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Probiotic Potential and Gluten Hydrolysis Activity of *Lactobacillus brevis* KT16-2

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Abstract

Celiac disease (CD) is a chronic autoimmune disease that occurs in genetically predisposed individuals. Gluten-hydrolyzing probiotic bacteria are promising for alleviating symptoms in individuals with CD. Therefore, in this study, the gluten hydrolysis ability and probiotic potential of *Lact. brevis* KT16-2 were determined. *Lact. brevis* KT16-2 formed proteolysis zones on gluten and gliadin agar plates, in which gluten and gliadin were used as the only nitrogen sources. SDS-PAGE analysis showed that *Lact. brevis* KT16-2 completely hydrolyzed peptides ranging from 28 to 66 kDa in 8 h. Then, the survival of the strain in bile salts, in simulated gastric juice and at low pH was determined. Additionally, the antioxidant and antimicrobial substance production, autoaggregation, hydrophobicity and antibiotic resistance of the strain were investigated. API-ZYM test kits were used to determine the enzymatic capacity of the strain. *Lact. brevis* KT16-2 had the ability to hydrolyze wheat gluten. It was able to survive in a broad pH range (pH 2–8), in bile salts (0.3–1%), and in simulated gastric juice. It had the ability to autoaggregate (59.4%), and the hydrophobicity (52.7%) of the strain was determined. In addition, this strain was capable of producing antimicrobial peptides against test bacteria, including antibiotic-resistant bacteria. Cell-free supernatants (CFS) of the strain had high antioxidant activity (DPPH-71.0% and ABTS-54.1%). The results of this study suggest that *Lact. brevis* KT16-2, which can hydrolyze gliadin and has many essential probiotic properties, has the potential to be used as a probiotic supplement for individuals with CD.

Keywords Celiac disease · Gluten · Lactic acid bacteria · Probiotics

Introduction

"Celiac disease (CD) is a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals" [1]. The main immunogenic component in gluten is gliadin peptides. The immune response caused by the digestion of gliadin leads to inflammation of the small intestine and destruction of villi in the intestinal walls. Due to atrophy of villi, the absorption of nutrients, vitamins, and minerals is impaired [2]. It has been reported that approximately 20 g/day gluten is consumed in the Mediterranean diet. Gluten is also found in products such as flavors, food dyes, emulsifiers, lipsticks, shampoos, toothpastes, lotions, brewer's yeast, ice creams, sweets, and confectionery [3]. At present, there is no method for the treatment of celiac patients. However, it is difficult to maintain a gluten-free diet since many ready-made foods contain cereal flours, even in small amounts, because of cross-contamination of foods, and inadequate food labeling [4]. Additionally, a strict diet that will last a lifetime negatively affects both the psychological and economical aspects of patients and reduces their quality of life. Gluten-free products are more expensive than products for regular consumer groups [5]. Various studies are underway to develop alternative or supplementary treatments for a gluten-free diet. Some of these alternatives include the use of probiotics and prolyl endopeptidase (PEP) and the suppression of tissue transglutaminase [6, 7].

Gluten consumption, microbiota dysbiosis, infections, and antibiotic use have been reported to be the primary triggers of CD. Probiotics, which breakdown gluten into non-antigenic peptides for susceptible individuals, are promising for both relieving/alleviating symptoms in individuals

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with CD and improving beneficial microbiota in the host intestine. In addition, probiotic use is thought to be the safest of the new treatment modalities recommended for CD individuals [8].

Studies on therapeutic use have demonstrated the ability of representatives of genus *Lactobacillus* to hydrolyze gluten, reducing its inflammatory effects [9–11]. The results obtained from animal models of CD have shown that probiotic bacteria have positive effects on disease pathology with different mechanisms of action [8].

Probiotics are described by the FAO/WHO [12] as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" and are considered safe [13]. Most of the bacteria used as probiotics are lactic acid bacteria (LAB). The term probiotic can be subcategorized into probiotic medicines, probiotic foods (foods, food components, and dietary supplements), direct-fed microbes, and genetically modified probiotics [14].

Probiotics release various bioactive metabolic products called "postbiotics" which are effective in modulating the host microbiome. They are effective in the modulation of the host microbiome [15]. However, the beneficial biological activities of a particular probiotic in the host may vary according to the host's age, gender, and physiological status [16]. Additionally, the positive effects of probiotic bacteria on host health are species-specific and even strain-specific [17, 18]. In other words, it can be said that each LAB strain is unique in terms of its probiotic properties. Therefore, in this study, the ability of the strain *Lact. brevis* KT16-2 isolated from an artisanal cheese to hydrolyze gluten and its potential as a probiotic were determined.

Materials and Methods

Identification of the *Lact. brevis* KT16-2 Strain by 16S rRNA Sequencing

The *Lact. brevis* KT16-2 strain used in this study was isolated from Kargı Tulum cheese as a part of our previous project. Genotypic identification of this strain was performed as follows. GeneMATRIX Bacterial & Yeast DNA isolation kit (EurX, Poland) was used for genomic DNA isolation according to the supplier's specifications. DNA quantity and quality were spectrophotometrically determined with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) device and confirmed by agarose gel electrophoresis. The gene regions targeted for species identification were amplified using universal 16S rRNA bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primers [19]. The PCR products were generated using an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 57 °C for 45 s, elongation at 72 °C for 1 min, and final extension at 72 °C for 5 min. A single-stage PCR procedure was performed to reproduce the approximately 1470 base region. PCR reaction was performed with FIREPol® Taq DNA polymerase enzyme (Solis BioDyne, Estonia). After PCR, a single band was obtained on agarose gel and it was confirmed that the PCR process was successful. The PCR product was purified by following the manufacturer's procedures with the ExoSAP-IT ™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) purification kit. For Sanger sequencing, the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit were used (Applied Biosystems). The readings obtained with the 27F and 1492R primers were contiguous to form a consensus sequence. CAP contig assembly algorithm was used in BioEdit software to perform this process. The obtained sequences were compared with those available in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information website (https://www.ncbi.nlm.nih.gov/).

Cell Suspension (*CS*) and Cell-Free Supernatant (*CFS*) Preparation from *Lact. brevis* KT16-2

Lact. brevis KT16-2 was inoculated (1% v/v) into De Man, Rogosa and Sharpe (MRS; Sigma-Aldrich, Darmstadt, Germany) broth (pH = 5.8) and incubated aerobically for 24 h at 37 °C. Then, the culture was centrifuged at 4400*g* for 10 min at 4 °C (Sigma, Shropshire SY4 5NU, UK), and the supernatant and cell pellet were collected. The CFS was then filtered through a 0.22-µm pore size filter (Sigma-Aldrich). This CFS was named *Lact. brevis* KT16-2-*CFS*. The *Lact. brevis* KT16-2 pellet was washed twice with physiological saline (PBS) by centrifugation (4400*g* for 10 min at 4 ℃), and the density was adjusted to 10^8 CFU/mL with PBS according to the 0.5 McFarland standard (BioMérieux, Marcy l'Etoile, France). This cell suspension was named *Lact. brevis* KT16-2-*CS*.

