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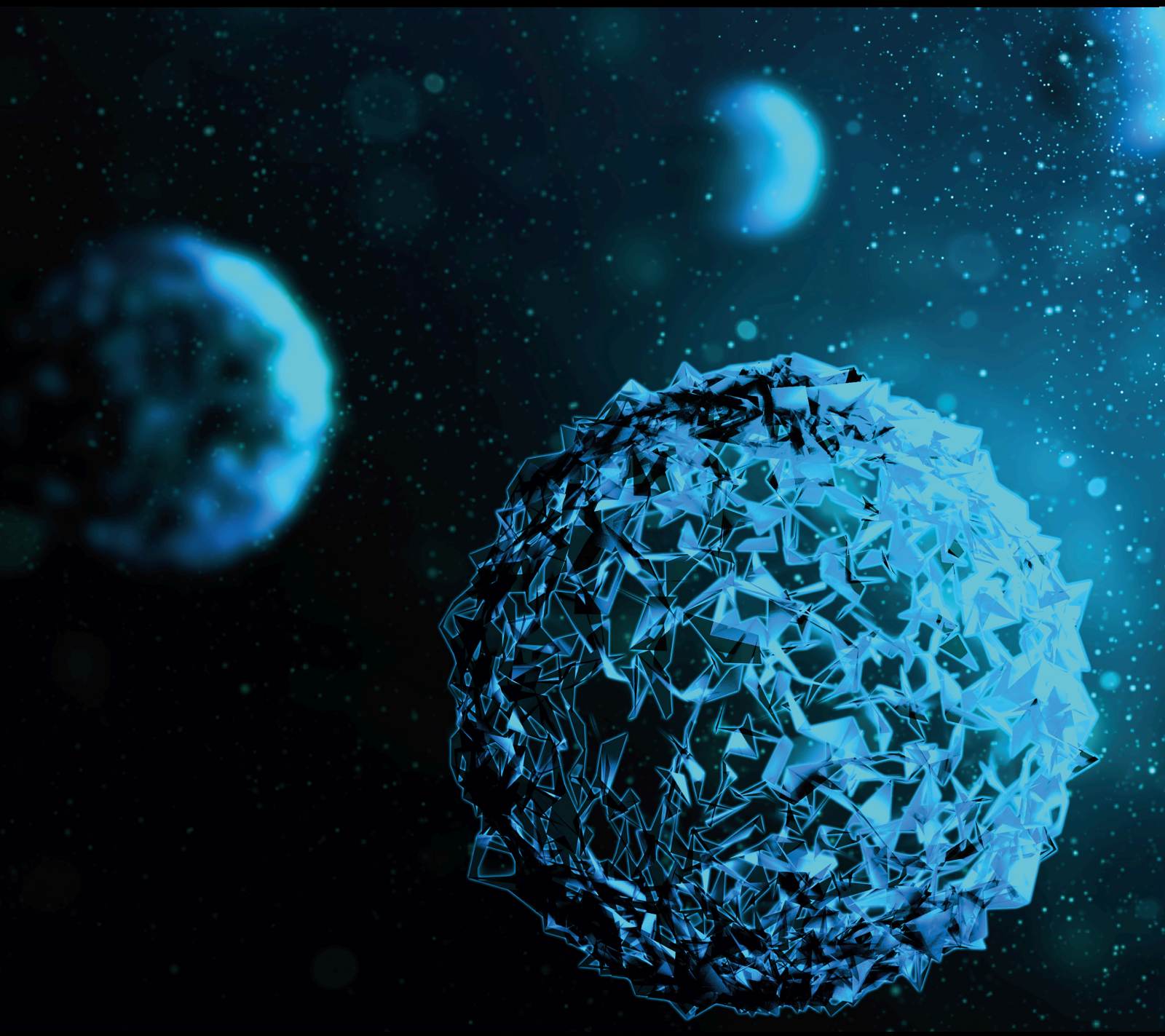
Food Microbiology 2020

Lead Guest Editor: Marta Laranjo
Guest Editors: María de Guía Córdoba, Teresa Semedo-Lemsaddek, and Maria Eduarda Potes



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Editorial

Food Microbiology 2020

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Food microbiology studies the microbiota of food products. It is a very diverse area within the field of microbiology because it deals with microorganisms that may have both beneficial and deleterious effects on food quality and safety, comprising fermentative, probiotic, spoilage, and pathogenic bacteria, moulds, yeasts, and other microorganisms.

The survival and growth of these diverse microorganisms is very much influenced by a wide range of environmental factors, which include the hugely diverse composition of the different food matrices.

Among the submitted manuscripts that pretty much covered all aspects of food microbiology, eight research articles were selected by external experts to enter this annual special issue of BioMed Research International.

Salameh et al. did a comparative study on the volatile oils of *Micromeria fruticosa serpyllifolia* plants, growing in different regions of Palestine, screening for their antioxidant and antimicrobial activities.

Gebru and Sbhatu studied a collection of probiotic lactic acid bacteria (LAB) isolated from Korean Kimchi and Ethiopian spontaneously fermented Teff and evaluated the effect of LAB on the phenolic content of Teff during fermentation.

The work by Su and coworkers evaluated the water-soluble polysaccharides of *Cordyceps kyushuensis*, a parasitic fungus, for their immunomodulatory and antioxidant actions and concluded that these could be considered potential candidates for functional foods and therapeutic agents.

Hassan and colleagues studied the probiotic properties of lactobacilli isolated from traditional Pakistani yoghurt and evaluated the antimicrobial activity of their bacteriocins against foodborne pathogens, such as *Staphylococcus aureus* and *Acinetobacter baumannii*.

