is readily metabolized by these microorganisms, providing the necessary energy and carbon skeletons for biosynthetic processes. In the case of *B. subtilis*, glucose is converted through glycolysis, and the tricarboxylic acid (TCA) cycle, leading to the production of α-ketoglutarate, a key intermediate that is subsequently aminated to form glutamic acid[33]

Similarly, *E. faecium* utilizes glucose efficiently, enhancing its growth and metabolic activity, which in turn boosts glutamic acid production. The presence of glucose in MRS broth not only supports robust microbial growth but also optimizes the yield of glutamic acid, making it an essential component for industrial fermentation processes [34].

The production of glutamic acid by *B. subtilis* and *E. faecium* varies significantly between intracellular and extracellular environments. Intracellularly, glutamic acid is synthesized as part of the cellular metabolism, primarily through the TCA cycle and subsequent amination of α-ketoglutarate. However, the majority of the produced glutamic acid is secreted extracellularly, where it accumulates in the surrounding medium. This secretion process is facilitated by specific transport proteins and efflux

systems that actively transport glutamic acid across the cell membrane [33]. In *B. subtilis*, the secretion is often linked to the presence of a high concentration of glutamic acid within the cell, triggering the activation of these transport mechanisms [34].

Similarly, *E. faecium* utilizes a comparable mechanism, ensuring efficient export of glutamic acid to maintain cellular homeostasis and optimize production yields. This differential distribution underscores the efficiency of these microorganisms in producing and secreting glutamic acid, making them valuable for industrial applications[35].

Using sucrose as the sole carbon source in MRS broth significantly impacts glutamic acid production in *B. subtilis* and *E. faecium.* Sucrose is metabolized through glycolysis, providing essential intermediates for the TCA cycle, which are crucial for the biosynthesis of glutamic acid. Studies have shown that sucrose not only enhances the overall yield of glutamic acid but also stimulates the expression of genes involved in its production, such as those encoding glutamate dehydrogenase and glutamate synthase. In *B. subtilis*, the presence of sucrose has been linked to increased transcriptional activity of the pgs operon, which is responsible for poly-γ-glutamic acid synthesis, a pathway closely related to glutamic acid production .Similarly, in *E. faecium,* sucrose induces the upregulation of genes associated with glutamate metabolism, thereby boosting glutamic acid output [6].This stimulatory effect underscores the potential of sucrose as an effective carbon source for optimizing glutamic acid production in industrial fermentation processes.

Sucrose enhances glutamic acid production in Enterococci more effectively than in *B. subtilis* due to several scientific reasons as it has a glucansucrase enzyme, that efficiently hydrolyzes sucrose into glucose and fructose, which can be metabolized efficiently to produce glutamic acid. Additionally, sucrose provides growth factors that enhance Enterococci's growth and metabolic activity, leading to higher cell density and increased glutamic acid production. Meanwhile, the energy-conserving sucrose utilization pathways in *B. subtilis* consume more ATP compared to those in Enterococci, making sucrose less efficient for glutamic acid production in *B. subtilis*[36,37].

Exposure to ultraviolet (UV) radiation has been shown to significantly enhance glutamic acid production in *B. subtilis* and *E. faecium*. UV treatment induces mutations that can lead to the overexpression of genes involved in glutamic acid biosynthesis, such as those encoding glutamate dehydrogenase and glutamate synthase. In *B. subtilis*, UV irradiation has been reported to increase the activity of the pgs operon, which is crucial for poly-γ-glutamic acid synthesis, thereby indirectly boosting glutamic acid production [9], Similarly, in *E. faecium*, UV exposure enhances the transcriptional activity of genes associated with glutamate metabolism, resulting in higher extracellular glutamic acid levels [32].UV radiation's ability to stimulate gene expression and metabolic pathways demonstrates its potential as a strategy for maximizing the production of glutamic acid in industrial fermentation processes.

