Probiotics and Antimicrobial Proteins (2021) 13:993–1004
https://doi.org/10.1007/s12602-021-09759-7

Evaluation of GABA Production and Probiotic Activities of *Enterococcus faecium* BS5

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Accepted: 22 February 2021 / Published online: 10 March 2021

Abstract

Gamma-aminobutyric acid (GABA) is a principal inhibitory neurotransmitter in the central nervous system and is produced by irreversible decarboxylation of glutamate. It possesses several physiological functions such as neurotransmission, diuretic, and tranquilizer effects and also regulates cardiovascular functions such as blood pressure and heart rate in addition to playing a role in the reduction of pain and anxiety. The objective of this study was to evaluate the GABA producing ability and probiotic capability of certain lactic acid bacteria strains isolated from dairy products. Around sixty-four bacterial isolates were collected and screened for their ability to produce GABA from monosodium glutamate, among which nine isolates were able to produce GABA. The most efficient GABA producer was *Enterococcus faecium* BS5. Further, assessment of several important and desirable probiotic properties showed that *Ent. faecium* BS5 was resistant to acid stress, bile salt, and antibiotics. *Ent. faecium* BS5 may potentially be used for large-scale industrial production of GABA and also for functional fermented product development.

Keywords Gamma-aminobutyric acid · Lactic acid bacteria · Dairy products · Screening and production · Probiotics

Introduction

Gamma-aminobutyric acid (GABA) is a widely distributed non-protein amino acid. It is produced from glutamate through irreversible alpha-decarboxylation by glutamate decarboxylase (GAD). GABA acts as a major neurotransmitter in the mammalian nervous system [1]. It has diuretic, anti-depression, and tranquilizing effects [2]. It induces hypotension and regulates growth hormone secretion. It has been used in the regulation of physiological disorders, including epilepsy, convulsions, Parkinson's disease, Alzheimer's disease, and Huntington's chorea. GABA is widely distributed in the environment [3] and has notable physiological functions as an anti-stress molecule [4] inhibiting the spread of cancer [5] and preventing diabetes [6].

Microbial-derived GABA regulates visceral hypersensitivity [7], and also, in different parts of the GI tract, GABA and GABA receptors have functions, that focus on the regulation of GI motility and inflammation [8]. Supplementation of GABA regulates intestinal functions including intestinal immunity, intestinal amino acid profiles, gut microbiota [9], and the promotion of jejuna SIgA secretion. This could be linked to the T cell-dependent pathway and altered structure of gut microbiota as well as metabolism [10].

Furthermore, GABA is a bioactive component in pharmaceuticals as well [11]. These numerous value-added benefits of GABA on human health have recently gained increasing interest in the food industry. Many GABA-enriched functional foods are currently being developed such as tea leaves that are anaerobically treated, water-soaked rice germs, red mold rice, tempeh like fermented soybeans, and dairy products such as yogurt, fermented milk products, and cheese [12]. Therefore, the due acknowledgement should be given to the mass production of GABA and its subsequent use in the modern food industry as a bioactive food ingredient.

The screening of GABA-producing lactic acid bacteria (LAB) and the production of GABA-enriched food by LAB are currently being actively considered and investigated.

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Some researchers reported GABA-producing LAB strains from different sources, including *Lactobacillus brevis* from kimchi [13–15], *L. brevis* from pacoai [16], *L. paracasei* from Traditional fermented fish [17], *L. plantarum* from kimchi [18], *L. sakei* from jeot-gal, Korean fermented seafood [19], *Lactococcus lactis* from kimchi [20], and *L. farciminis* from fishery products [12]. The food industry needs to screen and find out whether various LAB have the ability to produce GABA because acid production, taste, and flavour formation ability, etc., are specific profiles of LAB. These profiles are regarded as essential factors in the use of LAB as starters in fermented food production [21].

Among the various GABA producing microbes, *L. brevis*, *L. plantarum*, and *L. futsaii* [18, 22, 23] have been assessed for their probiotic characteristics, making a good choice as a starter ingredient for functional GABA-enriched foods. These probiotic bacteria are thus widely used as starter cultures in the food industry [24]. They have to survive in diverse conditions such as the acidic condition in the stomach and in the presence of bile acids in the intestines to assimilate in the intestinal tract. These diverse conditions represent the essential parameters for selecting probiotics [25]. Also, different bacterial strains affect the host through different mechanisms of action [26, 27]. Therefore, the probiotic ability was shown to be species dependent. Selection of probiotic strains based their ability to modulate the very specific physiological properties such as GABA production is essential for demonstrating a probiotic health effects.

Numerous studies have reported LAB belonging to the genera *Lactobacillus* and *Lactococcus* as the most GABA producing organisms, while a few studies on the genus *Enterococcus* have also been published. Considering the above, the current study aimed to evaluate the capability of LAB isolated from dairy products to produce γ-aminobutyric acid (GABA), and also analyse the role of this LAB as a probiotic.

Materials and Methods

Ten samples of dairy products such as milk, commercial curd, and yogurt were collected from different places in Coimbatore, Tamil Nadu. The collected samples were stored in sterile bottles and transported in cooler boxes to the laboratory. The bacterial population was isolated by serial dilution of samples using sterile peptone physiological saline (1% peptone, and 0.85% w/v NaCl), and after incubation, plates with 30–100 colonies were enumerated and colonies with distinct morphological differences were selected and then purified using MRS agar media (Himedia, Mumbai, India). These isolates were stored in MRS broth (Himedia, Mumbai, India) containing 50% sterile glycerol at − 80 °C. The isolates were subcultured in MRS broth at 37 °C for 24 h and used as inoculum for the following experiments.

