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ORIGINAL ARTICLE

Evaluating the antibacterial, antibiofilm, and anti-toxigenic effects of postbiotics from lactic acid bacteria on *Clostridium difficile*

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ABSTRACT

Background and Objectives: The most common cause of healthcare-associated diarrhea is *Clostridium difficile* infection (CDI), which causes severe and recurring symptoms. The increase of antibiotic-resistant *C. difficile* requires alternate treatments. Postbiotics, metabolites produced by probiotics, fight CDI owing to their antibacterial capabilities. This study aims to evaluate the antibacterial, antibiofilm, and anti-toxigenic potential of postbiotics in combating CDI.

Materials and Methods: GC-MS evaluated postbiotics from *Bifidobacterium bifidum* and *Lactobacillus plantarum*. Disk diffusion and broth microdilution determined *C. difficile* antibacterial inhibition zones and MICs. Microtiter plates assessed antibiofilm activity. MTT assay evaluated postbiotics anti-viability on HEK293. ELISA testing postbiotic detoxification of toxins A and B. Postbiotics were examined for *tcdA* and *tcdB* genes expression using real-time PCR.

Results: The most identified *B. bifidum* and *L. plantarum* postbiotic compounds were glycolic acid (7.2%) and butyric acid (13.57%). *B. bifidum* and *L. plantarum* displayed 13 and 10 mm inhibition zones and 2.5 and 5 mg/ml MICs against *C. difficile*. *B. bifidum* reduced biofilm at 1.25 mg/ml by 49% and *L. plantarum* by 31%. MTT assay showed both postbiotics had little influence on cell viability, which was over 80%. The detoxification power of postbiotics revealed that *B. bifidum* decreased toxin A and B production more effectively than *L. plantarum*, and also their related *tcdA* and *tcdB* genes expression reduction were statistically significant ($p < 0.05$).

Conclusion: Postbiotics' ability to inhibit bacterial growth, biofilm disruption, and toxin reduction makes them a promising adjunctive for CDI treatment and a good solution to pathogens' antibiotic resistance.

Keywords: *Clostridium difficile*; Biofilm; Postbiotics; Probiotics; Toxicity

INTRODUCTION

An alarming worldwide health issue is the emergence of infections caused by *Clostridium difficile*, a Gram-positive spore-forming anaerobe bacterium, that is the causative agent of several antibiotic-associated diarrheal diseases, which are induced by treatment with antibiotics or by disruption of the normal gastrointestinal flora (1). These syndromes are collectively known as *C. difficile* infections (CDI), and include pseudomembranous colitis, and toxic megacolon, and may lead to chronic infection, shock, and death (2). Colon infection caused by *C. difficile* may be very dangerous, particularly in older individuals

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and in patients who have an imbalance in their gut microbiota due to exposure to antimicrobial drugs (3). The life cycle of this microorganism is impacted by antimicrobial drugs, the human immune system, and the host microbiota and its related metabolites (4).

The most prevalent clostridial toxins, namely toxin A and toxin B, are the primary agents that are responsible for generating symptoms in CDI (5). Both toxins are cytotoxic and proinflammatory, cause the actin cytoskeleton to disintegrate and tight junctions in human intestinal epithelial cells to degenerate. This results in the big intestine suffering serious damage and collecting of liquids (6). The *tcdA* and *tcdB* genes, situated inside a pathogenicity locus known as the PaLoc and span a distance of 19.6 kb, are responsible for encoding these chemicals, which this specific genetic region has three other additional genes including *tcdR*, *tcdC*, and *tcdE*, which are responsible for the production and release of toxins from the cell (7). Additionally, both toxin A and toxin B are cytotoxic and pro-inflammatory, which disrupt the actin cytoskeleton and impair tight junctions in human intestinal epithelial cells, which ultimately leads to fluid collection and significant damage to the large intestine (8). It has been shown in more recent times that the toxins are responsible for the release of inflammatory cytokines from mast cells and macrophages, in addition to epithelial cells, which ultimately results in further fluid secretion and inflammation of the digestive tract (9).

It is interesting to note that in addition to causing damage to the digestive tract, toxin B is also cardiotoxic and has the potential to induce bleeding in the lungs, which suggests that this toxin may have more overall effects than was previously believed (10). CDI is characterized by several symptoms, which the most prominent are diarrhea, inflammation, and tissue death, that caused by the toxins, which led to complicated chain of reactions to take place inside the host cells (11). To conduct exact epidemiological analysis, as well as to effectively implement management and preventive measures, accurate identification of toxins is completely necessary (12).

Conventional antibiotic treatments are successful against several bacterial diseases; nevertheless, they often make the dysbiosis of microorganisms that are already present in the body worse (13). This may lead to repeated infections caused by *C. difficile* as well as the spread of microorganisms that are resistant to treatments (14). Because of the detrimental effects of antibiotic usage in the process of generating sickness, as well as the rising resistance of this microorganism to medications and the developing issue of antibiotic resistance in this bacterium, researchers are looking into novel ways to combat this microorganism (15, 16). One of the innovative ways that shows promise is the use of postbiotics, which have shown that they have the potential to be effective in the fight against infections (17, 18).