The comparative analysis of glutamic acid production in *B. subtilis* and *E. faecium* under different conditions reveals distinct responses to UV treatment and sucrose supplementation. In *B. subtilis*, UV irradiation significantly enhances glutamic acid production, likely due to the induction of mutations that upregulate genes involved in its biosynthesis, such as those encoding glutamate dehydrogenase and glutamate synthase. This effect is more pronounced than the use of sucrose as the sole carbon source in MRS broth, which, while beneficial, does not stimulate the same level of gene expression or metabolic activity [9]. Conversely, in *E. faecium*, sucrose proves to be a more effective stimulant for glutamic acid production. The presence of sucrose in the growth medium enhances the transcription of genes associated with glutamate metabolism, leading to higher yields of glutamic acid compared to UV treatment [6].These findings suggest that while UV irradiation is a potent enhancer for *B. subtilis*, sucrose is more effective for *E. faecium*, emphasizing the value of specific strategies for improving the production of glutamic acid by microorganisms. The production of glutamic acid can be quite costly due to the complex processes involved. However, extracting glutamic acid from probiotics can be a more economical alternative. Probiotics have been shown to produce glutamic acid through fermentation processes. This method can be more cost-effective due to the lower production costs and the use of readily available raw materials. [38].

Additionally, using probiotics for glutamic acid production can have other benefits, such as improved viability of the microorganisms and potential health benefits for humans and animals3. This approach not only reduces costs but also aligns with sustainable and environmentally friendly practices[39]. Researchers have examined the nutrient needs for synthesizing glutamic acid and discovered that they differ depending on the strain. The productivity and

quality of glutamic acid were affected by several parameters, including carbon, nitrogen sources, ionic strength, aeration, agitation, and medium Ph [40]. Different concentration of carbon sources, including ribose, sorbitol, manitol, fructose, glucose, sucrose, and lactose, have an impact on production of glutamic acid by lactic acid bacteria (LAB) strains cultivated in MRS medium. [6].

Conclusion

In conclusion, microbial production of glutamic acid, particularly through fermentation processes using bacteria like *E. faecium* and *B. subtilis*, presents a promising solution. Among various microorganisms, *B. subtilis* and *E. faecium* are recognized as predominant producers of glutamic acid, efficiently synthesizing it under controlled fermentation conditions. Using glucose, as the sole carbon source in MRS broth, plays a crucial role in this process by providing the necessary energy and carbon skeletons for biosynthetic pathways. Also, using sucrose as the sole carbon source in MRS broth significantly impacts glutamic acid production, demonstrating the importance of modified techniques to enhance the production of microbial glutamic acid for industrial applications. UV radiation emerges as a potent enhancer of glutamic acid production in both *B. subtilis* and *E. faecium.* The induced mutations from UV treatment lead to the overexpression of key biosynthetic genes, significantly boosting glutamic acid yields. In *B. subtilis*, this is achieved through increased activity of the pgs operon, while in *E. faecium*, UV exposure enhances the transcription of genes related to glutamate metabolism. For future research, appropriate nitrogen sources for Glu synthesis will be selected with various nitrogen sources (ammonium sulfate, ammonium chloride, soybean meal, potassium nitrate, urea, and yeast extract) as nitrogen plays an important role in fermentative cultivation of glutamic acid-producing bacteria.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study did not use animals and therefore no animal ethics certificate was issued, but rather it was based on the use of milk and its products.

TABLE 1. Types and numbers of collected samples from local market

Sample type	Number of samples collected
Raw milk	20
Cottage cheese	15
Locally produced yogurt	20
Commercial yogurt	15

TABLE 2. Primer sequences and thermal profile used in the analysis of *B.subtilis* and*E. faecium* under study[15,16]

Microorganism	Primer sequence	Amplicon size (bp)	Thermal profile
B. subtilis **(*EN1* gene)[15]**	5\-CCAGTAGCCAAGAATGGCCAGC-3\ 3\-GGAATAATCGCCGCTTTGTGC-5\	1311bp	1 cycle of 94 °C for 5 min, 10 cycles of 94 °C for 30 sec, 70 °C for 20 sec, 74 °C for 45 sec followed by 1°C decrease of the annealing temperature every cycle for 25 cycles, 94°C for 30 sec, 60°C for 20 sec, 74°C for 45 sec and a final extension at 74°C for 10 min.
E. faecium **(*ddl* gene)[16]**	5\- GCAAGGCTTCTTAGAGA-3\ 3\- CATCGTGTAAGCTAACTTC-5\	550 bp	Initial denaturation at 94°C for 2 mins, 94°C for 1 min, at first cycle, 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, at the next 30 cycles, and 72°C for 10 min.