Screening of GABA Producing Isolates

Thin-Layer Chromatography: GABA producing ability of the isolates and the positive control *L. plantarum* DM5 [28] were evaluated in bacterial supernatants following the method of Choi et al. [29] with some modifications of Kim and Kim [30]. The bacterial isolates were grown in MRS broth containing 2% monosodium glutamic acid (MSG) at 37 °C for 48 h. The cell-free supernatant (extracellular GABA) obtained by centrifugation (10,000 g, 4 C for 10 min) was filter-sterilized using a 0.2-µm syringe filter and stored for further analysis.

The collected bacterial cells (intracellular GABA) were washed three times with 0.9% NaCl and resuspended in 20 ml of phosphate buffer (pH 8.0). Then the cell suspension was treated in an ice bath with sonication (500 W, 20 min). The homogenate was centrifuged for 15 min at 8000 g at 4 °C, and the supernatant was collected for further analysis [31]. Four microlitres of extracellular GABA and intracellular GABA, along with 1 mg/ml of GABA standard (Sigma-Aldrich Co, St. Louis, MO, USA, cat No-03835) and MSG (Sigma Aldrich, Bengaluru, India) solution, was spotted on a silica TLC plate (Aluminium sheet silica gel 60 F_{254} Merck Germany). GABA separation by TLC was performed using a solvent mixture (1butanol:acetic acid:distilled water 3:2:1 v/v/v). When the solvent front had reached within about 1 cm of the top end of the adsorbent (after 20 to 30 min), the plate was taken out of the developing chamber, the location of the solvent front marked, and the solvent was evaporated from the plate. GABA spots were identified after spraying the plates with 2% (w/v) ninhydrin and exposing the plates to a heat source for a few minutes [12]. The conversion rate of MSG to GABA was quantified using ImageJ software and analysed with one-way ANOVA.

High-Performance Thin-Layer Chromatography: Based on the TLC data, extracellular GABA (10 µl) of four isolates along with GABA standard, MSG, and culture of positive control were loaded with silica gel 60 F_{254} using a Hamilton syringe and run on a CAMAG Linomat 5 instrument. The mobile phase used was 1butanol:glacial acetic acid:water (3:2:1 v/v/v). The developed plate was viewed after spraying it with 2% ninhydrin in acetone and developing at 105 °C for 5 min. The scanned area of the samples was compared with the scanned area of the GABA standard. The GABA spots were viewed at 480 nm on HPTLC plates [32]. Quantification of the graph was performed using the area under curve analysis and analysed with one-way ANOVA.

Quantification of GABA by HPLC

GABA standard solution and extracellular GABA of the isolate (BS5) were derivatized with phenyl isothiocyanate (PITC) [33]. Aliquots of 1 ml GABA standard solution and culture supernatant were dried using a lyophilizer. About 100 µl of ethanol-water-triethylamine (2:2:1 v/v/v/) was added to the residue and was evaporated to dryness under vaccum. This residue was dissolved in 150 µl ethanol-water-triethylamine-PITC (7:1:1:1 v/v/v/v/) which was kept at room temperature for 20 min to form phenyl thiocarbamyl-GABA. Excess reagents were removed under vacuum. About 500 µl of mobile phase consisting of 80% solution A (1.4 mM sodium acetate, 0.1% triethylamine, and 6% acetonitrile) and 20% solution B (60% acetonitrile) was added to the dry residue, and the resultant solution was filtered using a 0.45-µm filter, and analysed with HPLC. The GABA analysis was performed using an HPLC system equipped with a gemini C18 column. The column was eluted at a flow rate of 1 mL/min for 50 min with a linear gradient of 0–100% solution B. A sample (20 µl) was injected and detected at a wavelength of 254 nm. A standard curve was created using known concentrations (250, 500, and 1000 ppm) of GABA standard and the quantity of GABA in the culture supernatant was measured from the standard curve and analysed with one-way ANOVA.

Morphological and Phenotypic Characterization

The selected isolate was grown overnight in MRS broth at 37 °C in a rotary shaker. Gram-staining was performed according to the method of Dussault [34]. FESEM was also carried out for morphological analysis. The genomic DNA of the isolate was extracted from the 12 h culture by the phenol-chloroform method [35]. The 16S rRNA gene of the selected organism was amplified by using the polymerase chain reaction (PCR) technique from a genomic DNA using the forward primer 5′-GAGTTTGATCCTGGCTCAG-3′ and the reverse primer 5′-ACGGCTACCTTGTTACGACTT-3′. PCR reaction was carried out by a TI thermocycler. The amplicon was obtained with a PCR cycling program of 96 °C for 5 min, 30 cycles 94 °C for 1 min, 48 °C for 1 min, and a final extension time of 72 °C for 2 min. The amplified product was visualized by electrophoresis separation in a 0.8% gel. The amplicon was eluted, purified, and sequenced commercially (Eurofins Genomics India Pvt. Ltd., Bengaluru). The partial sequence data of the 16S rRNA genes (956 bp) were deposited in the database of the National Center for Biotechnology Information (NCBI) with accession no MN394829. These sequence data were compared with the public database in Genbank using BLAST, closest known relatives were obtained and the phylogenetic tree was constructed based on the neighbour-joining method using MEGA version 7.0 [36].

Evaluation of High GABA Producing Isolate for Probiotic Attributes

Ent. faecium BS5 was selected based on quantification studies and subjected to a series of probiotic tests under in-vitro conditions to explore the probiotic potential of the isolate.