Postbiotics have gained the interest of academics as a new strategy to combat antibiotic resistance. These chemicals are either components of the structure or byproducts of the action of probiotics, which are beneficial microorganisms and include wide range of bacteria such as lactobacilli and bifidobacteria, which are Gram-positive, non-spore-forming rods and yeasts such as *Saccharomyces boulardii* and *Saccharomyces cerevisiae* that provide several health advantages for the body when taken in enough quantities (19, 20). Host immune function, intestinal barrier integrity, and microbial composition are all influenced by these bioactive compounds, which have different physiological effects on the host (21). Postbiotics therefore offer attractive prospects for medical treatment in various disease situations, including CDI, among many other conditions. To effectively treat CDI, postbiotics have many different and complex mechanisms of action, which contribute to their effectiveness (22, 23). These bioactive compounds have been shown to directly inhibit the growth of *C. difficile* as well as its negative consequences, thereby hindering its ability to establish itself and produce toxins. Postbiotics can prevent the pathogenic effects of this pathogen by neutralizing its toxins (24). They do this by affecting the various pathogenic mechanisms that this pathogen uses, such as the formation of biofilms, altering the expression of genes that produce toxins, and the toxins themselves to detoxify the toxins and control this microorganism (25). Therefore, the purpose of the present study was to investigate and validate the efficiency of postbiotics derived from *L. plantarum* and *B. bifidum* against toxigenic *C. difficile*, as well as evaluate the safety of postbiotics as a novel and alternative therapy option for patients with CDI.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The pathogen used in the present study was *C. difficile*

ATCC43255, obtained from the American Type Culture Collection. At the same time, the probiotics employed were *Bifidobacterium bifidum* and *Lactobacillus plantarum*, which were utilized for the production of postbiotics. The microorganisms were cryopreserved at a temperature of -80°C. *C. difficile* was sub-cultured by spreading a large amount of the frozen culture onto the brain heart infusion-supplemented (BHIS) agar (Merck, Germany) supplemented with 5 µg/ml yeast extract, 10 µg/ml sodium taurocholate, 0.1% (w/v) L-cysteine, and 8 µg/ml cefoxitin and incubated in anaerobic condition at 37°C for 24 h. At the same time, the probiotics were cultured in Man Rogosa Sharpe (MRS) agar medium (Merck, Germany) at 37°C for 24 hours in a CO_2 incubator and these fresh cultures were then employed in the tests.

Preparation of postbiotics. An aliquot of the *B. bifidum* and *L. plantarum* cultures obtained from the frozen stock was introduced into the falcon-containing MRS broth (Merck, Germany) containing 0.6% yeast extract (Sigma-Aldrich). The cultures were then incubated overnight at 37°C under shaking conditions. Following the incubation, the cultures were centrifuged at 6000 rpm at 4°C for 10 minutes. The supernatants were then sterilized by passing through a filter with a pore size of 0.22 µm (Millipore Inc., Billerica, USA). The resulting supernatants were then divided into aliquots and stored at a temperature of -20°C until they were used in subsequent assays (26).

GC-MS analysis of postbiotics. The GC-MS analysis of postbiotics derived from *B. bifidum* and *L. plantarum* and contents was conducted utilizing Shimadzu QP-5050 apparatus and a Gas Chromatograph GC-17A coupled with an HP-5 capillary column (phenylmethyl siloxane, 30 m 0.25 mm internal diameter) and a mass spectrometer ranging from 50 to 600 m/z. As the carrier gas, helium was utilized at a split ratio of 1:30 and a flow rate of 1 ml/min. The specified operating temperatures for the injector and detector were 250 and 280°C, respectively. The column's temperature was anticipated to increase linearly at a rate of 5°C/min from 60°C to 250°C, where it would remain for ten minutes. The retention indices were calculated by making use of the retention durations of the n-alkanes that were injected before the sample injection, all the while ensuring that the chromatographic conditions remained the same. The retention durations of the n-alkanes that were injected into the injection were employed to calculate the retention indices for samples that were going to be injected under identical chromatographic circumstances. After comparing the mass spectra acquired from Willey (nl7) and the Adams Library, the contents were identified and studied using gas chromatography/mass spectrometry, and identification and analysis took place after the comparison (27, 28).