TABLE 3. Number and percentage of identified *B. subtilis* and *Enterococci* strains isolated from collected samples

Sample type	Total	Number of isolated *B. subtilis* strains	%	Number of isolated *E. faecium* strains	%
Raw milk	20	0	0	0	0
Cottage cheese	15	0	0	3	20
Locally produced yogurt	20	0	0	4	20
Commercial yogurt	15	1	6.7	0	0
Total	70	1	1.4	7	10.0

TABLE 4. Result of qualitative analysis of Glu produced by isolated strains expressed by intensity (cps) using LC-MS/MS

	Strain	ECG	ICG
1	B1*	3.7E4	6880
2	E1**	3.8E4	4898
3	E2	3.9E4	4872
4	E3	4.0E4	3080
5	E4	3.9E4	2396
6	E5	3.2E4	1672
7	E6	3.3E4	1944
8	E7	3.0E4	1770

(ECG)Extracellular Glutamic acid, (ICG)Intracellular Glutamic acid, *B=*B. subtilis* **E=*E. faecium*

TABLE 5. The effect of strains on production of Glu

Sample	ECG	ICG
	Mean ± SD	**Mean ± SD**
B1	3388.9 ± 1912.0^{b}	102.5 ± 55.0^{b}
E1	8201.1 ± 4340.8^{a}	132.3 ± 92.7^{a}
E2	6926.7 ± 2351.0^{a}	111.3 ± 160.5^{ab}
E3	7728.9 ± 1994.9^{a}	23.0 ± 34.5^{c}

(ECG)Extracellular Glutamic acid and (ICG)Intracellular Glutamic acid. Means with the same letters with each column of the trait are non-significant different (P<0.05)

TABLE 6. Results of statistical analysis of data obtained from production of ECGand ICG using treatments under study

	B1			E1			E2			E3		
	G	**S**	**UV**	**G**	**S**	**UV**	**G**	**S**	**UV**	**G**	**S**	**UV**
ECG	1527^{e}	2817^{de}	5823^{bcde}	5900^{bcde}	12837^{a}	5867^{bcde}	5207^{cde}	9980^{abc}	5593^{bcde}	7130^{bcd}	10083^{ab}	5973^{bcde}
ICG	39.8^{fe}	112^{cd}	155.7^{c}	39.8^{fe}	112^{cd}	245^{b}	0^{f}	9^{f}	325^{a}	0^{f}	0^{f}	68.9^{de}

(G) Standard production environment with Glucose, (S) Chemical stimulation with Sucrose, (UV) Stimulation using UV light. Means with the same letters with each raw of the trait are non-significant different (P<0.05).

(B1)*B. subtilis*, (E1) *E. faecium* 1, (E2) *E. faecium* 2, (E3) *E. faecium*3.

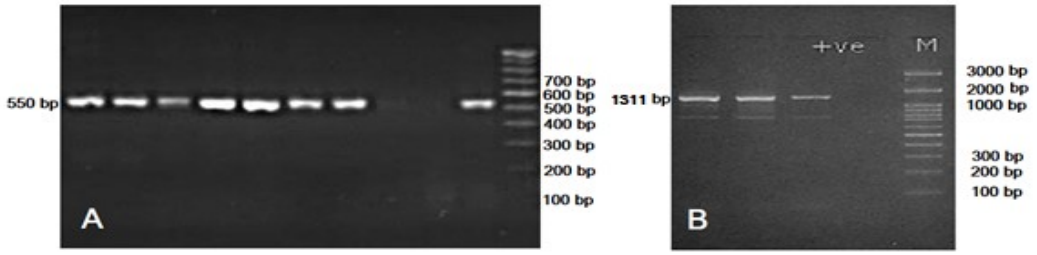

Fig. 1. (A) Gel electrophoresis for *ddl* gene of *E. faecium* at 550 bp, (B) Gel electrophoresis showing specific band for *EN1* gene of *B. subtilis* at 1311 pb.