Tolerance to Low pH and Bile Salt

The selected GABA producing *Ent. faecium* BS5 was characterized to identify its major probiotic features, such as tolerance to low pH, and bile salt based on the method of Ahire et al. [37]. *Ent. faecium* BS5 was grown in MRS broth overnight at 37 °C and pelleted at 8000 g for 5 min at 4 °C. Cells were rinsed twice with sterile phosphate buffer saline (PBS, pH 7.3) and resuspended in 1 ml PBS. *Ent. faecium* BS5 was diluted in PBS (1:100) of varied pH (1, 2, 3, and 4) and then incubated at different time intervals (0, 1, 2, and 3 h) at 37 °C. The viability of the bacterial cells in terms of CFU ml^{-1} on the MRS agar plate was determined. After 3 h of incubation, the survival of the isolate in different pH was also reported in percentage.

Bile salt tolerance of *Ent. faecium* BS5 was determined by inoculating the bacterial isolate in MRS broth containing 2.5, 5.0, 7.5, and 10% of bile salt and incubated at 37 °C for 3 and 6 h. The growth medium with 0% bile salt was used as a control. The treated cells were then evaluated by an absorbance microplate reader by recording the absorbance at 595 nm. Data were subjected to two-way ANOVA for bile and time.

Hydrophobicity Assay

Cell surface hydrophobicity of *Ent. faecium* BS5 was evaluated based on the method of Thapa et al. [38]. Chloroform and ethyl acetate were used to detect the surface hydrophobicity of the isolates. The overnight grown cells were centrifuged, washed with PBS three times, and resuspended in 10-ml Ringer's solution, and OD was measured as control (A_0) at 600 nm. In the tested sample, the cell suspension was mixed with an equal volume of solvent by vortexing for 2 min and held at room temperature for 30 min. The aqueous phase was removed and absorbance measured at 600 nm (A_1). The formula $(1 - A_1/A_0) \times 100$ was used to measure the hydrophobicity of bacterial adhesion to the solvent.

Autoaggregation Assay

Autoaggregation assay of *Ent. faecium* BS5 was carried out with minor modifications to the method of Patel et al. [39]. Cells from the overnight grown culture were collected by centrifugation and washed three times with PBS (pH 7.3). The pellets were resuspended to obtain OD_{595} 0.5, and 4 ml

of the cell suspension was gently vortexed for 10 s and incubated for 2 h at 37 °C. The supernatant was removed after incubation, and absorbance was measured at 595 nm using a UV–Vis Spectrophotometer. Autoaggregation assay was expressed in percentage using the following formula: $1 - (A_t/A_0) \times 100$, where A_t represents the cell suspension absorbance at time $t = 2$ h and A_0 for absorbance at t = 0.

Hemolytic Activity

Hemolytic activity was evaluated by streaking *Ent. faecium* BS5 on MRS blood agar plates and incubating for 48 h at 37 °C. Partial hydrolysis and a greenish zone (α-hemolysis), a clear zone around the colonies (β-hemolysis), or no reaction (γ-hemolysis) were observed for a hemolytic reaction [40].

Antibiogram

Antibiotic susceptibility of *Ent. faecium* BS5 was determined using antibiotic diffusion discs. *Ent. faecium* BS5 was inoculated in MRS broth and incubated for 24 h at 37 °C. Sample culture (100 µl) was spread on nutrient agar plates, and a disc dispenser was used to apply antibiotic discs to plates. The plates were incubated at 37 °C and observed after 24 h of inoculation. The antibiotics (Himedia) used were tetracycline (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), penicillin G (10 µg), streptomycin (300 µg), rifamycin (5 µg), kanamycin (30 µg), and erythromycin (15 µg). The diameter of the inhibition zone was measured (mm), and the antibiotic sensitivity was recorded based on their activity as different grades [41].

Antimicrobial Activity Assay

The antimicrobial activity of *Ent. faecium* BS5 was investigated against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Candida albicans* by the agar well diffusion method [42]. Isolate was grown overnight in MRS broth at 37 °C for 24 h, and the cell-free supernatant (CFC) pH was adjusted to 6.5 by adding 1 N NaOH. Briefly, aliquots of the supernatant in different volumes were loaded into wells in nutrient agar plates seeded with the indicator strain at a final concentration of 0.5% (v/v). The antibiotic disc was used as the positive control and water as the negative control. Then the plates were incubated at 37 °C for 24 h, and after incubation, the diameter of the inhibition zones around the well was measured.

Statistical Analysis

All experiments were conducted in triplicates with data expressed as mean ± standard deviation (SD). Results were tested for analysis of variance (ANOVA) using OriginPro 8.0 software to determine the statistical differences between the mean of the samples at the level of significance, $p < 0.05$.

Results and Discussion

Lactic Acid Bacteria from Dairy Products

The most interesting and practical group of bacteria for GABA production from dairy products are *L. s paracasei*, *L. plantarum* [43], and *L. lactis* [44], all of which possess special physiological activities and are generally regarded as safe. Therefore, using LAB as cell factories for GABA production offers a wide range of opportunities in the development of foods with potentially beneficial effects. In the present study, LAB was isolated from the dairy products and screened for GABA production and the highest GABA producing isolate was further assessed for its probiotic characteristics.