Determination of inhibition zone using disk diffusion method. To determine whether or not the postbiotic extract has antibacterial properties against *C. difficile*, the disk diffusion method was used. *C. difficile* with 0.5 McFarland turbidity was lawn on Mueller-Hinton agar (Merck). Sterile blank disks with a diameter of 6 millimeters were thoroughly saturated with 150 µl of postbiotics extract with 10 mg/ml concentration. The disks were then placed on the surface of Mueller-Hinton agar plates previously cultured with *C. difficile*. Additionally, for the control and comparison, blank, vancomycin, and metronidazole disks were integrated into the experiment. After that, the plates were cultured anaerobically at 37°C for 24 h. Following the designated incubation period, the plates were inspected to determine the presence or absence of inhibition zones around the disks. To determine the diameter of the inhibitory zones, a calibrated ruler was used for measuring. The presence of inhibition zones served as evidence of antibacterial activity, with bigger zones suggesting a greater degree of efficacy of postbiotics against the studied pathogen (29).

Determination of minimum inhibitory concentration (MIC). The MIC of the postbiotics extract against *C. difficile* was determined using the broth microdilution technique. This approach included utilizing 96 well microplates and Mueller-Hinton broth medium. A series of dilutions of postbiotics ranging from 10 to 0.039 mg/ml were prepared. Subsequently, a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL) of *C. difficile* was introduced into each well and subjected to a 48-hour incubation at 37°C in an anaerobic environment. The MIC was determined by observing each well for the presence or absence of turbidity. This allowed us to identify the lowest concentration of the postbiotics extract that inhibited 99% of the bacterial inoculum, indicating the microorganism's growth inhibition. The positive control used in the experiment was Vancomycin. The sub-

MICs are used to evaluate the range of effectiveness and efficiency of the postbiotics against pathogens for genomic analysis and the prevention of biofilm formation (30).

Effect of postbiotics on toxin A and toxin B production using ELISA. The *C. difficile* was subcultured in a BHI medium and placed in an environment without oxygen, where it was kept at a temperature of 37°C for 5 days. The concentration of toxins was quantified in the supernatants collected after centrifugation of the cells at 6000 rpm for 20 minutes and the supernatant containing toxins was passed through a 0.22 µm pore size filter to remove cells and debris. The levels of toxin A and B were assessed using a commercially available ELISA kit (*C. DIFFICILE* TOX A/B IITM, TechLab, USA), following the directions provided by the manufacturer. To assess the impact of postbiotics on the synthesis of toxin A and toxin B by *C. difficile*, the pathogen was grown in a BHI medium with and without two different sub-MIC concentrations of the postbiotics. After incubating the culture anaerobically for 24 hours, the supernatants were collected and centrifuged. The mentioned ELISA kit was then utilized to quantify the production of toxins A and B by *C. difficile*. The amount of toxin production was compared before and after the addition of postbiotics. The ELISA spectrophotometer was calibrated using air as a reference at a wavelength of 620 nm, and the OD was then measured at 450 nm. The toxin A and B generation by *C. difficile* was quantified as a percentage of the OD450 values for the filtrates of cultures treated with postbiotics and those left untreated and ultimately, the percentage of toxins production analyzed before and after postbiotics addition (31-33).

Effect of postbiotics on expression of *tcdA* and *tcdB* genes. The extraction of total RNA from *C. difficile* was performed using the RNXplus kit (SinaClon, Iran). Following precipitation, bacterial cells were dissolved in 1 ml of ice-cold RNXplus solution, which contains Guanidinium and phenol. Then, 200 ml of chloroform was added and the mixture was gently shaken. The resulting mixture was maintained on ice for 15 minutes and subsequently centrifuged at 12000 rpm at 4°C for 15 minutes. The liquid phase was moved to a new tube and an equal amount of isopropanol was added. After gently mixing and incubating on ice for 15 minutes, the mixture was spun at 12000 rpm at 4°C for 15 minutes. The liquid on top was discarded, and the remaining material was washed with 1 ml of 75% Ethanol. The mixture was then spun at 7500g, and the solid material at the bottom was dried and dissolved in 25 ml of Diethylpyrocarbonate (DEPC) water (SinaClon, Iran). The quality of RNA was assessed by determining the OD260/280 ratio, which should be greater than 1.9. Additionally, gel electrophoresis was performed. The DNAse treatment was performed by introducing 5 units of DNAse enzyme (DNAbiotech, Iran) and allowing it to incubate at a temperature of 37°C for 1 hour. The process was halted by the addition of EDTA and further incubation at a temperature of 65°C for 10 minutes. The cDNA synthesis was performed using an easy cDNA synthesis kit)Parstous, Iran). 1 ml of RNA was combined with 2 ml of random primers and 5 ml of DEPC water. The mixture was then incubated at a temperature of 70°C for 5 minutes. Afterward, the mixture was transferred to a cold environment. Subsequently, 4 ml of 5X reaction buffer, 20 U of Ribonuclease inhibitor, 2 ml of dNTP (10 mM), and 200 U of MMulv RT enzyme (Fermentas, Germany) were added to the mixture. The resulting mixture was then incubated at a temperature of 42°C for 60 minutes. The reaction was halted by incubating at a temperature of 70°C for 10 minutes. The process of Real-time PCR was conducted using allele-specific primers obtained from Bioneer, a company based in Korea. The particular details of the primers used seen in Table 1. The RT-PCR experiment was conducted using the Rotor-Gene 3000 instrument (Corbett Research, Australia) and analyzed with Real-Time Analysis Software Roter-Gene Version 6.1 (Corbett Research, Australia). The real-time PCR amplifications were conducted using 20 ml reactions containing 2X QuantiTect SYBR Green PCR master mix (Takara, Japan). This master mix includes HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, 2.5 mmol/L $MgCl_2$, deoxyribonucleotide triphosphate (dNTP) mix, and fluorescent dyes. Additionally, the reaction mixture consisted of RNase-free H_2O (Sigma-Aldrich, Germany), 0.6 Mmol/L primer (Bioneer, Korea), and 2 ml of the respective template DNA dilution. The experiments were conducted twice. The RNA quantities were quantified using a NanoDrop NDe1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RT-PCR experiment was optimized with an initial activation step at 95°C for 3 minutes, followed by 40 cycles of de-