Jaja et al. reported a high-level contamination of meat with *Staphylococcus aureus* and *Escherichia coli* resistant isolates, highlighting the public health consequences associated with the consumption of these unhygienic food products.

The article by Tenea described the role of peptide extracts from native LAB as promising food antimicrobials against *Salmonella enterica*.

Finally, Luo and colleagues described the beneficial effect of *Bacillus megaterium*-coated diets on the growth, digestive enzyme activity, and gut microbial diversity of Songpu mirror carp.

The authors come from nine different countries, one European (United Kingdom) and eight non-European, namely, Ethiopia, Pakistan, Saudi Arabia, South Africa, Nigeria, China, State of Palestine, and Equator.

We are happy to launch this special issue, which includes eight manuscripts that reported new findings mainly on the antimicrobial and antioxidant activities of food microbiota or their secondary metabolites. We hope that the readers of *BioMed Research International* find this Food Microbiology 2020 special issue of relevance to their research field.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The team of guest editors wish to thank the authors of the published manuscripts for their contributions and the anonymous reviewers for their invaluable involvement in the evaluation process.

Marta Laranjo
María de Guía Córdoba
Teresa Semedo-Lemsaddek
Maria Eduarda Potes

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Research Article

Probiotic Properties of *Lactobacillus helveticus* and *Lactobacillus plantarum* Isolated from Traditional Pakistani Yoghurt

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Probiotic bacteria are of utmost importance owing to their extensive utilisation in dairy products and in the prevention of various intestinal diseases. The objective of this study was to assess the probiotic properties of bacteriocin-producing isolates of *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yoghurt. In this study, ten bacteriocin-producing isolates were selected to screen for the probiotic property. The isolates showed resistance to acidic pH (6-6.5), bile salt (0.01-1%), and 1-7% NaCl salt and showed good growth at acidic pH and antibacterial activity against ten different foodborne pathogens. Interestingly, these isolates were proved to be effective against *Actinobacter baumannii* but least effective against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. A few isolates were found to be resistant to some antibiotics like vancomycim, gentamycin, erythromycin, streptomycin, and clindamycin. Our results provide strong evidence in favour of traditional Pakistani yoghurts as a potential source of bacteriocin-producing bacteria with an added benefit of the probiotic property. Specifically, LBh5 was considered a good probiotic isolate as compared to other isolates used in the study. Further extensive research should be done on isolation and characterisation of probiotic isolates from local fermented foods, and then, these isolates should be used in the development of probiotic enriched food supplements in Pakistan.

1. Introduction

Probiotic is a well-known term used for "live microorganisms" when added as supplements in food, which provide many health benefits. In the food industry, lactic acid bacteria (LAB) are the most promising group of bacteria that have been consumed fearlessly as they are generally regarded as safe (GRAS) [1]. In fact, most of them are natural inhabitants of dairy products like cheese and yoghurt and considered native microflora in dairy products [2, 3]. They are present

naturally or added purposely to food products for enhancing their flavour and aroma and for the maintenance of human gut microflora. Searching fermented food items for probiotics has been a very practical and convenient approach for preservation as it does not invite objections against chemical additives being a part of the intake [4]. The present study includes *Lactobacillus*, the probiotic genus popular for many medical applications such as decreasing enteric infections, intestinal tumors, and cholesterol levels, folate production, treating cardiovascular diseases, metabolic disorders, lactose intolerance, and boosting the immune system [5–9]. Moreover, such bacteria release anticarcinogenic and antimicrobial substances in addition to organic acids such as lactic acid, benzoic acid, and acetic acid [5, 10]. The antimicrobial peptides known as bacteriocins are also defining characteristics of these bacteria, which efficiently inhibit the growth of certain foodborne pathogens and spoilage bacteria [11]. Among lactobacilli, *Lactobacillus plantarum* and *Lactobacillus helveticus* are two probiotics commonly used as a starter culture in several fermentations, especially in the fermentation of dairy products [4]. *Lactobacillus helveticus* shows profound proteolytic activity and is also reported as a good inhibitor of angiotensin-converting enzyme, which has a role in alleviating hypertension. *Lactobacillus plantarum* is an indigenous gut inhabitant and therefore is considered more compatible with the gut environment [12, 13].

The antimicrobial ability maintained by a probiotic is attributed to the production of organic acids (acetic acid and lactic acid), protein metabolites, hydrogen peroxide, cyclic dipeptides, enzymatic effects, and certain antimicrobial peptide-bacteriocins [14, 15]. Lately, isolation and characterisation of bacteriocins are of interest to microbiologists as they could be a better candidate to replace antibiotics and synthetic preservatives [4]. In the past decades, many *Lactobacillus* species were reported for their probiotic potential and exhibited antagonistic activity against various microbes, but very few studies have been done on bacteriocin-producing potential probiotic strains isolated from a dairy product.