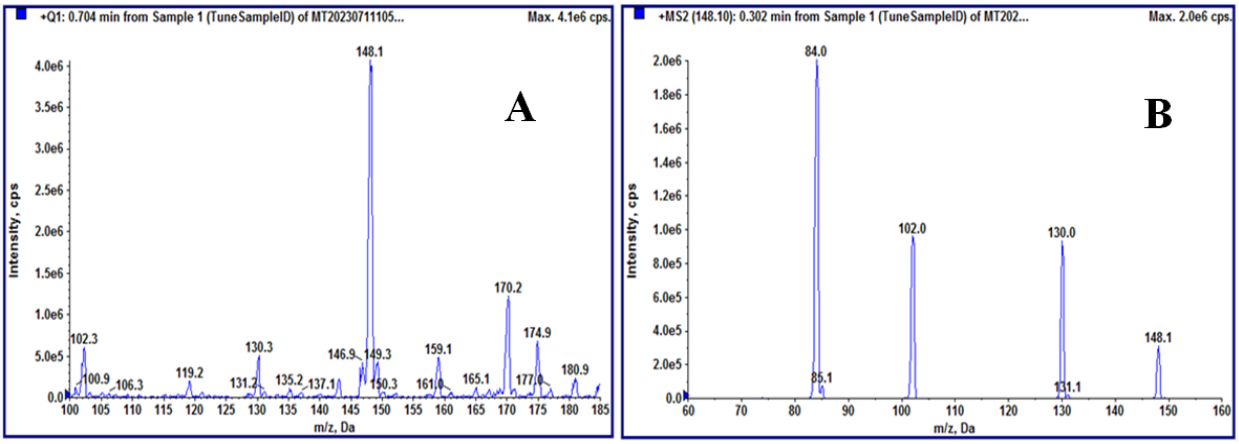

Fig. 2. LC-MS/MS chromatogram of identification of Glu as produced by one of the tested strains (A) Q1 showing m/z peak (B) MS2 showing parent and daughter ions.

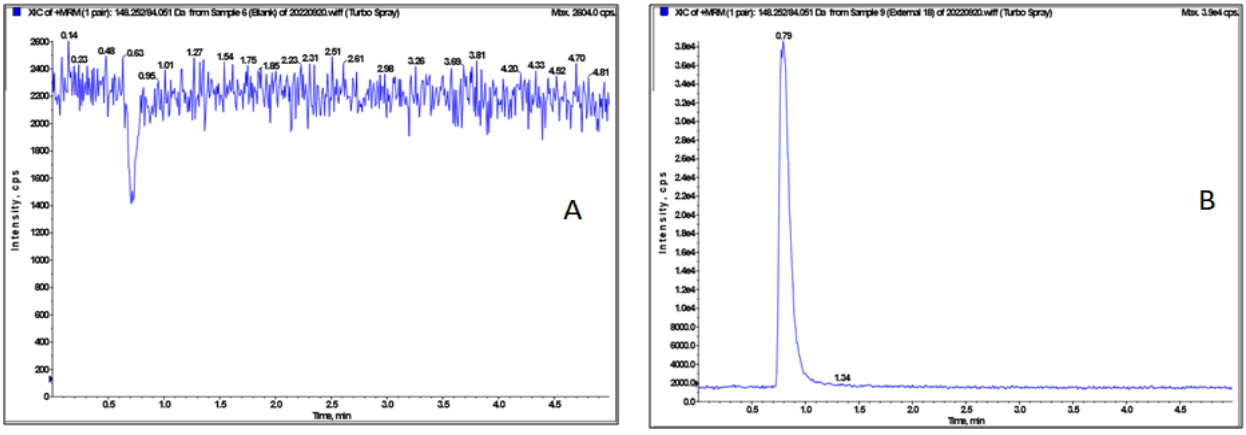

Fig.3. (A) Blank Sample(B) LC separation Retention Time (RT) of Glu in positive ion mode.