Screening of GABA Producing Isolates for Extracellular GABA Production Using TLC and HPTLC

Screening of different microorganisms capable of producing GABA is important for the food industry because this type of GABA production offers natural GABA, a bioactive agent that modulates the health features and provides the consumer with new and attractive food products. The 64 isolates selected from MRS agar plates were screened for extracellular and intracellular GABA production with 2% MSG. Based on TLC spots, 9 isolates were screened for the final TLC run that produced the dark spot indicating extracellular GABA compared with intracellular GABA production. Earlier, Kim et al. [45], Ko et al. [46], Lee et al. [47], and Villegas et al. [48] have reported the highest GABA production when its culture supernatants were analysed by TLC. Figure 1 shows the TLC profile of the supernatants obtained in the present study. The isolates with a visible band of GABA were DM5 (positive control), BS5, BS1, BS2, BS3, and BS18. Their Rf values range from 0.51 to 0.53, similar to that (Rf = 0.52) of the GABA standard. Significant production of GABA was shown with BS1, BS2, BS3, and BS5 compared with other samples after spot quantification, and hence, these isolates were chosen for further experiments.

In the previous research, Pradeep et al. [32] confirmed GABA from millets and legumes by HPTLC, and therefore, in our study, the culture supernatants of four isolates were subjected to HPTLC with the solvent system (1butanol:glacial acetic acid:water). The spectra of all the isolates and the standard are shown in Fig. 1. All the isolates showed a peak corresponding to the GABA