Isolating potential probiotic strains has been practiced for centuries and is still of interest because of its industrial and medicinal value [16]. Probiotic strains must have the ability to tolerate the stress environment contained in the gastrointestinal system. Their viability in harsh conditions like the presence of bile, gastric juices, and NaCl, low pH in the stomach, adhesion to the intestinal lining, and antibiotics contributes to their efficacy [8, 17]. Their resistance to environmental stress and their bacteriocin producing ability would aid the food industry in establishing safe preservation strategies. In Pakistan, yoghurt is a famous dairy product obtained by fermentation of milk. It is consumed daily by a large population as it possesses nutritional value; plus, it is a significant source of beneficial bacteria [17]. Yoghurt from different countries has been investigated for the isolation of potential probiotics. Literature shows that a distinctive microbiome has also been reported from yoghurt of different cities of Pakistan, such as Lahore, Faisalabad, Karachi, Islamabad, and Peshawar. These isolates include *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, and *Leuconostoc mesenteroides* CYG362 [18, 19] from Lahore; *L. acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus paracasei* subsp. *paracasei*, *L. bulgaricus*, *L. delbrueckii*, *L. plantarum*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, and *Streptococcus thermophilus* from Faisalabad [20–24]; *L. bulgaricus* from Islamabad [25]; *Pediococcus pentosaccus*, *L. delbrueckii*, *L. plantarum*, *L. helveticus*, and *Pediococcus acidilactici* from Peshawar; and *Lactobacillus curvatus* KIBGE-IB44 from Karachi [26, 27]. These microbes are also included in commercial probiotic yoghurt in Pakistan [28]. In Pakistan, *L. helveticus* and *L. plantarum* are rarely isolated from yoghurt as compared to other species; thus, their probiotic potential is barely determined.

In our previous study [29], we isolated bacteriocin from two lactobacilli, *L. plantarum* and *L. helveticus*, which effectively inhibited foodborne pathogens. In this study, we extended our research and evaluated the probiotic potential of bacteriocin-producing species, as very few bacteriocin producing probiotic species were described earlier. This study provides potent probiotics that could be used in food preservation and storage strategies to prevent certain spoilage bacteria and thus reduces the economic loss experienced by the food industry.

1.1. Contributions. Our contributions in this study are as follows:

(i) In an earlier study, an analysis of the bacteriocin properties of *Lactobacillus* species (isolated from traditional yoghurt) was done. Among different isolates, *Lactobacillus* species *L. helveticus* and *L. plantarum* were involved in the production of bacteriocins. In the current study, these two species were evaluated for probiotic properties. The comparative analysis of probiotic properties on both species showed that *L. helveticus* was the most suitable probiotic species as compared to *L. plantarum* against foodborne pathogens

(ii) Among these two species, *L. helveticus* was seldom found in dairy products. It was the first time that these bacteriocin-producing isolates have been isolated from a yoghurt sample from Peshawar KPK while *L. plantarum* has been isolated a few times from dairy products. These isolates could further be subjected to various in vivo approaches to find the actual target of these isolates. Furthermore, these isolates could be assessed for the production of the unusual proteolytic spectrum and other inhibitors

(iii) The remarkable probiotic properties of these two bacteriocin-producing species and the comparison of *L. helveticus* and *L. plantarum* make this study distinctive and interesting for readers

2. Materials and Methods

2.1. Bacterial Species Used in This Study. Ten clinically identified foodborne pathogens used in this study were ordered

from the Bacteriology Lab, Department of Microbiology at the Faculty of Sciences, Punjab University, Pakistan. The included species used in this study are *Acinetobacter baumanni*, *Bacillus subtilis*, *Enterococcus faecium* DO, *Escherichia coli*, *Klebsiella pneumoniae*, *Methicillin-resistant Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Salmonella paratyphi A*, *Staphylococcus aureus*, and *Streptococcus pyogenes.* The same bacterial isolates were used in a previous study by Hassan et al. [29].

2.2. Isolation, Screening, and Identification of Bacteriocin-Synthesising Species. The bacteriocin-producing isolates were obtained from screening 50 traditional Pakistani yoghurt samples used in the previous study conducted by Hassan et al. [29].

2.3. Growth Rate Study. A starter culture of 1 mL of a 24 hrs old culture of *Lactobacillus* bacterial isolates was inoculated in 100 mL of nutrient broth and MRS broth (Merck, Darmstadt, Germany) in an anaerobic jar (BD GasPak®100 System, Becton Dickinson®, USA) with a fresh Thermo Scientific®Oxoid®AnaeroGen®.2.5 L sachet. The anaerobic jar was in a New Brunswick™ Excella®E24 Incubator Shaker at 37°C at 100 rpm [30]. Bacterial growth was monitored spectrophotometrically at 600 nm (A_{600}) at 0, 2, 4, 6, 8, 10, 12, and 24 hrs. The measurements were carried out using an Agilent Cary 5000 Ultraviolet-to-Visibleto-Near-Infrared (UV-Vis-NIR) spectrophotometer (Agilent Technologies, Petaling Jaya, Malaysia) [31].

2.4. Evaluation of Probiotic Properties. To evaluate the probiotic potential of bacteriocin-producing isolates, the following properties were considered: tolerance to low pH, bile salts , NaCl, antibiotic susceptibility, antimicrobial activity, and response to gastroduodenal stimuli.

2.4.1. Low pH Tolerance. A 1 mL bacterial culture was inoculated into eight tubes, each containing 9 mL MRS broth adjusted to varying pH ranges from 1 to 8. After 4, 6, 12, and 24 hours of incubation at 37°C, the growth rate was calculated by considering the optical density (OD) values measured at 600 nm.

Uninoculated media was taken as a negative control.

2.4.2. NaCl Tolerance. Isolates were drawn in MRS broth having different NaCl concentrations ranging from 1 to 7%, incubated at 37°C for 4, 6, 12, and 24 hrs at 37°C. Growth was determined by measuring the optical density of the broth at 600 nm. A tube without NaCl was run as a negative control.

2.4.3. Bile Salt Tolerance. MRS broth was supplemented with different concentrations of bile (Ox-gall): 0%, 0.05%, 0.1%, 0.3%, 0.6%, and 1%, and was inoculated with lactobacilli to investigate bile salt tolerance. ODs measured at 600 nm were used to measure cell growth after 0, 2, 4, 6, 12, and 24 hrs at 37°C.