Gluten and Gliadin Hydrolysis Tests

Gluten hydrolysis with Gluten Agar (Glu-A): This test was performed twice using *Lact. brevis* KT16-2-*CS* or *Lact. brevis* KT16-2-*CFS*. Wheat gluten (G5004; Sigma-Aldrich) was used as the sole nitrogen source in Glu-A. Glu-A medium contained the following ingredients (Sigma-Aldrich): wheat gluten (9% w/v), glucose 2% (w/v), KH_2PO_4 1% (w/v), K_2HPO_4 1% (w/v), Tween 80 0.1% (v/v), and agar 1.5% (w/v), pH 6.2 [20]. In the first test, *Lact. brevis* KT16-2-*CS* (10^8 CFU/mL) was spotted (5 µL) on the surface of Glu-A and incubated at 37 °C for 48 h. At the end of the incubation period, the Glu-A plates were stained

with Coomassie Blue Brilliant R-250 dye (Sigma-Aldrich); 5 g/L Coomassie Blue Brilliant R-250 + 500 mL/L methanol + 92 mL/L acetic acid) for 1 min, and the excess dye was removed. After that, washing solution (250 mL/L ethanol, 50 mL/L acetic acid) was added to the Glu-A plates and allowed to stand at room temperature overnight. The presence of unstained zones showing gluten hydrolysis under and around the colony was evaluated as a positive result [21–23]. In the second test, *Lact. brevis* KT16-2-*CFS* was used, and a gluten hydrolysis assay was performed as described above.

Gliadin hydrolysis test with Gliadin Agar (Gli-A): Gliadin from wheat was purchased from Sigma-Aldrich (G3375) and used as the sole nitrogen source in Gli-A. A stock solution of gliadin at a concentration of 2 mg/mL was prepared in 60% ethanol and then used to prepare Gli-A and Gli-B at a final concentration of 0.2%. The components of Gli-A were as follows: gliadin (0.2% w/v), glucose 2% (w/v), KH_2PO_4 1% (w/v), K_2HPO_4 1% (w/v) (w/v), Tween 80 0.1% (v/v), and agar 1.5% (w/v), pH 6.2. KT16-2-*CS* (10^8 CFU/mL) was spotted (5 µL) on the surface of Gli-A and incubated at 37 °C for 48 h. At the end of the incubation period, the Gli-A plates were stained as described above in the gluten hydrolysis section with Coomassie Blue Brilliant R-250 dye. Then, the presence of a hydrolytic halo surrounding the colony was checked [24].

Two LAB strains that were isolated in our laboratory and never showed gluten hydrolysis before were used as negative controls in these tests. *Pseudomonas aeruginosa* possesses enzymes that cleave gluten peptides [25]; therefore, *Ps. aeruginosa* (ATCC 2783) was used as a positive control in tests performed with Glu-A and Gli-A plates. An uninoculated part of the plate served as a sterility control.

Gliadin hydrolysis test with Gliadin Broth (Gli-B): Gli-B contained the same components as Gli-A without the addition of agar. *Lact. brevis* KT16-2-*CS* (10^8 CFU/mL) was inoculated into tubes containing 10 ml of Gli-B and incubated at 37 °C for 48 h. At predetermined intervals (0, 2, 4, 8, 24, and 48 h), samples were taken, and supernatants were prepared and stored as described above until sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

SDS-PAGE

Gliadin hydrolysis was determined in supernatants from *Lact. brevis* KT16-2 cultured in Gli-B. SDS-PAGE (Mini Protean Tetra XL system; Bio-Rad, Hercules, CA, USA) was performed by the method of Laemmli [26] using a 5% stacking gel and 12% acrylamide gel. Aliquots of supernatant (20 µL) were diluted 1:1 with sample buffer and incubated at 100 °C for 5 min, and the supernatants were used to load the gels. The separating gel was run at a constant current of 200 V for approximately 3 h. The gels were stained with Coomassie Brilliant Blue R-250 for 24 h. A wide range of protein markers (mol wt, 6.5–205 kDa; Sigma-Aldrich) was used to estimate the molecular weight of the proteins on the gels.

Some *in vitro* Probiotic Characteristics of *Lact. brevis* KT16-2

Survival at different pH levels: Survival of *Lact. brevis* KT16-2 in acidic or alkaline medium was determined according to Elcioglu and Kunduhoglu [27]. Briefly, the pH of the MRS broth was adjusted to 1, 2, 3, 4, 5, 6, 7, or 8 with concentrated HCl or 5 M NaOH. *Lact. brevis* KT16-2-*CS* (10^8 CFU/mL) was added (10 µL) to each tube and incubated at 37 °C for 4 h. At 1-h intervals, a 20 µL sample was taken from each tube and then spotted onto the surface of MRS agar plate, and colony growth was checked. *Lact. brevis* KT16-2 grown in MRS broth (pH 5.8) was used as a growth control for survival tests.

Survival in bile: *Lact. brevis* KT16-2-*CS* (10^8 CFU/mL) was inoculated into MRS broth supplemented with 0.3, 0.5, or 1.0% (w/v) Ox-bile (70168; Sigma-Aldrich) and incubated for 6 h at 37 °C. At 1-h intervals, a 20 µL sample was taken from each tube and then spotted onto the surface of an MRS agar plate, and colony growth was checked [27].

Survival in simulated gastric juice: Simulated gastric juice was prepared by suspending pepsin (1000 U/mL) in 100 mL of 0.5% NaCl and adjusting the pH of the medium to 2.0 with concentrated HCl. *Lact. brevis* KT16-2-CS (10^8 CFU/mL) was inoculated into simulated gastric juice and incubated at 37 °C for 4 h [27].

Cell surface hydrophobicity: The *in vitro* cell surface hydrophobicity of *Lact. brevis* KT16-2 cells was measured spectrophotometrically (at 580 nm; Shimadzu, Kyoto, Japan) according to Tamang et al. [28]. The cell surface hydrophobicity percentage (H%) of *Lact. brevis* KT16-2 cells partitioning into n-hexadecane (Sigma-Aldrich) was calculated using the following equation:

$$H\% = [(A0 - A)/A0) \times 100],$$

where *A*0 and *A* are the absorbance values of the aqueous phase before and after contact with n-hexadecane, respectively. Cell surface hydrophobicity was considered weak at 0–20%, moderate at 21–50%, and strong at > 50%, as explained by Rosenberg and Gutnick [29].