Table 1. Primers used for Real-time PCR assay

Gene	Primers sequence	Expected amplicon size (bp)
tcdA	F: 5´ AATGATGTTACCTAATGCTCCTTC 3´	311
	R: 5´ AGTAAGTTCCTCCTGCTCCATC 3´	
tcdB	F: 5´ CCAGCTAATACACTTGATGAAAACC 3´	432
	R: 5´ TTTCTTCACCTTCTTCATTTCCT 3´	
23SrRNA	F: CCTATCGGCCTCGGCTTAG	100
	R: AGCGAAAGACAGGTGAGAATCC	

naturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds for the *tcdA* and *tcdB* genes, extension at 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes. The normalization of all the results was achieved by utilizing a housekeeping gene, specifically 23srRNA. The expression of *tcdA* and *tcdB* genes was evaluated and compared before and after adding postbiotics to demonstrate genomic-level detoxification of these metabolites (34, 35).

Effect of postbiotics on biofilm formation. The impact of postbiotics on the formation of biofilms by *C. difficile* was assessed using a microtiter plate assay. The experiment was conducted using a 96-well polystyrene microtiter plate (SPL Life Sciences). A suspension was formed by combining 90 µl of Tryptic Soy Broth (TSB) (Sigma-Aldrich) and 10 µl of bacterial suspension with an optical density (OD600) of 0.10 in each well. This resulted in a concentration of 10^7 colony-forming units per milliliter (CFU/ml) in each well. Within each well, a volume of 100 µl of postbiotics was administered at sub-MIC doses. Subsequently, the plate was put in an incubator under anaerobic conditions at a temperature of 37°C and a carbon dioxide concentration of 5% for 24 hours. The *Pseudomonas aeruginosa* strain ATCC 22924 was used as a positive control to assess biofilm development. The negative control, referred to as the blank control, consisted of TSB mixed with 0.5% glucose. In addition, *C. difficile* is employed as a control to compare the effects of postbiotics before and after their inclusion. After the incubation period, the liquid culture was cautiously extracted, and each well underwent three cycles of rinsing with normal saline solution at room temperature. After 20 minutes of fixation in 150 µl of methanol, the biofilms were discarded. To facilitate the drying process, the wells were placed upside down for around 30 minutes. After being left at room temperature for 15 minutes, the biofilms were treated with 150 µl of crystal violet (Ghatran Shimi) solution in water (0.1%). Subsequently, the biofilms were rinsed with slow-flowing tap water to remove any residue. After positioning the microtiter plates on tissue paper, they were allowed to air-dry (36).

Cytotoxic potential of postbiotics. Based on the results of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT test (Sigma-Aldrich) performed on the normal cell line human embryonic kidney 293 (HEK293) obtained from the Cell Bank at Razi Vaccine and Serum Research Institute in Iran, the effects of the postbiotics with concentrations of 0.3125, 0.625, 1.25, 2.5, and 5 mg/ml of each postbiotic on the viability of the cells were evaluated. At a density of 7×10^3 cells per well, the cells were planted on a 96-well culture plate in Roswell Park Memorial Institute (RPMI 1640) (Gibco) 10% media. This was done to attain 80% confluency before the addition of postbiotics. The postbiotics were then filtered through 0.22 µm membranes. For HEK293 treatments, the cells with a volume of 180 µl were subjected to a treatment with 20 µl of postbiotics, which was performed in triplicate. Using the MTT test, the cells were processed after being subjected to a treatment for forty-eight hours at a temperature of 37°C. We did not include postbiotics in the group that served as the control. The control group and the postbiotic-treated group were both subjected to a treatment of 10 µl of MTT at a concentration of 5 mg/mL. Following this, the cells were cultivated in a CO_2 incubator for 4 hours. In the subsequent step, the cell culture fluid was replaced with 1% DMSO, and the 96-well plate was examined using an ELISA plate reader (StatFax 2100) at a wavelength of 570 nm. At a wavelength of 570 nm, the absorbance of formazan dye was determined with the use of an ELISA plate reader (37). It was determined that the rate of cell viability may be calculated using the following equation:

$$\text{Cell viability (\%)}: \frac{\text{OD postbiotic treated}}{\text{OD control}} \times 100$$

Statistical analysis. The data were presented in the form of the mean ± standard deviation. The data underwent one-way analysis of variance (ANOVA) utilizing SPSS software (version 26). The Duncan test was employed to determine if there were significant differences between the means at a 95% confidence level ($P < 0.05$). The procedures were conducted in triplicate or more.