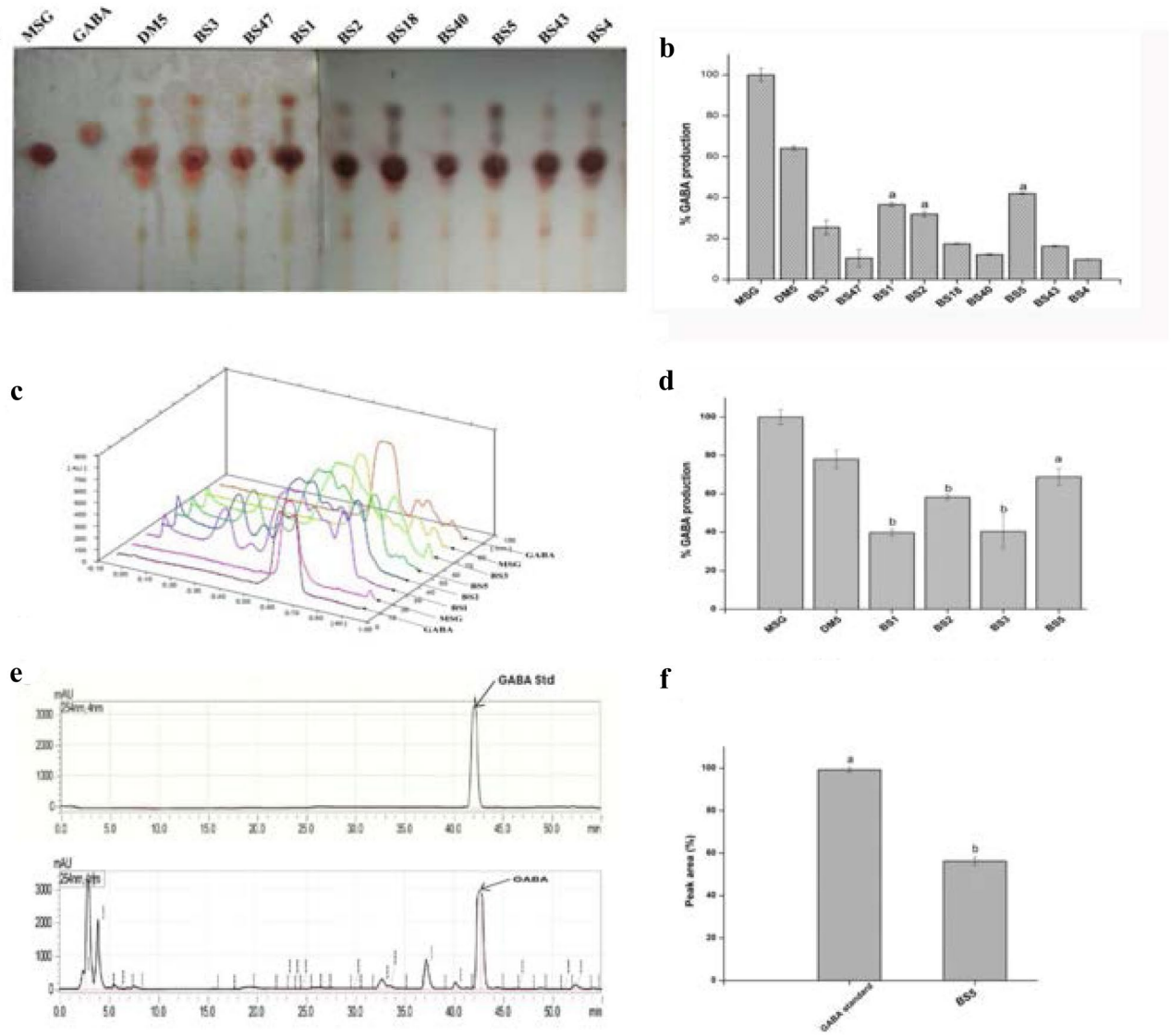

Fig. 1 **a** TLC chromatogram showing GABA spots produced by the dairy isolates. The development solvent consists of 1butanol:acetic acid:water (3:2:1, v/v/v). The chromatogram was viewed after spraying the plates with a 2% ninhydrin solution and developing at 105 °C for 5 min. **b** Estimation of the amount of GABA produced from MSG by the isolates for screening and selection of the best isolate. **c** High-performance thin-layer chromatogram of GABA standard (1 mg/ml) and cell culture supernatants. The mobile phase used was 1butanol:glacial acetic acid:water (3:2:1 v/v/v). The developed plate was viewed after spraying with 2% ninhydrin in acetone and developing at 105 °C for 5 min. **d** Determination of quantity of GABA produced by the selected isolates using MSG as the substrate. **e** HPLC profile of GABA standard (1 mg/ml) and supernatant from BS5 in MRS medium. Mobile phase: 80% solution A (1.4 mM sodium acetate, 0.1% triethylamine, and 6% acetonitrile) and 20% solution B (60% acetonitrile). The column was eluted at a flow rate of 1 mL/min for 50 min and detected at a wavelength of 254 nm. Data are representative of three independent experiments. **f** Comparison of GABA concentration in terms of area under peak between GABA standard and the isolate BS5. The data represent the mean ± SD of three independent experiments and one-way ANOVA was performed with $p < 0.05$

standard, with an Rf value of 0.60. After analyzing the area under the curve, we found that the highest production of GABA was achieved with BS5 and there was no significant difference in GABA production with BS1, BS2, or BS3.

Quantification of GABA by HPLC

The GABA contents of the isolate BS5 were quantified using HPLC (Fig. 1). It was observed that the HPLC chromatogram of the culture supernatant of the presumptive

GABA-producing isolate BS5 exhibits a peak that shows the same retention time (42 min) as that of GABA standard. The sample peak area was measured and compared with the calibration curve of the standard to quantify the GABA concentration of the isolate. In a similar study, Zmari et al. [49] confirmed GABA production in ethanolic extract (30 mg/ml) of germinated brown rice by HPLC whereby a retention time of 49 min was obtained. Tajabadi et al. [50] reported that *L. plantarum* Taj-apis 362 showed the highest GABA production (1.76 mM) as measured by HPLC.

General Phenotypic Characteristic of GABA Producing Bacteria

The highest GABA producing isolate BS5 was characterized based on morphological and biochemical analysis. The isolate BS5 formed creamy, circular, and opaque colonies on MRS agar plates and was seen as gram-positive cocci shape under the microscope. Further, the FESEM image of the isolate BS5 affirmed the cocci shaped morphology as shown in Fig. 2. Genetic identification by 16S rRNA sequencing of