Autoaggregation assay: Bacterial cells from an overnight culture were harvested by centrifugation (5000×*g*, 20 min, 4 °C) and washed twice with PBS (pH = 7.1). The absorbance (*A*600 nm) was adjusted to 0.25 to standardize the number of bacteria (10^7–10^8 CFU/mL). *Lact. brevis* KT16-2-*CS* was vortexed for 30 s, and the OD_{600} was measured at time 0 (*A0*) using a UV/visible spectrophotometer

(Shimadzu). Then, the OD of the same suspension was recorded (*At*) after resting for 4 h at 37 °C without vortexing. Autoaggregation percentage (AA%) was calculated as follows:

$$AA\% = (1 - At/A0) \times 100,$$

where At is the absorbance at 600 nm at 4 h and A0 is the absorbance at 600 nm at time 0 [30].

Safety Evaluation for *Lact. brevis* KT16-2

Deoxyribonuclease (DNase) production: *Lact. brevis* KT16-2 was inoculated onto DNase agar (Sigma-Aldrich) and incubated at 37 °C for 24–48 h. Then, plates were poured with 1 N HCl. The presence of transparent zones around the colony was evaluated as DNase-positive [31].

Hemolysin production: *Lact. brevis* KT16-2 was inoculated into sheep blood agar (Nutrient agar; Sigma-Aldrich, supplemented with 5% defibrinated sheep blood) and incubated at 37ºC for 24–48 h. The presence of clear-colorless zones around the colony was evaluated as β-hemolysis [32].

Coagulase production: A *Lact. brevis* KT16-2 colony was taken from blood agar and suspended in FTS drops on both ends of a slide. Rabbit plasma (Sigma-Aldrich) was then placed into one of these drops. The observation of agglutination within 5 s was a positive reaction. If agglutination was not seen within 2 min, the test result was negative [31].

Antibiotic Susceptibility Test

The disc diffusion method [33] was used to determine the sensitivity of the *Lact. brevis* KT16-2 strain to therapeutic antibiotics. Ten different antibiotics (Bio-Rad) were used in this study: streptomycin, erythromycin, gentamicin, chloramphenicol, tetracycline, rifampicin, amoxicillin, penicillin G, vancomycin, and nalidixic acid.

Enzymatic Profile of *Lact. brevis* KT16-2

To determine the enzymatic capacity of *Lact. brevis* KT16-2, API-ZYM kits (BioMérieux) were used according to the manufacturer's instructions.

Carbohydrate Fermentation Profile of *Lact. brevis* KT16-2

With an API 50 CHL test kit (BioMérieux), the ability of *Lact. brevis* KT16-2 to ferment 49 carbohydrates was determined. API 50 CHL kits were used according to the manufacturer's instructions.

Antimicrobial Activity of *Lact. brevis* KT16-2-*CFS*

In this assay, the antimicrobial peptide production potential of *Lact. brevis* KT16-2 was determined. The antimicrobial activity of *Lact. brevis* KT16-2-*CFS* was determined against some clinical pathogens and foodborne pathogens; these indicator test bacteria are listed in Table 4. To avoid the antimicrobial effects of organic acids and hydrogen peroxide; the pH of the *Lact. brevis* KT16-2-*CFS* was adjusted to 6.5 with 5 M NaOH and catalase (final concentration of 1 mg/mL; Sigma-Aldrich) was added. Then, the solution was filtered through a 0.22-µm pore size filter (Sigma-Aldrich). Catalase-free *Lact. brevis* KT16-2-*CFS* was used as the control series. The antimicrobial activity of CFS treated with pepsin (1 mg/mL, Sigma-Aldrich) and trypsin (1 mg/mL, Sigma-Aldrich) was also included in the study as a control group to show that the antimicrobial effect of the CFS in our study was due to protein compounds. An agar well diffusion assay (AWDA) was used to determine the antimicrobial activity of *Lact. brevis* KT16-2-*CFS* as described previously by Abanoz and Kunduhoglu [34]. *Listeria monocytogenes*, *Micrococcus luteus*, and *Ps. aeruginosa* were incubated at 30 °C, and other test bacteria were incubated at 37 °C for 24 h. The diameter of the clear inhibition zone around the well was measured.

Antioxidant Activity of *Lact. brevis* KT16-2-*CFS*

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (RSA) assay: The total free radical scavenging capacity of *Lact. brevis* KT16-2-*CFS* was estimated according to a previously reported method [35]. Briefly, 2.9 mL of freshly prepared DPPH (Sigma-Aldrich) solution (0.06 mM in methanol) was incubated with 0.1 mL of *Lact. brevis* KT16-2-*CFS* for 30 min in the dark, and the absorbance at 517 nm was measured. For the blank control, 2.9 mL of methanol was used in place of the sample. Uninoculated MRS broth served as a blank. The readings were recorded in triplicate, and the average absorbance value was calculated. Butylated hydroxytoluene (BHT; Sigma-Aldrich) and ascorbic acid (100 µg/mL; Sigma-Aldrich) were used as synthetic and natural standards. The percentage of RSA was calculated as follows [36]:

$$RSA(\%) = (1 - A\ sample\ /\ A\ control) \times 100,$$

where A control is the absorbance of the blank and A sample is the absorbance of *Lact. brevis* KT16-2-*CFS*

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) assay: The free radical scavenging activity of *Lact. brevis* KT16-2-*CFS* was determined by the ABTS radical cation decolorization assay reported by Re et al. [37]. This

assay was based on the ability of different substances to scavenge ABTS radical cations in comparison to a standard antioxidant. The radical solution was prepared mixing 7 mM ABTS (Sigma-Aldrich) with 2.45 mM potassium persulfate (1:1, v/v). Reaction mixture was prepared as: 0.9 mL of ABTS• + solution and 0.1 mL of CFS (sample), and measurements were taken at 734 nm after 1 min. BHT and ascorbic acid were used as standard antioxidants. The readings were recorded in triplicate, and the average absorbance value was calculated. The antioxidant activity was calculated by using the following equation [38]:

$$(\%) = ((AB - AA)/AB) \times 100,$$

where AB is the absorbance of ABTS radical + methanol and AA is the absorbance of ABTS radical + *Lact. brevis* KT16-2-*CFS*/standard.

Statistical Analysis

Experiments were replicated at least three times. One-way analysis of variance (ANOVA) was used to calculate standard deviations of the test results using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

The KT16-2 strain was subjected to amplification and sequencing of 16S rRNA; the KT16-2 strain showed 100% identity with the 16S rRNA sequences reported for *Lact. brevis* BP-1.4 (MF191679.1), indicating that this strain was *Lact. brevis.* The NCBI accession number of our *Lact. brevis* KT16-2 strain is MN334209.1. The KT16-2 strain used in this study was isolated from artisanal Kargı Tulum cheese as a part of our previous project. This result agrees with literature data in which *Lact. brevis* is the common LAB species found in the microbiota of fermented cheeses [39–42].