RESULTS

Analysis of postbiotics compounds using GC-MS. The GC-MS analysis of the postbiotics of *L. plantarum* and *B. bifidum*, gained the major constituents of postbiotics, shown in the Table 2. Succinic acid is a dicarboxylic acid that has attracted the attention of researchers in recent years due to its antibacterial properties, and due to its presence in probiotic bacteria, it has multiplied the importance of studying these microorganisms (38). The antibacterial activity of succinic acid comes from its ability to disrupt essential microbial processes and cell structures, among which we can mention the disruption of cell membrane integrity, in which succinic acid can penetrate the bacterial cell membrane and cause disturbance in the integrity of the membrane and disturb the cell homeostasis and finally cause the death of the pathogen (39). This acid can also interfere with the metabolic pathways necessary for the growth and survival of bacteria and prevent the proliferation of pathogens by inhibiting enzymes involved in energy production and biosynthetic processes (40). In the same way as succinic acid, benzoic acid is a simple aromatic carboxylic acid that possesses considerable antibacterial characteristics. Because of the low pH, benzoic acid is mostly found in its undissociated form, which makes it easier for it to diffuse across the membranes of bacteria and degrades once when reaches the cell, causing the pH of the intracellular space to decrease and interrupting important functions that are carried out by pathogens (41). Pyrrolo[1,2-a]pyrazine-1,4-dione derived from the postbiotic of *L. plantarum*, a compound that can stop DNA gyrase from working, which is an important enzyme for replication and transcription of DNA in bacteria. This would stop bacteria from making DNA and growing. This substance also stops bacteria from making peptidoglycan, which breaks the cell wall of the bacteria and makes it more likely to be killed by osmotic stress (42).

Inhibition zone in *C. difficile* growth by postbiotics. The effect of postbiotics activity against *C. difficile* was determined by measuring the inhibition zone diameter that surrounds the disk containing postbiotics of *B. bifidum* and *L. plantarum.* A smaller or nonexistent inhibition zone implies that there is either very little or no antimicrobial activity of postbiotics, while a bigger inhibition zone indicates that there is a higher amount of antimicrobial activity that postbiotics show. When postbiotics from *B. bifidum* and *L. plantarum* were added, there was an increase in the width of the inhibitory zone in comparison with the control group, which was a blank disk with no postbiotics, which no inhibition zones were seen. Also, the antibiotic disks of vancomycin and metronidazole were used as a positive control, which produced inhibition zones around the disks. The diameter of inhibition zones around the disks containing the postbiotics of *B. bifidum* and *L. plantarum* were 13 and 10 mm against *C. difficile*, respectively, which showed that the antibacterial effects of postbiotics derived from *B. bifidum* are stronger than *L. plantarum* (Fig. 1).

MIC analysis of postbiotics against *C. difficile*. The MIC values of postbiotics were determined using the microdilution technique, and the results differed based on the specific postbiotics being studied. The MIC values of each postbiotic are crucial for investigating the mechanism of action of these products and their capacity to hinder the development of biofilms at sub-MIC concentration thresholds. The MIC of

Table 2. The most obtained metabolites identified in postbiotics using GC-MS

No.	*B. bifidum*	Percentage (%)	*L. plantarum*	Percentage (%)
1	Glycolic acid	7.2	Butyric acid	13.57
2	Succinic acid	6.7	Hydroxyacetone	5.3
3	Stearic acid	6.35	Lactic acid	5.11
4	Butyric acid	4.9	Benzoic acid	2.41
5	5-Aminovaleric acid	3.18	Pyrrolo[1,2-a] pyrazine-1,4-dione	1.7

Fig. 1. Zones of inhibition resulting from disk diffusion test of postbiotics against *C. difficile*

postbiotics generated from two distinct probiotics, *B. bifidum* and *L. plantarum*, against *C. difficile*, are 2.5 mg/ml and 5 mg/ml, respectively. The findings indicate that *B. bifidum* had more efficacy in suppressing the pathogen compared to the other one, and was capable of inhibiting *C. difficile* at a lower dose.