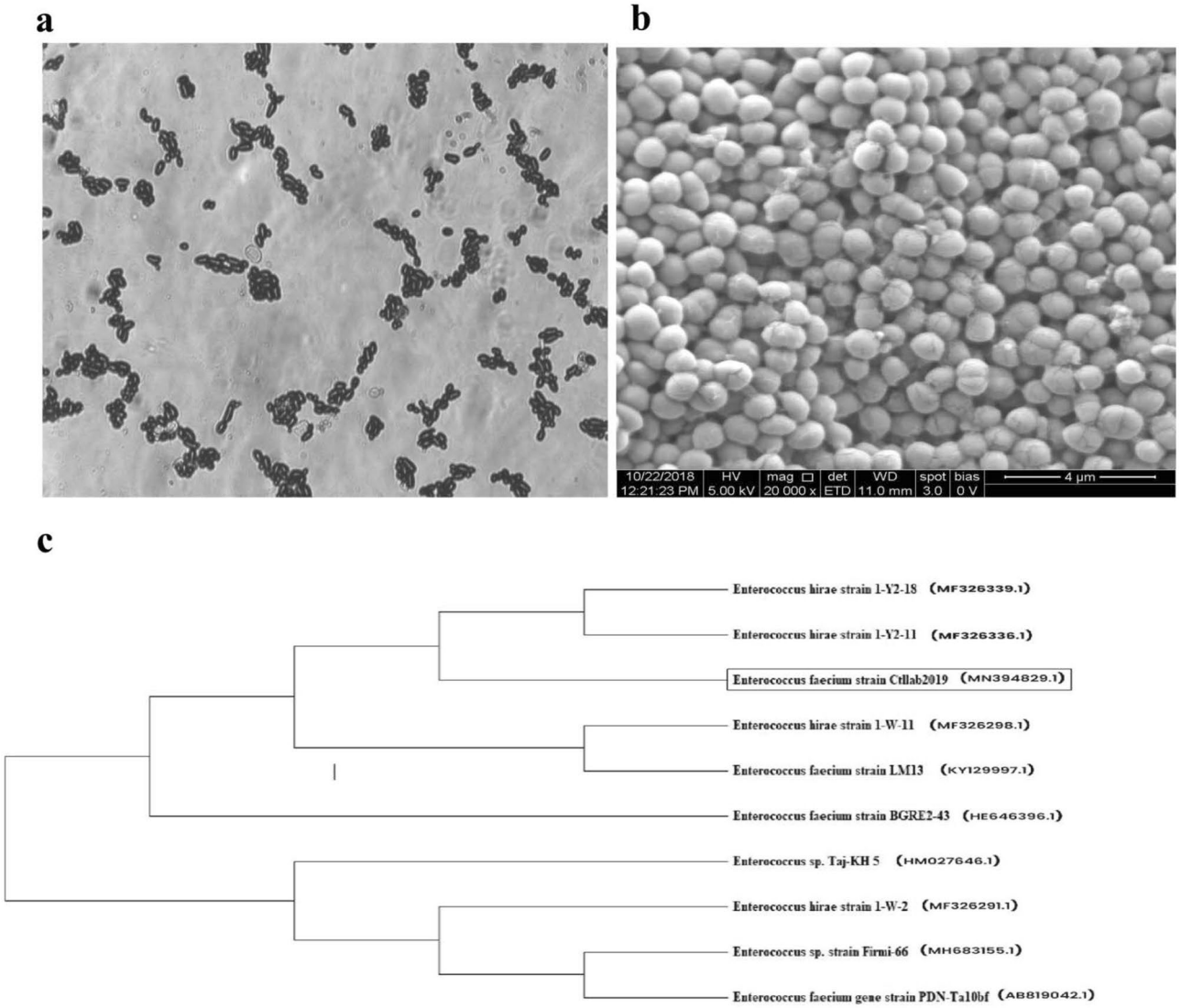

Fig. 2 **a** Morphology of the GABA producing isolate, BS5 viewed under 60X magnification of the phase-contrast microscope. **b** Representative FESEM of the isolate BS5 showing the cocci shape of the bacterium. **c** Phylogenetic tree analysis of the 16S rRNA gene sequence of the isolate *Ent. faecium* BS5 (NCBI Accession no. MN394829) using the neighbour-joining method. The bacterial isolate was named based on its alignment to the homolog bacterial species. The bacterial isolate identified in this study has been represented in the box

the isolate showed that BS5 belonged to the genus *Ent. faecium*. The partial sequences of *Ent. faecium* were submitted to the NCBI GenBank nucleotide sequence database, and the accession number MN394829 was obtained. The phylogenetic tree of the isolate BS5 (Accession no MN394829) with the most closely related *enterococci* was constructed using MEGA 7 software with neighbour-joining method (Fig. 2).

Evaluation of the Highest GABA Producing *Ent. Faecium* BS5 for Probiotic Attributes

The prerequisite of a good probiotic is its ability to harbour and colonize the intestinal tract. Microorganisms are subjected to a variety of stresses during transit in the gastrointestinal tract, such as the action of enzymes in the oral cavity, the acidic environment of the stomach, and high bile concentration in the intestine to colonize, and thus, probiotics must survive these stresses and exert health benefits [51]. The isolates must be able to resist an acidic gastric environment with bile salt-resistance and epithelial cell adhesion. Before being used as a probiotics, their adherence to the appropriate surface and survival in the gastrointestinal tract has to be confirmed in vitro [52]. Furthermore, their safety and other functional features such as antibiotic resistance and antimicrobial activities should also be checked before they are commercially explored for human consumption. In this study, the highest GABA-producing isolate *Ent. faecium* BS5 was selected for further evaluation of probiotic attributes. All these experiments were performed under in vitro conditions, and predicting the designated isolate *Ent. faecium* BS5 may also mimic it`s in vivo activity when fed to the consumer.

Tolerance to Low pH and Bile Salt

A probiotic must be tolerant to both bile and acid to benefit human health [53]. Stomach pH will rise to pH 3 if food is present, while in the stomach the pH of secreted HCl is 0.9 [54]. For entering into the small intestine through the stomach, probiotics need to survive at a pH value less than 3 [55]. Therefore, the ability of *Ent. faecium* BS5 to withstand acid stress at pH 1.0, 2.0, 3.0, and 4.0 was evaluated which showed the survival ability of *Ent. faecium* BS5 in the gastrointestinal tract. *Ent. faecium* BS5 showed the highest survivability of 76% and 78% in pH 3 and 4, respectively, after 3 h of incubation. The minimum survivability of 25% was observed in pH 2 and at pH 1; the isolate was not able to survive. Akalu et al. [56] have reported the survival rate (more than 80%) of *Lactobacillus* isolated from traditionally fermented Ethiopian beverage and food (*Shamita* and *Kocho*) at pH 2.5 and 3 for 3 and 6 h, respectively. Oh and Jung [57] reported that *Pediococcus* and *Lactobacillus* species isolated from Omegisool, a Korean traditional fermented millet alcoholic beverage can survive 3 h in pH 2 and pH 3.

Bile salt alters the structure of the cell membrane and is, therefore, harmful to living cells. Bile tolerance is a vital requirement for the survival and growth of LAB in the small intestine [58]. The viability percentage of cells with different concentrations of bile salt was carried out for 0, 3, and 6 h to determine the survival ability of *Ent. faecium* BS5 in the gastrointestinal tract. Two-way analysis of variance (ANOVA) revealed a significant difference in the survivability of *Ent. faecium* BS5 at 0, 3, and 6 h incubation at all concentrations of bile (Fig. 3). The survivability of *Ent. faecium* BS5 was significantly reduced at 10% bile compared with 2.5% bile. In conclusion, *Ent. faecium* BS5 was able to tolerate and survive in different concentrations of bile, in agreement with the earlier reports showing that the *Lactobacilli* and *pediococcus* possess a high tolerance to bile salt [59, 60] and the different rates of survival for *L. plantarum* strains indicate that survival of bacteria in the bile media is strain-dependent [61, 62].

Cell Hydrophobicity Assay and Autoaggregation Assay

The adhesion property can provide information about the ability of probiotics to colonize and may modulate the immunological system of the host. One of the factors that contribute to the adhesion of bacterial cells to host tissues is cell hydrophobicity [63]. Many mechanisms about the colonization of the gastrointestinal tract involving microbial adhesions and surface hydrophobicity have been identified. In vitro analysis of microbial hydrophobicity which participates in microbial adhesion to ethyl acetate and chloroform was performed to investigate probiotic bacteria. The results revealed that GABA producing *Ent. faecium* BS5 is found to adhere to ethyl acetate at 45.09% and chloroform at 43.42% (Fig. 3). Cell hydrophobicity is involved in microbial-microbial interactions and may help isolate *Ent. faecium* BS5 to maintain its residence in the GI tract.