Negative control was also run.

2.4.4. Antibiotic Susceptibility Test. The agar well diffusion method was used to test antibiotic susceptibility against frequently used antibiotics. The antibiotics which were used in this study against all ten isolates with the same concentrations are ampicillin (Amp) (2 mg/L), gentamycin (Gen) (16 mg/L), erythromycin (Ery) (1 mg/L), and clindamycin (CLI) (4 mg/L). The concentration of kanamycin used for the *L. plantarum* isolates was 64 mg/L and for the *L. helveticus* isolates 16 mg/L. The two antibiotics tetracycline (Tet) at 32 mg/L and chloramphenicol (CL) at 8 mg/L were only tested against *L. plantarum* isolates. The two antibiotics streptomycin (SM) at 16 mg/L and vancomycin (Van) at 2 mg/L were only tested against *L. helveticus* isolates. The stock solution of the antibiotics was made in distilled water according to guidelines from the Clinical and Laboratory Standards Institute (CLSI) and the European Food Safety Authority (EFSA) [32, 33]. Distilled water was run as a control.

2.4.5. Antibacterial Activity. A cell suspension of the *Lactobacillus* species was prepared in MRS broth by comparing the turbidity with 0.5 McFarland solution. Test microorganisms were cultured in nutrient broth and swabbed onto Muller Hinton Agar (MHA) plates (Merck, Darmstadt, Germany). Isolates were incubated for 24 hrs and then centrifuged at 12,000 × g for 10 min, and 50 μL of supernatant was loaded in the well made on MHA and incubated at 37°C for 24 hrs. The antibacterial activity was determined against both gram-negative and gram-positive pathogens.

2.4.6. Response to Stomach-Duodenal Stimulus. The response of the tested LAB to the stomach-duodenal stimulus was evaluated in vitro by the method described in Pinto et al. [34]. It was performed on overnight bacterial culture 10-fold dilutions to determine the OD_{600} and cell number of each dilution. Bacterial survival was determined by measuring the OD_{600} at 0, 2, 4, 6, 8, 10, 12, and 24 hrs [35].

2.4.7. Arginine Hydrolysis Test. To perform the arginine hydrolysis test, MRS broth (without glucose and meat extract, supplemented with 0.3% arginine and 0.2% sodium citrate) was used [36].

2.5. Statistical Analysis. Statistical tools Pearson correlation and two-way ANOVA were applied to analyse the data. R program version 1.3.959, Graph Pad Prism version 8.4.3, and MS Excel 16.0 were used to calculate statistics.

3. Results and Discussion

3.1. Isolation and Identification of Lactobacillus spp. from Traditional Yoghurts. Bacteriocin-producing isolates were collected from yoghurt. From 12 different isolates, five isolates were identified as *L. helveticus*, and of the rest five were identified as *L. plantarum* on the basis of their colony morphology and various biochemical tests, and the remaining two isolates were identified as *E. coli* and *Enterococcus faecium.* The *L. helveticus* isolates were named LBh1, LBh2, LBh3, LBh4, and LBh5, and the *L. plantarum* isolates were named LBp1, LBp2, LBp3, LBp4, and LBp5.