An agar spot test was performed to determine the gluten hydrolysis ability of *Lact. brevis* KT16-2 on Glu-A plates. The cell suspension (KT16-2-*CS*) obtained from an *Lact. brevis* KT16-2 culture was used as the source of the enzyme that hydrolyzes gluten. Gluten was added to Glu-A as the sole nitrogen source. Five microliters of *Lact. brevis* KT16-2-*CS* or *Lact. brevis* KT16-2-*CFS* (data not shown) was added to a Glu-A plate. After incubation at 37 °C for 48 h, Coomassie Brilliant Blue was used to stain proteins in the Glu-A plates. The areas of the plates where *Lact. brevis* KT16-2-*CS* and positive control *Ps. aeruginosa* (Fig. 1a) were added were not stained, and other areas were stained with Coomassie Brilliant Blue. While *Lact. brevis* KT16-2-*CS* formed a halo with a diameter of 15.6 mm, the positive control *Ps. aeruginosa* formed a halo with a diameter of 15.9 mm on Glu-A plate. These unstained areas are indicative of gluten hydrolysis [21]. Two LAB strains used as negative controls in this test showed good growth on Glu-A plates but did not show a hydrolysis zone. Similar results were obtained from the agar spot test performed with Gli-A. While the *Lact. brevis* KT16-2 strain formed a halo with a diameter of 13.4 mm, the positive control *Ps. aeruginosa* formed a halo with a diameter of 13.0 mm on a Gli-A plate (Fig. 1b).

In our study, the gliadin-hydrolyzing ability of the *Lact. brevis* strain KT16-2 was also determined in liquid medium using Gli-B. Gli-B was inoculated with *Lact. brevis* KT16-2-*CS* (10^8 CFU/mL) and incubated at 37 °C for 48 h. A LAB strain that did not hydrolyze gliadin served as a negative control strain. Samples were taken from the Gli-B culture (0, 2, 4, 8, 24, and 48 h); the supernatants of the cultures were obtained and used for SDS-PAGE. The electrophoretic profiles of the supernatants obtained from *Lact. brevis* KT16-2 (lanes 2–7) and the negative control strain (lanes 8 and 9) are shown in Fig. 2. Among the different gluten subunits, α-gliadins are considered the most immunogenic, while γ-gliadins and glutenins are much less responsible for gluten intolerance. Gliadins can be separated into α-, β-, γ-, and ω-gliadins with molecular weights ranging from 30 to 74 kDa, as determined by SDS-PAGE. The molecular weights of ω gliadins are between 52 and 74 kDa, while α-, β-, and γ-gliadins have lower molecular weights, ranging from 30 to 51 kDa [43]. According to this information, our supernatant samples from Gli-B contain α-, β- and γ-, ω-gliadins. Through SDS-PAGE analysis, six peptides with molecular weights ranging from approximately 28 to 66 kDa were observed in lane 2 (0 h supernatant of KT16-2) and lane 8 (0 h supernatant of the negative control). The *Lact. brevis* KT16-2 strain completely hydrolyzed peptides ranging from 28 to 66 kDa in 8 h incubation period (lanes 5–7). The peptide bands in lanes 8 and 9 remained unchanged, showing that the negative control strain was unable to hydrolyze these peptides.

The first step in determining the probiotic potential of microorganisms is to determine their resistance to various physiological barriers in the digestive tract. Depending on the type and quantity of food, digestion can take up to 2–4 h in the stomach (pH 1.0–2.5), 5–6 h in the small intestine (pH = 6.6 ± 0.5), and 12–24 h in the large intestine (pH 7.0 ± 0.7) [44–46]. In other words, the pH in the gastrointestinal environment ranges from pH 1 to pH 8 during the digestive process. Therefore, the survival of *Lact. brevis* KT16-2 at pH 1–8 was determined. *Lact. brevis* KT16-2 did not survive at pH 1. However, the survival of the cells at pH 2 and 3 for 4 h was almost close to that of the control cells at pH 2 and 3. *Lact. brevis* KT16-2 showed good survival at the level of the control series at pH = 4–8. Additionally,