Toxin production alteration. ELISA was used in this experiment to determine the quantity of toxins A and B that were generated by *C. difficile* before and after the addition of two different sub-MIC concentrations of the postbiotics including 1.25 and 0.625 mg/ml for *B. bifidum* and 2.5 and 1.25 mg/ml for *L. plantarum.* A comparatively high concentration of toxins A and B was seen in the control group without adding postbiotics depending on high OD. Following the addition of postbiotics derived from *B. bifidum* and *L. plantarum*, there was a reduction in the concentration of both toxins A and B as compared to the control. Postbiotic treatment, led to a reduction in the generation of toxins A and B by *C. difficile*, as shown by the lowering of toxin concentrations that were seen during the experiment, which highlights the potential of these probiotics as therapeutic agents for the treatment of *C. difficile* infections. The results show that the postbiotic of *B. bifidum* had more ability to lower the both toxin A and B production power of the pathogen in comparison with *L. plantarum.* These results also show that both postbiotics are more effective against toxin B in comparison with toxin A (Fig. 2).

Alteration of the expression of *tcdA* and *tcdB* genes after postbiotics addition. The real-time PCR assay was done to investigate the effect of sub-MIC concentration of postbiotics for the *tcdA* and *tcdB* genes responsible for the production of toxins A and B of *C. difficile*. After adding both postbiotics obtained from *B. bifidum* and *L. plantarum*, the relative expression level of both genes decreased, which was statistically significant (P-value < 0.05). And also the effect of *B. bifidum* was more than the *L. plantarum* in decreasing the expression of both toxigenic genes (Fig. 3).

Antibiofilm effect of postbiotics against *C. difficile*. In the current research, we investigated biofilm formation by *C. difficile* using the microtiter plate method, and it was found that the control *C. difficile* had a reasonably high OD value before the administration of postbiotics, which indicated that this microorganism was able to establish a strong biofilm. In the examination of the results of the experiments, which we performed on the effect of different sub-MIC concentrations of postbiotics from different probiotics including *B. bifidum* and *L. plantarum*, were used against *C. difficile*, and it was found that both postbiotics were effective on the formation of biofilm, by inhibiting its formation, and it was observed that with the increase in the concentration of the postbiotics, the amount of biofilm formation decreased. Of the two mentioned postbiotics, the postbiotics of *B. bifidum* was the most effective in comparison with the other one, which inhibited 49% of biofilm formation in the concentration of 1.25 mg/ml, which *L. plantarum* inhibited 31% of biofilm formation in this concentration (Table 3).

Effect of postbiotics on cell viability. The MTT test was used to evaluate the impact that postbiotics produced from *B. bifidum* and *L. plantarum* had on the viability of HEK293 cells. At a time interval of 48 hours, the viability percentage values were determined by calculating the concentrations of each postbiotic at 0.3125, 0.625, 1.25, 2.5, and 5 mg/ml, respectively. The survival test of the normal HEK293 cell line in contact with postbiotics obtained from two distinct probiotics indicated that the survival rate of HEK293 cells was about above 80%, which had minimal influence on inhibiting the growth of these cells (Fig. 4).

DISCUSSION

The efficacy of antibiotic therapy for CDI is limited due to its potential to disrupt the bacterial equilibrium in the stomach, hence increasing the proba-

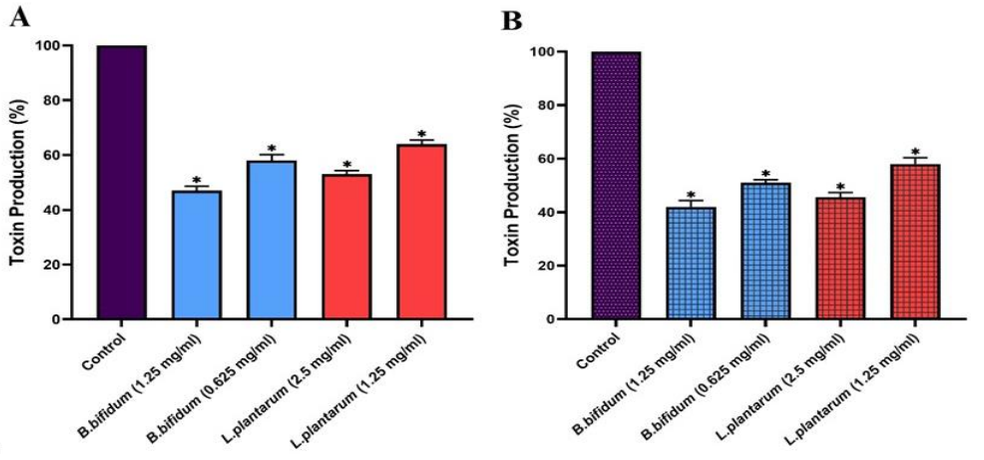

Fig. 2. Effect of different sub-MIC concentrations of postbiotics on *C. difficile* (A) toxin A and (B) toxin B production (*P<0.05)