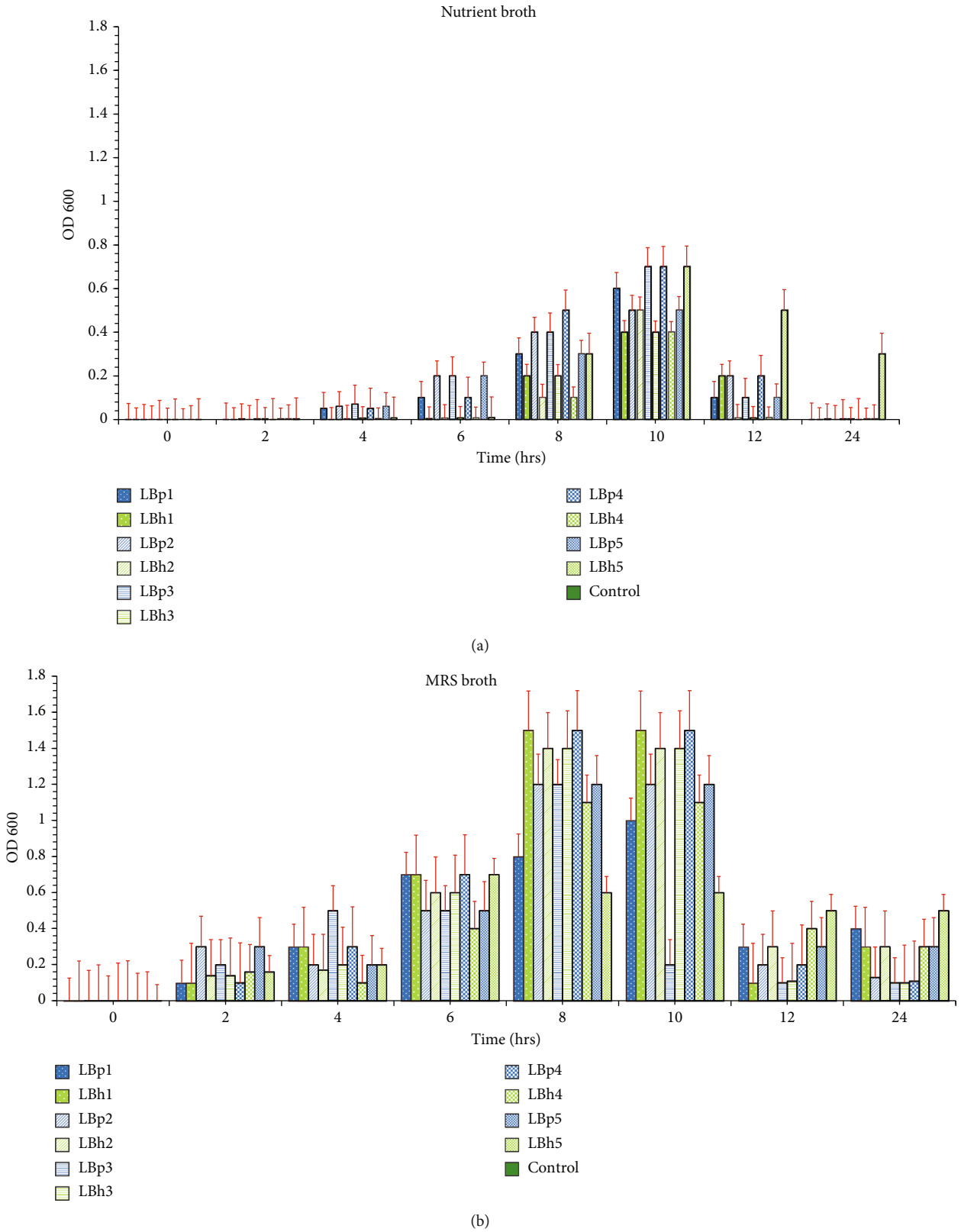
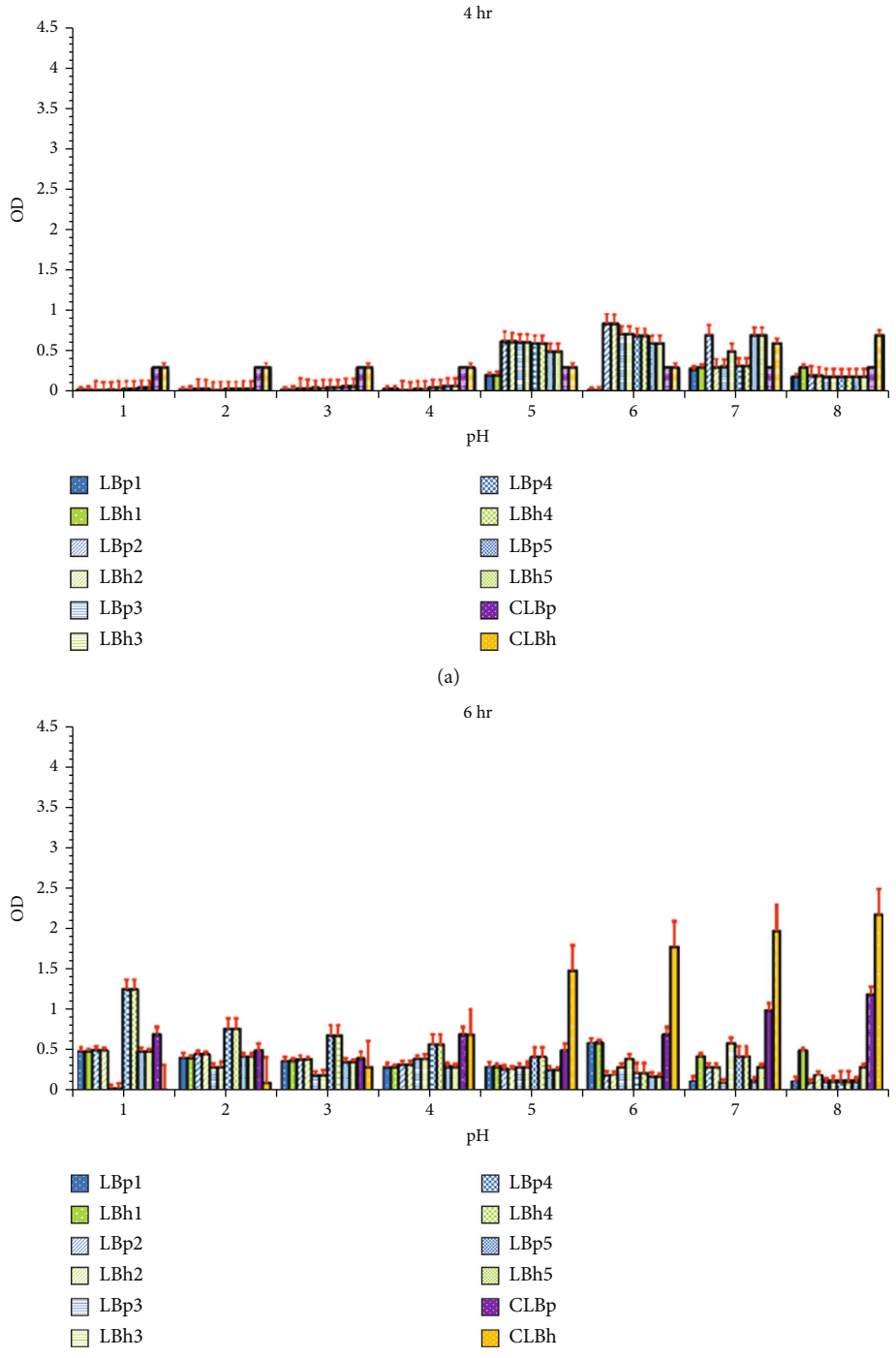


Figure 1: Growth curve of *L. plantarum* and *L. helveticus* isolates. (a) Nutrient broth. (b) MRS broth. LBp1-LBp5 are the *L. plantarum* isolates, and LBh1-LBh5 are the *L. helveticus* isolates.