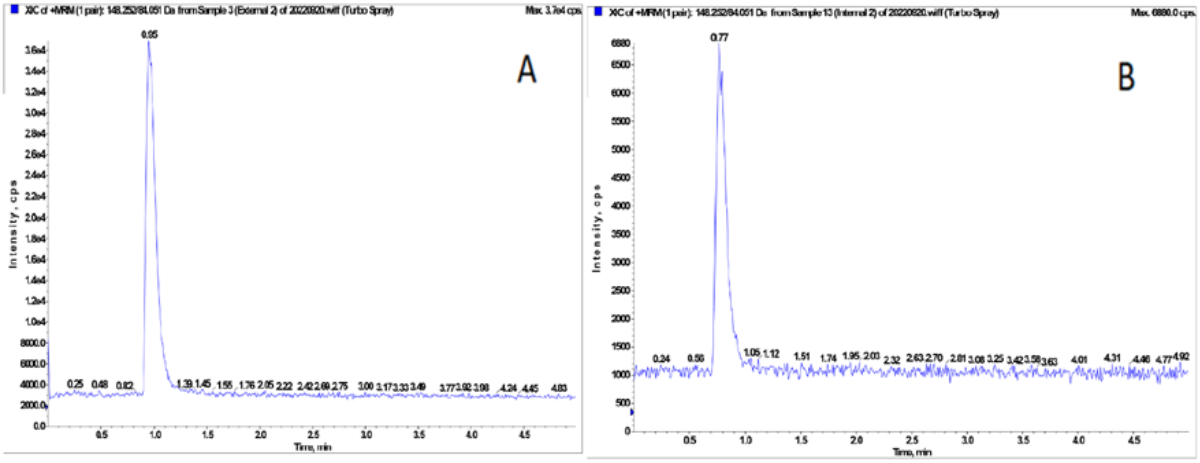

Fig.4. Chart Linearity, accuracy and standard curve of Glu standard solution.

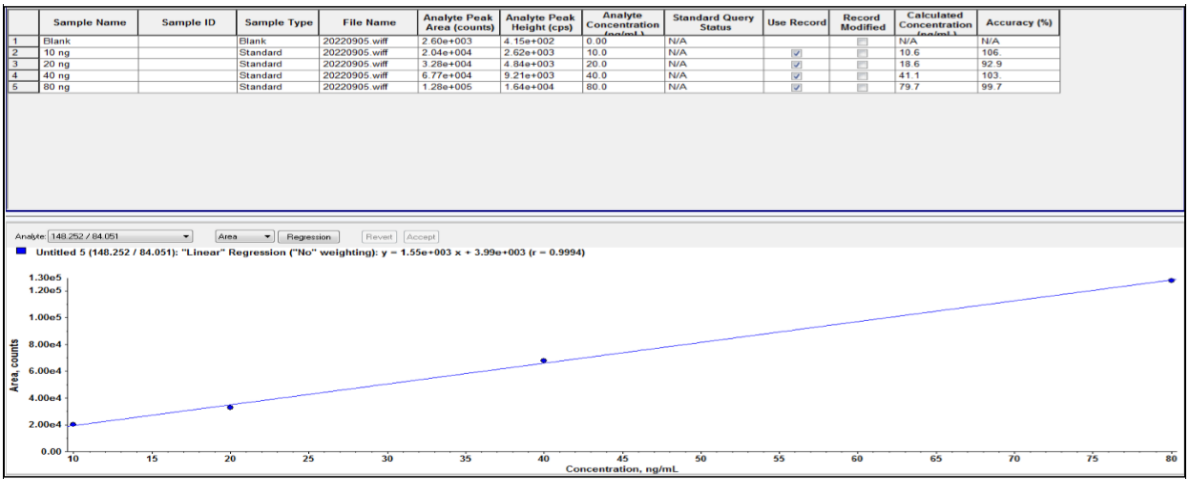

Fig.5. Chromatogram of qualitative analysis of extra and intra cellular Glu from isolated strains: A) Intensity of Extracellular glutamic acid, B) Intensity of Intracellular glutamic acid.