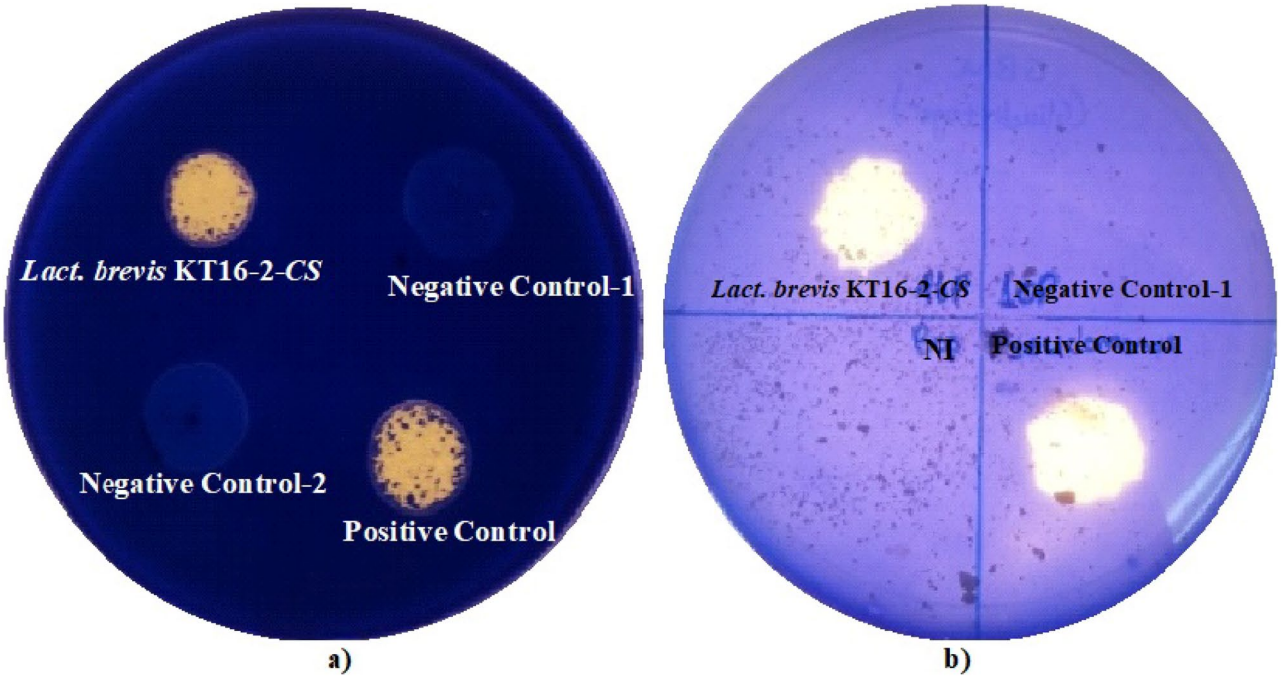


Fig. 1 Gluten and gliadin hydrolysis assays with Glu-A (**a**) and Gli-A (**b**) plates. Wheat gluten and gliadin were used as the sole nitrogen source in Glu-A/Gli-A. *Lact. brevis* KT16-2 cell suspension (*Lact. brevis* KT162-*CS*) was spotted on the surface of Glu-A/Gli-A and incubated at 37 °C for 48 h. At the end of the incubation period, Coomassie Brilliant Blue was used to stain proteins in the Glu-A/Gli-A plates. Evidence of gluten/gliadin hydrolysis is the presence of a transparent zone around the *Lact. brevis* KT16-2-*CS* after Coomassie Brilliant Blue staining. Two LAB strains that never showed gluten hydrolysis before were used as negative control strains (negative control-1 and negative control-2) in these tests. *Ps. aeruginosa* (ATCC 2783) was used as a positive control. An uninoculated part of the plate served as a sterility control (NI). **a** Gluten hydrolysis assay with Glu-A plate: there are hydrolysis zones formed by *Lact. brevis* KT16-2-*CS* and positive control strain. Although there was good growth observed with negative control strains inoculated areas on Glu-A plate, these strains did not form hydrolysis zones. **b** Gliadin hydrolysis assay with Gli-A plate: there are hydrolysis zones formed by *Lact. brevis* KT16-2-*CS* and positive control strain. However, there was no growth in negative control-1 strain on Gli-A plate

Lact. brevis KT16-2 maintained its stability at all bile salt concentrations tested in this study. In addition, the survival of *Lact. brevis* KT16-2 cells in simulated gastric juice at pH = 2 for 4 h was almost at a level close to that of the control series (Table 1).

Cell surface hydrophobicity and autoaggregation ability are essential parameters in determining the adhesion capacity of bacterial cells to the intestinal epithelium of the host and the potential for biofilm formation. It was determined that the cell surface of *Lact. brevis* KT16-2 was highly hydrophobic (52.7%), and the autoaggregation capacity was 59.4 after 4 h (Table 1).

The free radical scavenging activity of CFS obtained from *Lact. brevis* KT16-2 was tested by the DPPH and ABTS methods. The total free radical scavenging activity was determined to be 71.0% by the DPPH method. The percentage of scavenging ABTS radical cations was 54.0% (Table 1).

Microorganisms to be used as probiotics should not be virulent and should not carry resistance genes against antibiotics used in treatment. For this reason, virulence-related enzyme production of *Lact. brevis* KT16-2, such as DNase, coagulase, and hemolysin, was tested. As a result, it was observed that *Lact. brevis* KT16-2 does not produce these enzymes (Table 1).

In our study, the resistance of *Lact. brevis* KT16-2 to 10 different antibiotics was tested by the disk diffusion method [33]. *Lact. brevis* KT16-2 was found to be susceptible to most of the tested antibiotics except streptomycin, erythromycin, and nalidixic acid (Table 2).

The enzyme spectrum of *Lact. brevis* KT16-2 was found to be quite broad (Table 3). It also does not encode a carcinogenic enzyme, such as β-glucuronidase.

Bacteriocins are antimicrobial peptides synthesized ribosomally by bacteria and have been accepted as an essential criterion in the selection of probiotic strains [47]. Therefore, the antimicrobial effect of CFS obtained from *Lact. brevis* KT16-2 against various indicator test bacteria was determined. Catalase enzyme was added to the CFS of *Lact. brevis* KT16-2, and the pH was adjusted to 6.5 to eliminate the inhibitory effect of H_2O_2 and bacterial organic acids. Subsequently, the antimicrobial effect of *Lact. brevis*

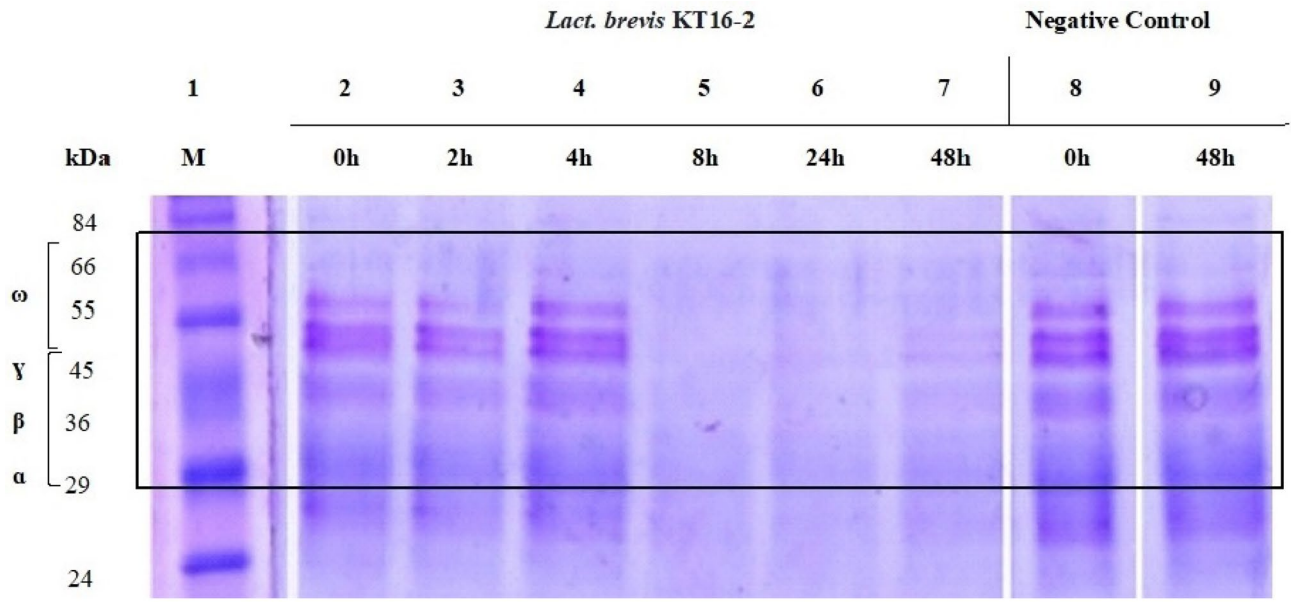


Fig. 2 SDS-PAGE profiles of gliadin peptides. Gliadin was used as the sole nitrogen source in gliadin broth (Gli-B). *Lact. brevis* KT16-2 was inoculated into Gli-B. Samples were taken from Gli-B culture at 0-, 2-, 4-, 8-, 24-, and 48-h intervals then their supernatants were prepared. Also, one LAB strain that was never showed gliadin hydrolysis before in Gli-A plates served as negative control. The electrophoretic patterns of gliadins showed that the intensity of the bands between 52–74 kDa and 30–51 kDa (shown inside the rectangle), related to the ω gliadins and α-, β-, and γ-gliadins [43], respectively, decreased (lanes 5–7). *Lact. brevis* KT16-2 completely hydrolyzed these peptides in 8-h incubation period (lanes 5–7). However, the peptide bands in lanes 8 and 9 remained unchanged. Lane 1, molecular weight marker; lanes 2–7, supernatants of *Lact. brevis* KT16-2; lanes 8 and 9, supernatants of negative control