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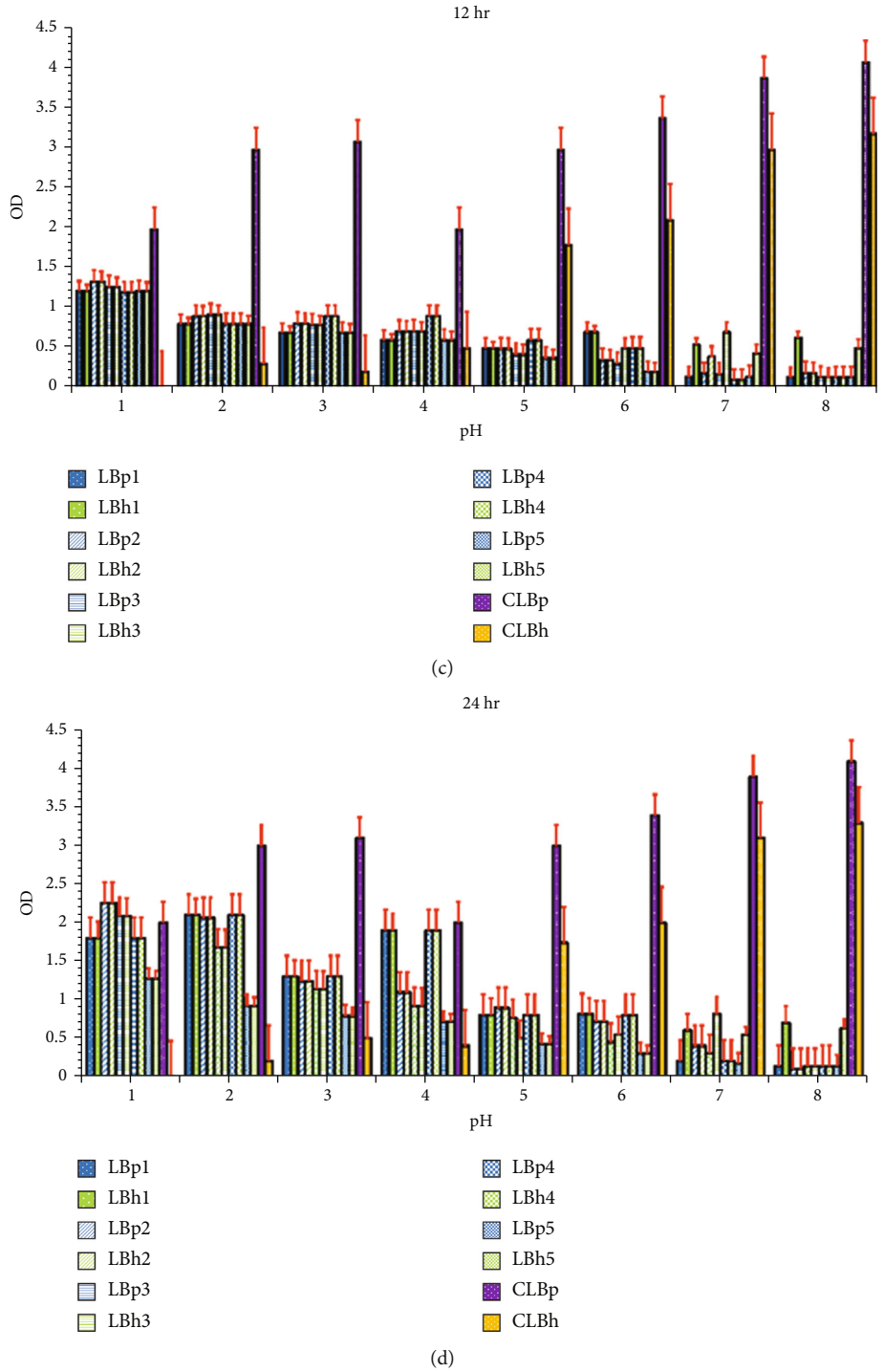
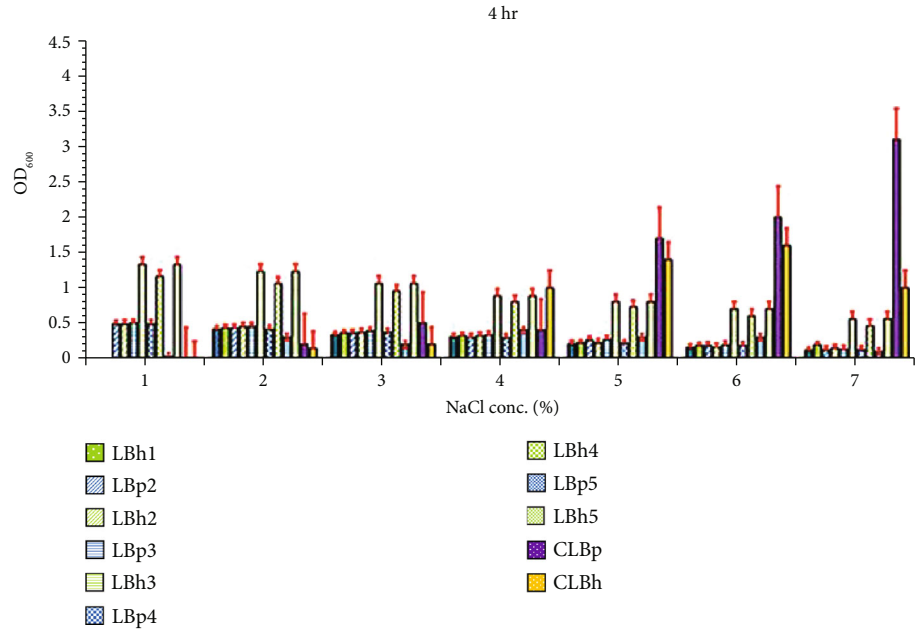