Autoaggregation is a significant bacterial feature in human and animal mucosa and bacteria with aggregation ability could adhere better onto the intestinal cells. Previous research has reported that some *Lactobacillus* [64, 65] and pathogens like *Fusobacterium nucleatum* [66] exhibit aggregation ability. *Ent. faecium* BS5 showed a noteworthy aggregation of 48%, and these findings are supported by previous data demonstrating a medium degree of aggregation of *E. faecalis,* and *E. faecium* [67]. However, aggregation varies among microbial species, and adhesive properties that lead to aggregation could potentially influence intestinal colonization.

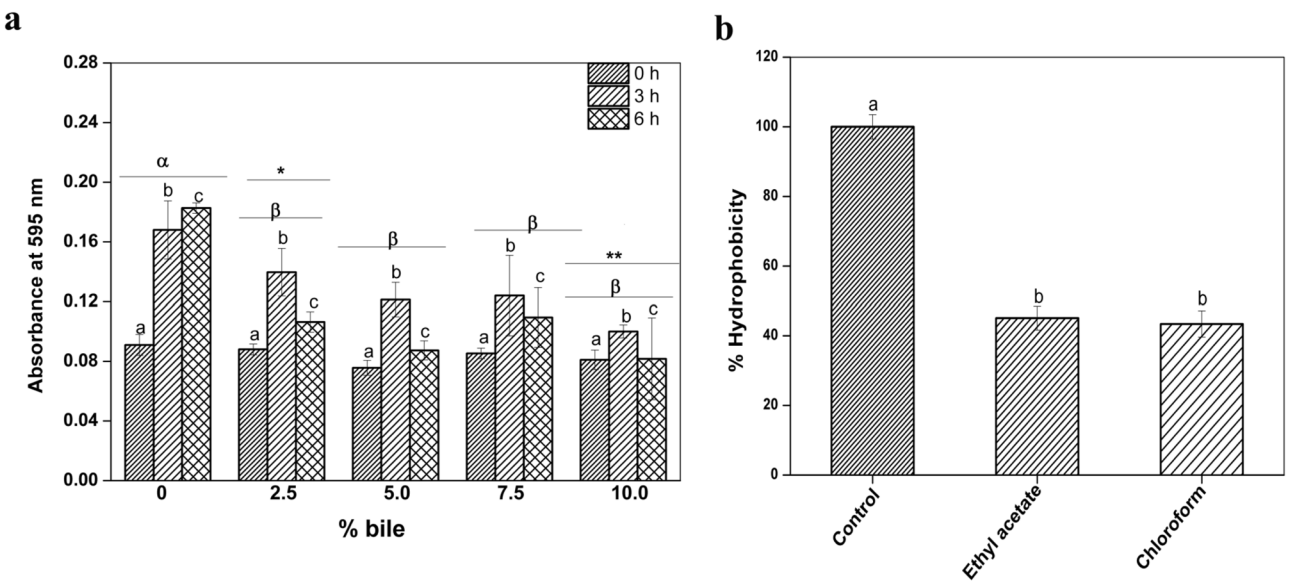

Fig. 3 **a** *Ent. faecium* BS5 showing bile salt tolerance grown in MRS broth supplemented with 0, 2.5, 5, 7.5, and 10% of bile. Growth was recorded by measuring the OD at 595 nm. **b** Cell surface hydrophobicity of *Ent. faecium* BS5 against ethyl acetate and chloroform. The data represent mean ± SD of three independent experiments. Two-way ANOVA for bile and one-way ANOVA for hydrophobicity were performed with $p < 0.05$

Hemolytic Assay

One of the significant safety criteria often used to evaluate potential probiotic strains is the hemolytic activity. In this study, a partial hydrolysis and greening zone (α -hemolysis), the clear zone around the colonies (β-hemolysis), and no reaction (γ-hemolysis) have been observed for hemolytic activity. *Ent. faecium* BS5 had shown no clear zone or greenish zone around their colonies on the blood agar plate which illustrates the non-hemolytic activity, and these results were following the earlier studies in which *lactobacilli* strains were shown to be nonhemolytic [68].

Antibiogram

If the use of antibiotics in medicines and foods eliminates probiotics, probiotics will no longer function [69]. So, survival in the presence of antibiotics is essential for probiotic strain. So we have investigated the tolerance of *E. faecium* BS5 against eight antibiotics and the results are shown in Table 1. *Ent. faecium* BS5 found to be resistant to erythromycin, kanamycin, streptomycin, ampicillin, and penicillin and sensitive to tetracycline, chloramphenicol, and rifamycin. These findings concur with the report by Zhou et al. [70] who claimed that *L. bulgaricus* and *S. thermophilus* strains tend to be strongly resistant to kanamycin, streptomycin, and ampicillin. According to a review by Mathur and Singh [71], different strains exhibit different levels of antibiotic resistance. Hence, due to its antibiotic resistance, *E. faecium* BS5 can survive in the environment containing high levels of antibiotic concentrations.