Table 1 Resistance to different physiological barriers and other probiotic characteristics of *Lact. brevis* KT16-2

Probiotic characteristics of *Lact. brevis* KT16-2			
Survival tests:			
	Survival at different pH levels (for 4h)	pH 1	–
		pH 2	++
		pH 3	++
		pH 4 - 8	+++
		Control*	+++
	Survival at bile salts for 6h	0.3%	+++
		0.5%	+++
		1.0%	+++
		Control*	+++
	Survival in simulated gastric juice (for 4h)	pH 2	++
		Control*	+++
Cell surface hydrophobicity (%)			52.7±1.4
Auto-aggregation (%)		1h	48.3±1.3
		2h	51.1±0.9
		4h	59.4±0.5
DPPH scavenging activity (%)			71.0±2.9
ABTS scavenging activity (%)			54.1±1.5
Virulence factors:			
	DNase production		np
	Coagulase production		np
	Hemolysin production		np

+++ good growth, ++ slightly weak growth, + weak growth, – no growth, *np* not produced

**Lact. brevis* KT16-2 grown in MRS broth (pH = 5.8), control for survival tests.

Table 2 Antibiotic susceptibility of the *Lact. brevis* KT16-2 strain determined with the disk diffusion assay

Mode of action	Antibiotics	Results
Protein synthesis inhibitors	Streptomycin (10 µg/disk)	R
	Erythromycin(15 µg/disk)	R
	Gentamicin (10 µg/disk)	S
	Chloramphenicol (30 µg/disk)	S
	Tetracycline (30 µg/disk)	S
	Rifampicin (5 µg/disk)	S
Cell wall synthesis inhibitors	Amoxicillin (25 µg/disk)	S
	Penicillin G (10 µg/disk)	S
	Vancomycin (30 µg/disk)	S
Nucleic acid synthesis inhibitor	Nalidixic Acid (30 µg/disk)	R

Resistant according to the CLSI breakpoints [33]

S susceptible, *I* intermediate, *R* resistant

Table 3 Enzymatic profiles of *Lact. brevis* KT16-2 scanned with the API-ZYM test kit

Enzymes		Enzymes	
Esterase (C–4)	1	Phosphohydrolase	1
Esterase lipase (C–8)	0	α-Galactosidase	0
Lipase (C–14)	0	β-Galactosidase	4
Leucine aminopeptidase	0	β-Glucuronidase	0
Valine aminopeptidase	3	α-Glucosidase	3
Cystine aminopeptidase	2	β-Glucosidase	1
Trypsin	2	N-Acetyl-β-glucosaminidase	0
Chymotrypsin	0	α-Mannosidase	0
Alkaline phosphatase	1	α-Fucosidase	0
Acid phosphatase	2	Control	0

0; 0 nmol, 1; 5 nmol, 2; 10 nmol, 3; 20 nmol, 4; 30 nmol, 5; ≥ 40 nmol

KT16-2-*CFS* was tested against a variety of gram-positive and gram-negative bacteria, including methicillin and/or vancomycin-resistant bacteria. As a result, *Lact. brevis* KT16-2-*CFS* showed antimicrobial effects against gram-negative test bacteria such as *Escherichia coli* and *Ps. aeruginosa* and gram-positive test bacteria such as *Staphylococcus aureus*, *M. luteus*, *L. monocytogenes*, *Enterococcus faecium*, and *Bacillus cereus*. It also showed antimicrobial activity against methicillin-resistant *Staph. epidermidis* and methicillin- and vancomycin-resistant *Staph. warneri* strains (Table 4). As a result, the CFSs treated with pepsin and trypsin enzymes lost their antimicrobial activity. The results of these experiments suggest that the compound (s) responsible for antimicrobial activity was in protein structure.

The carbohydrate fermentation profile of *Lact. brevis* KT16-2 was determined with an API 50CHL test kit. The strain was able to ferment 25 of the 49 types of carbohydrates tested (Table 5). These include nondigestible carbohydrates such as D-mannose, mannitol, sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, and arbutin. It was also able to ferment D-lactose and inulin.

Discussion

Gluten susceptibility is one of the most common food intolerances, with a global prevalence of approximately 5% and an increasing incidence [48]. The most immunogenic gluten peptide for celiac patients is proline- and glutamine-rich 33-mer peptides that are resistant to enzymatic proteolysis. Gluten-free nutrition forms the basis of CD. However, the complete removal of gluten from the diet is difficult due to the ubiquity of this protein complex, cross-contamination of foods, inadequate food labeling practices, and social constraints [48–50]. Gut dysbiosis is also known to directly or indirectly contribute to CD. Probiotics are thought to affect individuals with CD by three potential mechanisms. The first is to eliminate and/or reduce the causative agent of CD by hydrolyzing gluten proteins into small nonimmunogenic polypeptides, thereby preventing its onset. Second, probiotics protect the intestinal barrier by preventing the access of immunogenic polypeptides to the lamina propria. The third and most interesting is the role of the intestinal microbiome in homeostasis and regulation of both internal and adaptive immune systems [51].

Various *in vitro* and *in vivo* studies have been conducted to evaluate the effects of the use of probiotics on the prognosis of CD; these studies supported the addition of probiotics to the gluten-free diet because of their beneficial or preventive effects [52–56]. Probiotics in the intestine have inhibitory effects on the negative microbiota that causes intestinal dysbiosis due to various biological activities. For example, probiotics produce inhibitory agents against target pathogens, block their adhesion sites, compete for prebiotics and other nutrients, and degrade toxin receptors [51, 57]. Based on this information, in our study, the ability of the *Lact. brevis* KT16-2 strain to hydrolyze gluten and its probiotic potential were investigated using *in vitro* methods.