Figure 2: pH tolerance to *L. plantarum* and *L. helveticus* isolates: (a) 4 hours, (b) 6 hours, (c) 12 hours, and (d) 24 hours. The red bars show the standard error. LBp1-LBp5 are the *L. plantarum* isolates, LBh1-LBh5 are the *L. helveticus* isolates, CLBp was the control of *L. plantarum*, and CLBh was control of *L. helveticus*.

3.2. Growth Rate Study. Optical densities for isolates were measured every 2 hrs, i.e., 0, 2, 4, 6, 8, 10, 12, and 24 hrs, on MRS and nutrient broth simultaneously. After 6 hours, all the isolates used in the study reached an OD of approximately 0.7 in MRS broth, while in the nutrient broth, it took 10 hours, as shown in Figure 1. In the study of Cho et al. [37], the isolates of *L. plantarum* reached stationary phase after 10 hours. Balamurugan et al. [38] also reported that *L. helveticus* showed an elevated growth curve at around 16 hours in MRS broth, which was similar to our results as shown in Tables S1 and S2.



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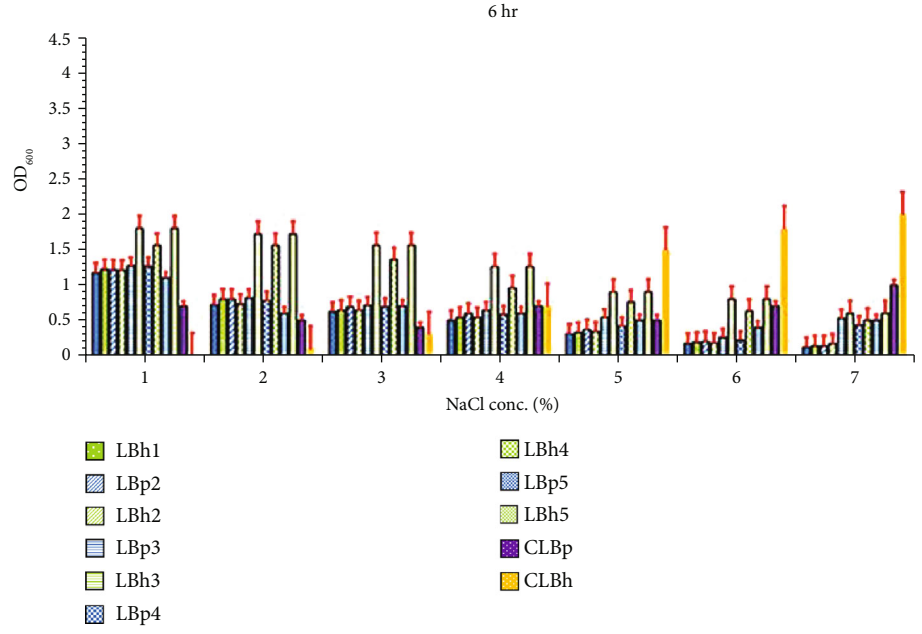