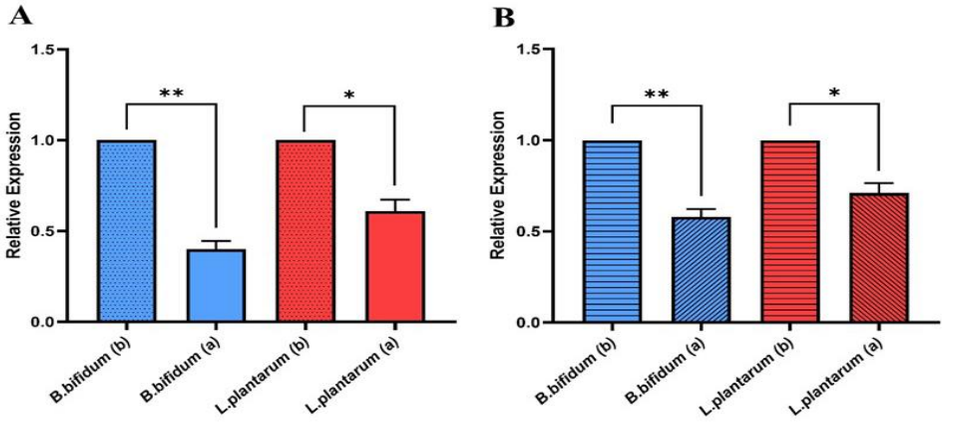

Fig. 3. (A) The relative expression of *tcdA* before and after postbiotics addition (B) The relative expression of *tcdB* before and after postbiotics addition (**P<0.01, *P<0.05); b, before postbiotic addition; a, after postbiotic addition.

Table 3. Biofilm formation reduction percentage by different sub-MIC concentrations of postbiotics against *C. difficile*

***B. bifidum* concentration**	**Biofilm reduction (%)**	***L. plantarum* concentration**	**Biofilm reduction (%)**
1.25 mg/ml	49%	2.5 mg/ml	44%
0.625 mg/ml	41%	1.25 mg/ml	31%
0.3125 mg/ml	32%	0.625 mg/ml	23%

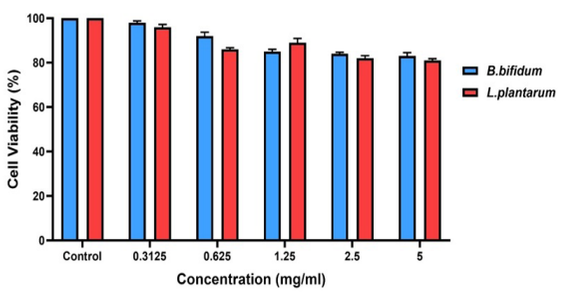

Fig. 4. The mean percentage of cell viability after MTT assay for different concentrations of postbiotics

bility of CDI recurrence (43). Extensive research is required to provide more pharmaceuticals to fight infections caused by the pathogen *C. difficile*, due to a notable rise in antibiotic-resistant strains and a shortage of effective therapies against this bacteria (44). One potential method for managing CDI is by eliminating *C. difficile* cells. As a result, the inhibitory activity of postbiotics was initially examined. The findings indicated that the postbiotics obtained from *B. bifidum* and *L. plantarum* effectively suppressed the development of *C. difficile*, with a MIC of 2.5

and 5 mg/ml, respectively. Despite the larger MIC of the postbiotic compared to the positive control, vancomycin, it is plausible that the postbiotic may contain multiple unpurified molecules. Postbiotics were analyzed using GC-MS and revealed the presence of compounds including succinic acid, benzoic acid and lactic acid. These compounds have the ability to penetrate the bacterial cell membrane, which leads to disruption of membrane integrity and disruption of cellular homeostasis, which ultimately, leads to the destruction of the pathogen (45). Previous work done by Wu et al. showed that *Enterococcus* by creation of bacteriocin, especially enterocin, which can create gaps in the cell membrane of target bacteria, releasing their essential organelles, which then kills the target bacteria (46). The research conducted by Lee et al. assessed the antibacterial activity of several lactic acid bacteria against *C. difficile*, including *Lactobacillus rhamnosus, Lactococcus lactis, Bifidobacterium breve,* and *Bifidobacterium lactis,* which *C. difficile* was incubated with several strains of lactic acid bacteria and it was shown that the postbiotics derived from the lactic acid bacteria strains described all shown antibacterial activity and *L. lactis* had the strongest antibacterial activity for a 24 hour period. This suggests that they might be used as an alternative therapy for treating gastrointestinal diseases associated with *C. difficile* (47).

The auto-generated biofilm matrix is recognized for safeguarding microorganisms by offering an enclosed habitat. Biofilm matrices consist mostly of extracellular polymeric substance (EPS), which predominantly consists of proteins, DNA, and polysaccharides. A primary role of a biofilm is to shield bacteria from adverse circumstances, especially antibiotics, especially in the context of an illness (48). The involvement of bacterial biofilms in antibiotic resistance has been extensively documented for several diseases. The resistance to antibiotics in biofilm may rise by a factor of 10 to 1,000 compared to planktonic bacteria (49). The prevalence of recurrent clostridial infections has risen, along with the emergence of drug-resistant strains of *C. difficile*. This has posed challenges in effectively managing CDI, since the commonly used medications metronidazole and rifampicin are no longer effective against these strains (50). In this work, we investigated the impact of different sub-MIC concentrations of postbiotics on the treatment of *C. difficile* infections. We observed that the pathogen exhibited varying percentages of biofilm development in response to different concentrations of postbiotic therapy. The present investigation found that *B. bifidum*, at a dosage of 1.25 mg/ml, was able to inhibit 49% of the biofilm formation of *C. difficile*. In comparison, *L. plantarum* inhibited 44% of biofilm development at a dose of 2.5 mg/ml. These results show that *B. bifidum* is more effective in preventing biofilm formation. A study by Yang et al. showed that the postbiotics obtained from B.breve have the ability to inhibit the formation of biofilms produced by *C. difficile* and its toxin production, which findings of that research are consistent with our study (51).