Lact. brevis KT16-2 showed good growth on Glu-A/Gli-A medium containing wheat gluten/gliadin as the only nitrogen source and hydrolyzed gluten and gliadin. In addition, according to SDS-PAGE analysis, it can be said that *Lact. brevis* KT16-2 is able to hydrolyze about 2 g/L gliadin within eight hours. Similar studies have reported that lactobacilli, including *Lact. brevis*, can hydrolyze gluten. The *Lact. brevis* strain 1Hd was isolated from Italian wheat sourdoughs by Di Cagno et al. [10], who found that this strain has peptidase enzyme activity. Gerez et al. [20] reported

Table 4 Antimicrobial activity of *Lact. brevis* KT16-2-***CFS*** with the agar well diffusion assay

	Inhibition zone diameter (in mm)		
Indicator strains	KT16-2-*CFS*	KT16-2-*CFS* + pepsin	KT16-2-*CFS* + tripsin
E. coli (LMG 8223) [a]	15±0.6	–	–
E. coli O157:H7 (ATCC 35150) [b]	–	nt	nt
Ps. aeruginosa (ATCC 2783) [b]	12±0.8	–	–
Salmonella sp. [d]	–	nt	nt
Staph. aureus (ATCC 25923) [b]	14±1.2	–	–
M. luteus (NRRL 1018) [c]	16±1.3	–	–
L. monocytogenes (LMG 13305) [a]	15±1.4	–	–
Ent. faecium (NRRL 2354) [c]	16±0.5	–	–
B. cereus (LMG 8821) [a]	12±1.1	–	–
*B. subtilis (*NRRL NRS 744) [c]	–	nt	nt
Staph. epidermidis [e] *(*methicillin-resistant)	20±1.2	nt	nt
Staph. warneri [e] (methicillin and vancomycin-resistant)	20±1.0	nt	nt
Staph. aureus [e] (methicillin and vancomycin-resistant)	-	nt	nt
Staph. haemolyticus [e] (methicillin and vancomycin-resistant)	-	nt	nt
Staph. epidermidis [e] (methicillin and vancomycin-resistant)	-	nt	nt
Staph. hominis [e] (vancomycin resistant)	-	nt	nt

- no inhibition, *nt* not tested

[a]BCCM/LMG: Bacteria Collection, Laboratorium Voor Microbiologie-Universiteit Gent, Gent-Belgium

[b]ATCC: American Type Culture Collection, Manassas, VA 20110-2209, USA

[c]NRRL: Agricultural Research Service, Culture Collection, Peoria. IL 61604, USA

[d]Obtained from Eskisehir Osmangazi University, Faculty of Medicine, Eskisehir-Turkey

[e]Obtained from Anadolu University, Faculty of Science, Eskisehir-Turkey

Table 5 Carbohydrate fermentation profile of *Lact. brevis* KT16-2 scanned with an API 50 CHL test kit

Carbohydrates utilization of *Lact. brevis* KT16-2					
Glycerol	–	Inositol	–	Inulin	+
Erythritol	–	D-mannitol	+	D-melezitose	+
D-arabinose	–	D-sorbitol	+	D-raffinose	+
L-arabinose	+	Methyl-alpha-D-mannopyranoside	+	Amidon (starch)	–
D-ribose	+	Methyl-alpha-D-glucopyranoside	–	Glycogen	–
D-xylose	+	N-acetylglucosamine	+	Xylitol	–
L-xylose	–	Amygdalin	+	Gentiobiose	–
D-adonitol	–	Arbutin	+	D-turanose	+
Methyl-beta-D xylopyrano-side	–	Esculin ferric citrate	+	D-lyxose	–
D-galactose	+	Salicin	+	D-tagatose	+
D-glucose	+	D-cellobiose	+	D-fucose	–
D-fructose	+	D-maltose	+	L-fucose	–
D-mannose	+	D-lactose (bovine origin)	+	D-arabitol	–
L-sorbose	–	D-melibiose	+	L-arabitol	–
L-rhamnose	–	D-saccharose (sucrose)	+	Potassium gluconate	–
Dulcitol	–	D-trehalose	+	Potassium 2-ketogluconate	–
				Potassium 5-ketogluconate	–

+ fermented, – not fermented

that the *Lact. brevis* strain could hydrolyze 135–9 kDa gluten peptides at rates ranging from 100 to 49.9%. In another study, the amount of gluten in sourdough fermented with a microbial mixture, including the *Lact. brevis* 14G strain, was found to decrease from 74.6 to 20.3 ppm after 48 h of fermentation (at 37 °C) [58]. De Angelis et al. [59] investigated the ability of VSL#3 commercial probiotic preparation to hydrolyze gliadin polypeptides during dough fermentation. As a result of SDS-PAGE analysis, they determined that VSL#3 (10^9 CFU/ml) completely hydrolyzed alpha 2-gliadin-derived epitopes at the end of 24-h fermentation.

In the next stage of our study, some probiotic properties of *Lact. brevis* KT16-2 were tested. The first features to be tested in the selection of probiotic bacteria are their survival at low acidity of the stomach and whether they are resistant to the levels of bile salt in the small intestine. The pH of the empty stomachs of healthy people ranges from 1 to 3, depending on the amount of food or liquid consumed, and may increase to pH 6 [59]. The pH profiles of the duodenum, small intestine, terminal ileum, cecum, and rectum were 2.0–3.0, 6.0, 6.0–7.4, 5.7, and 6.7, respectively [60]. Therefore, the bacteria to be used as probiotics must survive over a wide pH range. In our study, *Lact. brevis* KT16-2 was able to maintain viability for 4 h in simulated gastric juice (pH 2) and MRS broth, pH adjusted to 2–8. Bile salts released into the small intestine can breakdown the lipids and fatty acids of bacterial cell membranes, damaging them [61]. Therefore, the tolerance of LAB to bile salt is an important criterion affecting their probiotic potential. We determined that *Lact. brevis* KT16-2 survived at 0.3 and 1.0% bile salt over 6 h. In a similar study, 5 strains of *Lact. brevis* were determined to survive at pH 2.5 and 0.3% bile salt for 4 h [62]. In another study, the *Lact. brevis* LAP2 strain was reported to survive at pH 3.0, gastric juices (pH 3 and 4, for 3 h), and in bile salts (0.3% w/v, 24 h) [63].

The cell surface hydrophobicity of a probiotic is associated with its adhesion ability [64, 65]. In our study, *Lact. brevis* KT16-2 showed high affinity for n-hexadecane, and the cell surface hydrophobicity was 52.7%. The autoaggregation capacity was 59.4% (at the end of 4 h). In a similar study, Abouloifa et al. [66] tested the hydrophobicity, and autoaggregation capacities of 5 *Lact. brevis* strains, and the ranges of 15.1–34.7% and 12.1–30.5% were observed, respectively. Jang et al. [67] reported that the *Lact. brevis* KU15153 strain showed a high affinity for chloroform; its hydrophobicity was 69.25%, and its autoaggregation ability was 21.44%. Vasiee et al. [68] reported that the hydrophobicity percentage of four *Lact. brevis* strains was between 28 and 76%.