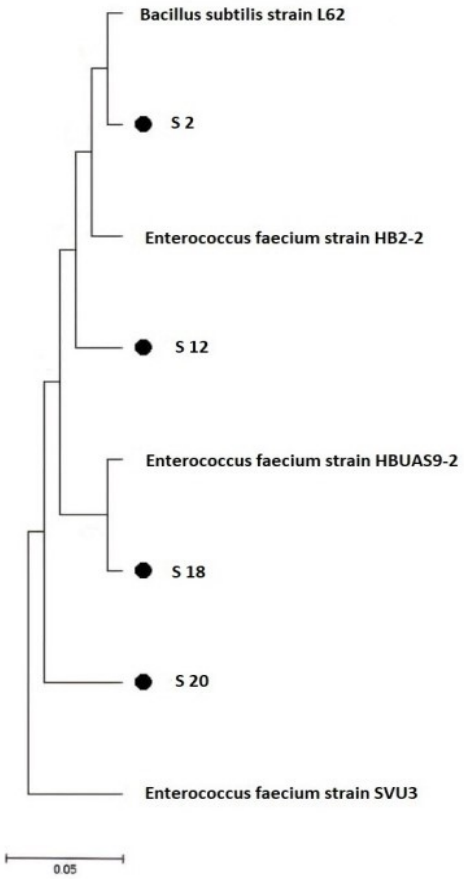

Fig.6. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of four bacterial isolates (Dark circles) with the closest hits obtained from the NCBI gene bank.

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أهمية وتقييم إنتاج حمض الجلوتاميك من بكتريا البروبيوتيك الشائعة

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الملخص

هدفت هذه الدراسة إلى تقدير إنتاج حمض الجلوتاميك بواسطة البكتيريا الحيوية (البروبيوتيك) وتقييم تأثير المعاملات على هذا الانتاج . حيث تم استخدام 70 عينة، بما في ذلك الحليب والجبن القريش والزبادي البلدى والزبادي التجاري. وتم تحديد بكتريا الباسيلس ساتلس وبكتريا انتيروكوكس فيشيم على أنهما البكتيريا الحيوية الرئيسية. فمن بين 70 عينة، تم الحصول على 7 سلالات من بكتريا انتيروكوكس فيشيم وسلالة واحدة فقط من بكتريا الباسيلس ساتلس بطرق التحاليل الميكروبيولوجيه التقليدية وتم تأكيدها بواسطة تفاعل البوليميراز المتسلسل, ثم تم استخدام جهاز الإل سى ماس ماس لتقدير إنتاج حمض الجلوتاميك نوعيًا، حيث أظهرت ثلاث سلالات من بكتريا انتيروكوكس فيشيم وسلالة واحدة من بكتريا الباسيلس ساتلس أعلى تقدير من بين باقى السلالات ، والتي تم تحليلها بعد ذلك عن طريق تسلسل الحمض النووي للتعريف الجزيئي,ثم تم قياس كمية إنتاج حمض الجلوتاميك الخلوي خارج وداخل الخلية من الاربع سلالات المختارة كأعلى تقدير لحمض الجلوتاميك غير المعالجة (التى نمت فى وسط مكمل بالجلوكوز) كميًا باستخدام تقنية جهاز الإل سى ماس ماس ومقارنتها بكمية حمض الجلوتاميك خارج وداخل الخليه من نفس السلالات وتحت نفس الظروف ولكن نمت فى وسط مكمل بالسكروز(معاملة 1) وتم تعريضها للأشعة فوق البنفسجية (معاملة 2).

أظهرت النتائج أن التحفيز بالأشعة فوق البنفسجية(معاملة 2) أظهر أكثر فعاليةوزيادة فى الكمية المنتجه لحمض الجلوتاميك الخلوي خارج وداخل الخلية في حالة بكتريا الباسيلس ساتلس وحمض الجلوتاميك داخل الخلايا في حالة بكتريا انتيروكوكس فيشيم إذا ما قورنت بالسلالات غير المعالجة (باستخدام الجلوكوز) أو السلالات المعالجه بمكملات السكروز(معاملة 1)، بينما أظهرت السلالات المعالجه بمكملات السكروز(معاملة 2) فعالية اكثر في إنتاج كمية حمض الجلوتاميك خارج الخلية في حال بكتريا انتيروكوكس فيشيم.

ومن هذه الدراسة، يمكن أن نستنتج أن التعرض للأشعة فوق البنفسجية(معاملة 3) لبكتريا الباسيلس ساتلس ونمو البكتريا فى وسط مكمل السكروز(معاملة 2) لبكتريا انتيروكوكس فيشيم له تأثير مثالي على الإنتاج الآمن والاقتصادي لحمض الجلوتاميك.

الكلمات الدالة: حمض الجلوتاميك، بكتريا انتيروكوكس فيشيم ، بكتريا الباسيلس ساتلس ، جهاز الإل سى ماس ماس ، السكروز، التعرض للأشعة فوق البنفسجية.