Antimicrobial Activity

Another desirable property of the selection of a suitable starter culture is antimicrobial activity against potential pathogens and spoilage organisms to ensure the development of healthy fermented foods [72]. Hence, the selected *Ent. faecium* BS5 isolate was examined for its antimicrobial activity using the agar-well diffusion method. CFS of *Ent. faecium* BS5 inhibited the growth of *E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* and *Ent. faecium* BS5 was inactive against *C. albicans* (Fig. 4). A similar result was reported by Bassyouni et al. [73] who showed that *Lactobacillus* species was able to inhibit *E. coli*, and *Staphylococcus sp.* Yuksekdag [74] found that LAB can inhibit the growth of pathogen and spoilage microorganisms through

Table 1 Antibiotic susceptibility and resistance pattern of the *Ent. faecium* BS5

Antibiotic	Concentration (μg)	Sensitivity (S)/resistance (R)
Tetracycline	30	S
Chloramphenicol	30	S
Ampicillin	10	R
Penicillin G	10	R
Streptomycin	300	R
Rifamycin	5	S
Kanamycin	30	R
Erythromycin	15	R

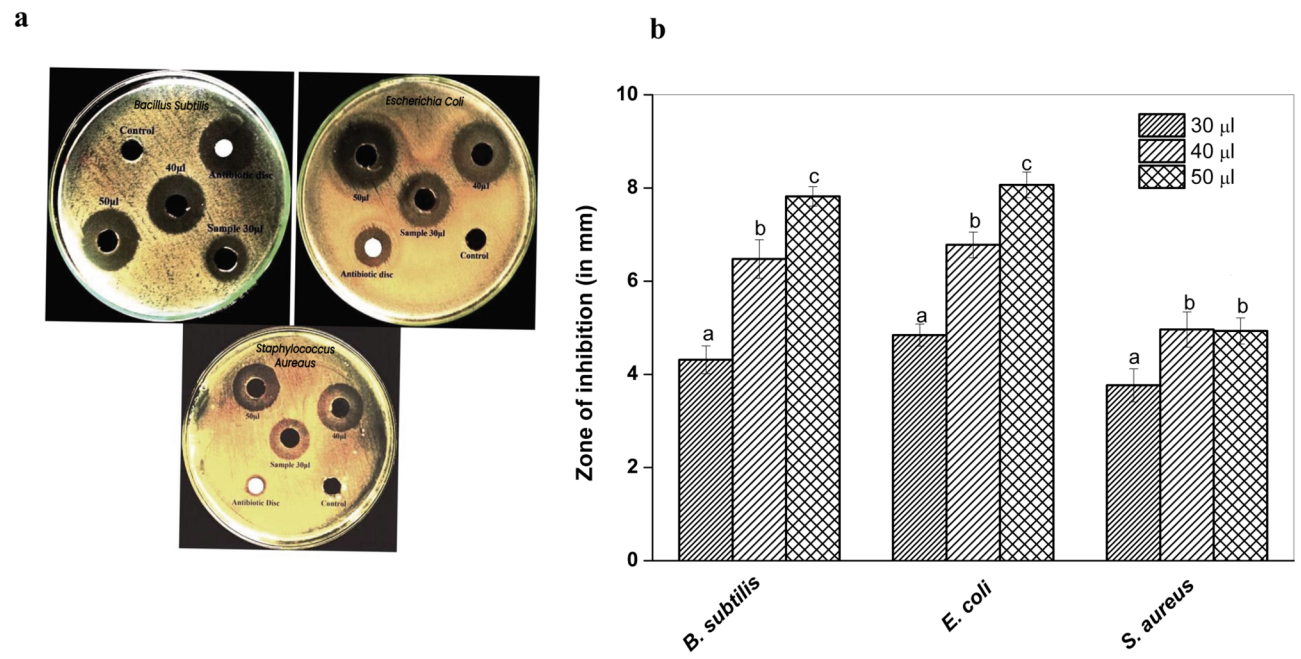

Fig. 4 **a** Antimicrobial activity of *Ent. faecium* BS5 against *B. subtilis*, *E. coli*, and *S. aureus* after 24 h of incubation at 37 °C. **b** Statistical analysis of zone of inhibition at different concentrations of the bacterial supernatant against test strains. The data represent mean ± SD of three independent experiments and one-way ANOVA was performed with $p < 0.05$

the production of antimicrobial substances such as organic acids, diacetyl, and hydrogen peroxide. Salminen et al. [75] also found that the ability to produce different antimicrobial compounds may be one of the important characteristics to prevent pathogen survival in the intestine and expression of a probiotic effect of a host.

Conclusion

Identification of strains with biochemical characteristics and a mode of action that can mediate specific health effects in the host underpins the scientific selection of the next generation of probiotic strains. Production of GABA functional foods or enriched foods capable of providing GABA is a key objective of research and development in the food industry. Furthermore, the isolation of GABA-producing strains from various fermented foods is an important natural method for functional food design. Such isolates can represent next-generation probiotics with a specific mode of action based on the potential gut: brain axis modulation and GABA producing ability.

Based on the knowledge of the beneficial effects of GABA and as confirmed by many studies, this paper reports the high capacity of gamma-aminobutyric acid production of nine isolates from dairy products, and the selected isolate BS5 was identified as *Ent. faecium* through biochemical tests and 16S rRNA sequencing. Our results recommend that *Ent. faecium* BS5 could be a good candidate as a probiotic starter culture strain in the food industry. Although in vivo investigations are needed, these preliminary findings show that the *Ent. faecium* BS5 can substitute chemical GABA with natural GABA. *Ent. faecium* BS5 also represents a promising next-generation probiotic and a good starter culture for the production of GABA rich cultured dairy products that can be used as a functional food.

Funding The second author received financial assistance from DST-WOS A(SR/WOS-A/LS-458/2018).

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human and Animal Participants The research does not contain any human or animal subjects for study.

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