Bacteria to be selected as probiotics should not have β-glucuronidase enzyme activity, which is associated with the induction of toxins, mutagens, and carcinogenesis [69]. Increases in β-glucuronidase in feces are thought to be associated with gastric cancer and inflammatory bowel disease [67]. In our study, *Lact. brevis* KT16-2 was not found to have β-glucuronidase enzyme activity. In addition, the β-galactosidase enzyme hydrolyzing lactose was found at high levels. Similar to our study, the enzymatic profile of the *Lact. brevis* S82 strain was determined, and it was determined that while the aminopeptidase, naphthol-AS-biphosphohydrolase, β-galactosidase, and β-glucosidase enzyme activities were high, β-glucuronidase enzyme activity was not present [66]. *Lact. brevis* KT16-2 has β-galactosidase and gluten-hydrolyzing enzyme activity, and the absence of β-glucuronidase increases its probiotic potential. In addition, the API 50CHL carbohydrate fermentation analysis showed that *Lact. brevis* KT16-2 has the ability to metabolize a number of nondigestible sugars and inulin, which are used as prebiotics.

Lact. brevis KT16-2-*CFS* showed an antagonistic effect on gram-positive (*Staph. aureus*, *M. luteus*, *L. monocytogenes*, *Ent. faecium*, and *B. cereus*) and gram-negative (*E. coli* and *Ps. aeruginosa*) bacteria. Furthermore, it also exhibited antimicrobial activity against methicillin-resistant *Staph. epidermidis* and methicillin- and vancomycin-resistant *Staph. warneri*. The loss of antimicrobial activity of CFSs treated with pepsin and trypsin showed that the substances responsible for antimicrobial activity are in protein structures. It can be concluded that *Lact. brevis* KT16-2 produces peptide or peptides with antimicrobial effect against test bacteria. Similar to our findings, the *Lact. brevis* FPTLB3 strain has been reported to produce bacteriocin-like antimicrobial peptides against *E. coli* MTCC 1563, *Ent. faecalis* MTCC 2729, *Lact. casei* MTCC 1423, *Lact. sakei* ATCC 15521, and *Staph. aureus* ATCC 25923 [70]. In a study by Gautam and Sharma [71], the *Lact. brevis* MTCC 7539 strain was found to exhibit antimicrobial activity against foodborne pathogens such as *Staph. aureus* and *L. monocytogenes.* Although bacteriocins are generally sensitive to proteolytic enzymes, some low molecular weight peptides have been reported to be resistant to various proteases [72]. It has been reported that resistance to proteases may result from the presence of cyclic peptides containing unusual amino acids in the active site of the bacteriocin [73]. Additionally, the activity of enzymes varies with exposure time, concentration, temperature, and pH. Furthermore, the type of enzyme and substrate specificity determine its ability to hydrolyze a protein [74]. Some studies have demonstrated that strains with proteolytic activity produce antimicrobially effective peptides. For example, Bacteriocin (BAC-IB17) produced by *B. subtilis* KIBGE-IB17 has been found to be antimicrobial against methicillin-resistant *Staph. aureus* strains. This bacteriocin remained stable against a variety of proteolytic enzymes (protease, proteinase-K, amylase, pepsin) but lost 50% of its activity after 2 h [72]. Singh et al. [75] reported that *Pediococcus pentosaceus* IE-3 produced

a low molecular weight antimicrobial peptide with broad spectrum antimicrobial activity resistant to proteases. Atasanova et al. [76] determined that *Lact. delbrueckii* subsp. *lactis* b38 and b24 strains with very high proteolytic activity produced peptides with antimicrobial effect against *Ent. aerogenes* and *E. coli*, respectively. García-Cano et al. [77] screened 137 LAB strains for their proteolytic and antibacterial activities. It was determined that 7 of 137 strains showed high proteolytic activity. Supernatants obtained from these strains showed antimicrobial effect against at least one of the test bacteria such as *E. coli*, *L. innocua*, *Staph. aureus*, and *Staph. epidermidis*.

The DPPH and ABTS scavenging capacities of *Lact. brevis* KT16-2-*CFS* were 71% and 54%, respectively. In similar studies, different antioxidant activity capacities were determined with different strains. Jang et al. [67] reported the DPPH radical scavenging and β-carotene bleaching inhibitory activity of *Lact. brevis* KU15153 strain, and the values of 44.1% and 71.6% were observed, respectively. Abouloifa et al. [66] reported that the DPPH and radical scavenging activities of 5 *Lact. brevis* strains were between 43.1 and 52.5%. For the lipid peroxidation and free radical scavenging activities of the *Lact. brevis* MG000874 strain, a high inhibition of 69.4–86.6% was observed [78]. It has been reported that factors such as cell wall composition, enzyme activity, and metabolite production are effective on antioxidant activity, and therefore, antioxidant capacity is strain-specific [67]. As a result, *Lact. brevis* KT16-2 is thought to be promising as a probiotic that can be used in the prevention of inflammation or inflammation-related problems. However, these results should be supported with *in vivo* assays.

Lact. brevis KT16-2 was tested for virulence factors such as DNase, coagulase, and hemolysin by phenotypic assays. These results indicated that this strain did not exhibit DNase, coagulase, or hemolytic activity. Similarly, Hasali et al. [79] reported that none of the 5 tested *Lact. brevis* strains showed β-hemolytic activity, whereas one strain showed α-hemolytic activity. In another study, Abouloifa et al. [66] reported that the *Lact. brevis* S82 strain did not have DNase and hemolytic activity. However, these phenotypical assays do not guarantee that the bacterium does not carry the specified virulence genes in its genome. Therefore, these results should be supported by further genotypic assays.

Recently, it has been reported that some LAB isolated from fermented foods carry resistance genes to antibiotics such as tetracycline, erythromycin, and vancomycin [80]. Probiotic bacteria should not carry antibiotic resistance genes that can be transferred horizontally to other microorganisms in the gastrointestinal microbiota. In our study, *Lact. brevis* KT16-2 was sensitive to most of the antibiotics tested, such as gentamicin, chloramphenicol, tetracycline, rifampicin, amoxicillin, penicillin G, and vancomycin, but resistant to streptomycin, erythromycin, and nalidixic acid. Therefore, the genetic determinants of antibiotic resistance of this strain should be determined before it is recommendation as a probiotic for human consumption.

Conclusion

Lact. brevis KT16-2 was able to hydrolyze gluten and gliadin. Additionally, *Lact. brevis* KT16-2 has many essential probiotic characteristics, such as the ability to survival at low pH, in bile salts and in simulated gastric juice. The cell-free supernatant of *Lact. brevis* KT16-2 showed antimicrobial activity against some foodborne and clinical pathogens used in this study. Additionally, it had antioxidant activity and showed high autoaggregation capacity and hydrophobicity. It has been concluded that *Lact. brevis* KT16-2, which can hydrolyze gliadin and has many basic probiotic properties, has the potential to be used as a probiotic supplement for individuals with CD. Supporting these features with *in vivo* experiments will be the subject of our future work.

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Compliance with Ethical Standards

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

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