In the process of CDI creation, the synthesis of toxins A and B by *C. difficile* is a significant contributor to the development of the disease. Because of the chemical qualities that they possess, these chemicals are responsible for causing damage and inflammation in the large intestine. Both of these toxins are powerful cytotoxic enzymes that cause damage to the mucosa that lines the human digestive tract (52). In order for *C. difficile* to establish itself in the gut, it has to first connect to the epithelial cells that are covered by a coating of thick mucus. Researchers have found both confirmed and potential supplementary virulence factors that may contribute to adhesion and colonization in the intestines, which include proteolytic enzymes and adhesins (53).

In addition, our investigation revealed that the presence of postbiotics resulted in a significant reduction in the production of toxin A and toxin B by *C. difficile*. This was demonstrated by the significant reduction in the amount of toxins produced by *C. difficile*. The results indicate that the postbiotic of *B. bifidum* has a greater capacity to decrease the production of both toxin A and B by the pathogen compared to *L. plantarum*. Specifically, it decreased the production of toxin A by 49% and 43% and the production of toxin B by 53% and 45% at concentrations of 1.25 mg/ml and 0.625 mg/ml, respectively, for both toxins. *L. plantarum* also reduces the synthesis of toxin A by 44% and 33%, and the production of toxin B by 48% and 42% at doses of 2.5 mg/ml and 1.25 mg/ml, respectively, for both toxins. Shen et al. discovered that *Lactobacillus casei* produces an antibiotic compound that hinders the development of *C. difficile* (54). Our data supports the findings of McFarland et al.'s investigation, which demonstrated the effectiveness of the probiotic strain *Saccharomyces boulardi* in preventing CDI by binding to *C. difficile* toxins A

and B (55).

The toxins A and B are generated through the expression of the *tcdA* and *tcdB* genes (56). In this study, the Ct value indicate that the expression levels of both genes were markedly increased. The addition of postbiotics produced from *B. bifidum* and *L. plantarum* resulted in an increase of 21.4 and 21.1 in the Ct values for the *tcdA* gene, and an increase of 25.1 and 24.7 in the Ct values for the *tcdB* gene, compared to the control group. According to the findings provided, the inclusion of postbiotics resulted in a reduction in the activity of the *tcdA* and *tcdB* genes in *C. difficile*. Moreover, the impact of *B. bifidum* was more pronounced compared to *L. plantarum* in terms of reducing the expression of both virulence genes. Yang et al. conducted a research in which they observed a progressive drop in the expression levels of *tcdA* and *tcdB* when the dosages of *B. breve* postbiotic were increased (57). When compared to the levels that were observed after 24 hours, the expression levels of *tcdA* showed a rise after 48 hours from the initial time point. A dose-response relationship was observed for *tcdB*, indicating that higher dosages of postbiotic resulted in a considerable weakening effect.

Administering postbiotics has shown promise effectiveness in reducing CDI. Postbiotics have a considerable inhibitory impact on *C. difficile* virulence factors by inhibiting pathogen growth, disrupting biofilm formation, and reducing toxin generation. The results indicate that postbiotics have the potential to be used as additional or alternative treatment agents for treating CDI. This offers a great opportunity for developing new ways to tackle this difficult healthcare-associated illness (58).

CONCLUSION

The field of postbiotics has promise in efficiently combating CDI via its detoxifying characteristics. These metabolites are composed of various bioactive substances such as short-chain fatty acids, peptides, and organic acids, which have antibacterial properties, modulate the host's immune responses, and improve the integrity of the intestinal barrier. Postbiotics have shown potential in decreasing the intensity of CDI, diminishing the likelihood of recurrence, and enhancing overall therapeutic results by targeting the pathways responsible for the illness. Furthermore, the ability of postbiotics to remove *C. difficile* toxins and decreasing the expression of their related genes, highlights their potential as a therapeutic approach to reduce the detrimental effects of toxin-induced injury to the intestinal epithelium. While the first findings are promising, more research is required to elucidate the specific pathways via which postbiotics detoxify *C. difficile* toxins and enhance their therapeutic use. Utilizing the detoxifying abilities of postbiotics in clinical settings is a potentially efficacious strategy to treat CDI and improve patient outcomes.

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