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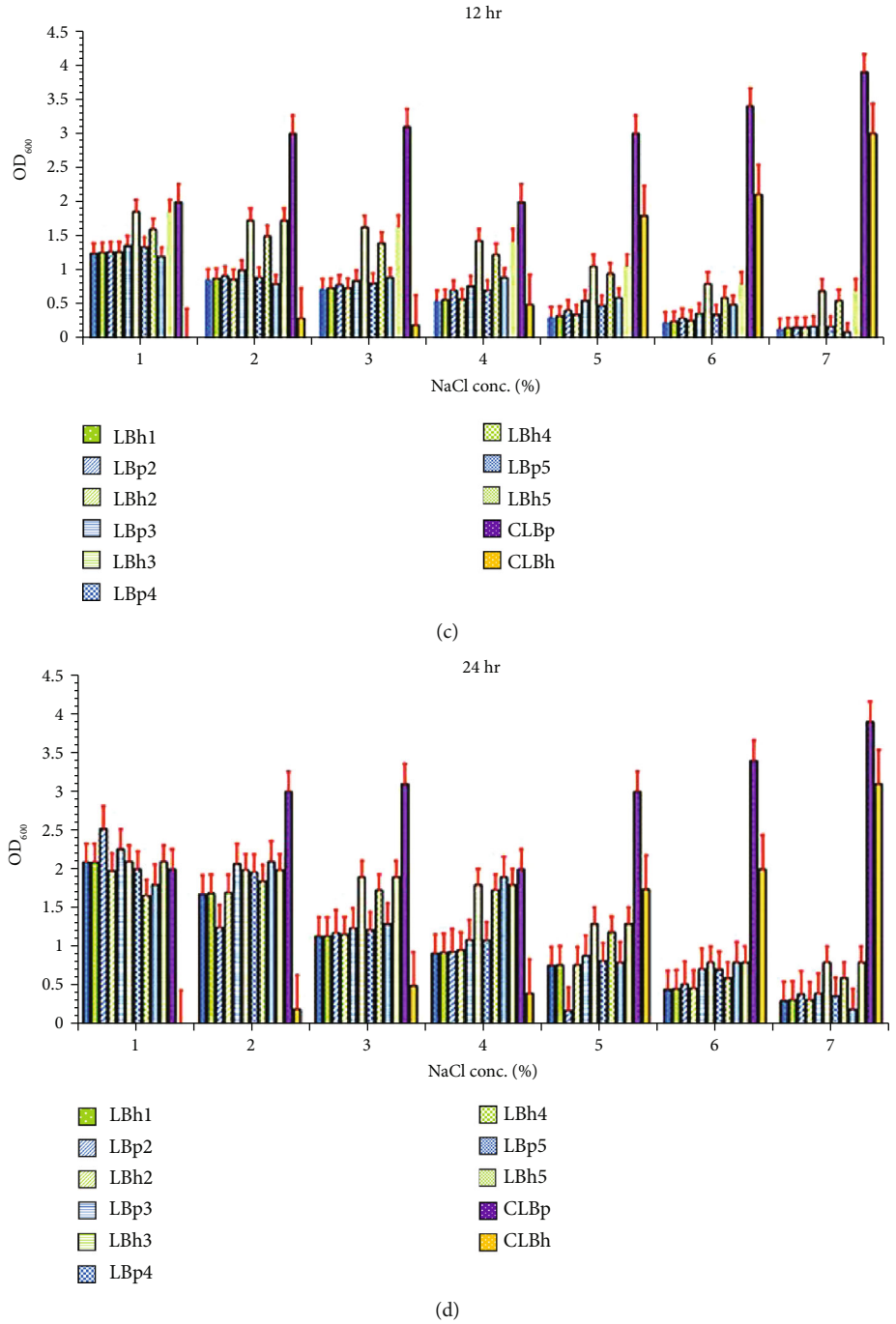
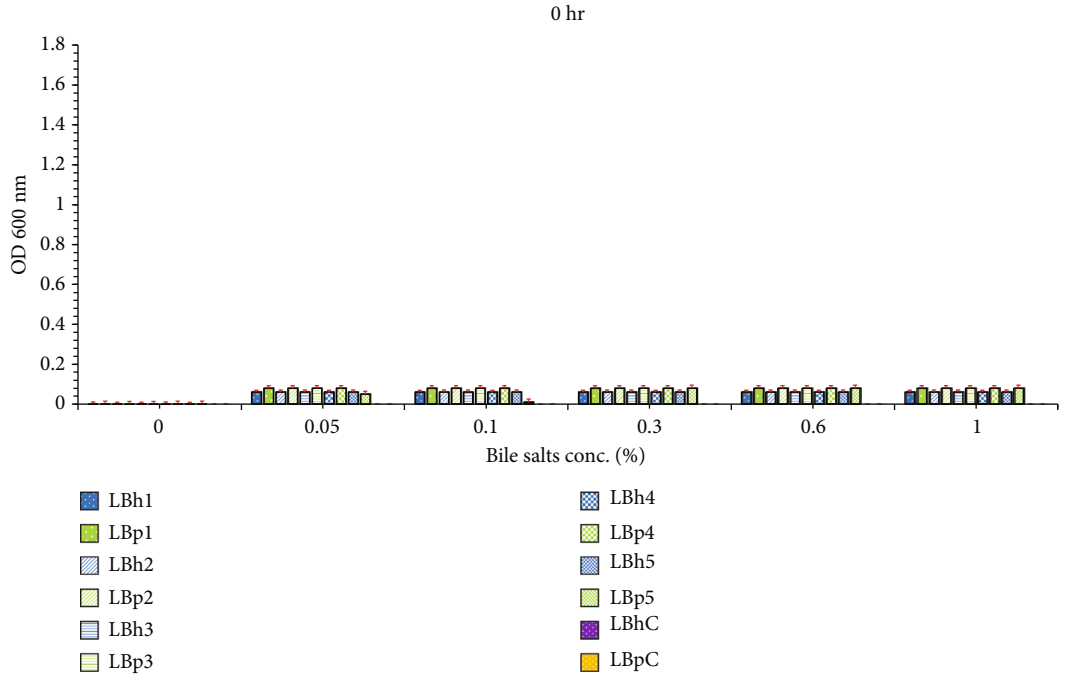


Figure 3: NaCl tolerance of *L. plantarum and L. helveticus* isolates: (a) 4 hours, (b) 6 hours, (c) 12 hours, and (d) 24 hours. The red bars show the standard error. LBp1-LBp5 are the *L. plantarum* isolates, LBh1-LBh5 are the *L. helveticus* isolates, CLBp was the control of *L. plantarum*, and CLBh was control of *L. helveticus*.

3.3. Evaluation of Probiotic Properties

3.3.1. pH Tolerance. A low pH tolerance test is essential to predict the survival of the isolates in the stomach environment. It was observed that *L. plantarum* isolates showed negligible growth up to pH 4, but at pH 5-6, an increase in growth rate was observed. The decline in the growth rate of *L. plantarum* isolates occurred at pH above 6; the growth rate declines drastically. Chakraborty and Bhowal [39] reported that *L. plantarum* showed maximum growth at pH 5-6, which was quite similar to our findings. *Lactobacillus helveticus* isolates showed tolerance up to pH 8. Guetouache and Guessas [40] had also reported that *L. helveticus* showed tolerance at alkaline pH. Most of the isolates showed a decrease in the tolerance towards pH up to 6 hours of incubation; after that, they showed a sharp decline in their growth rate, as shown in Figure 2. *Lactobacillus helveticus* isolates showed better tolerance to high pH as compared to *L. plantarum*.



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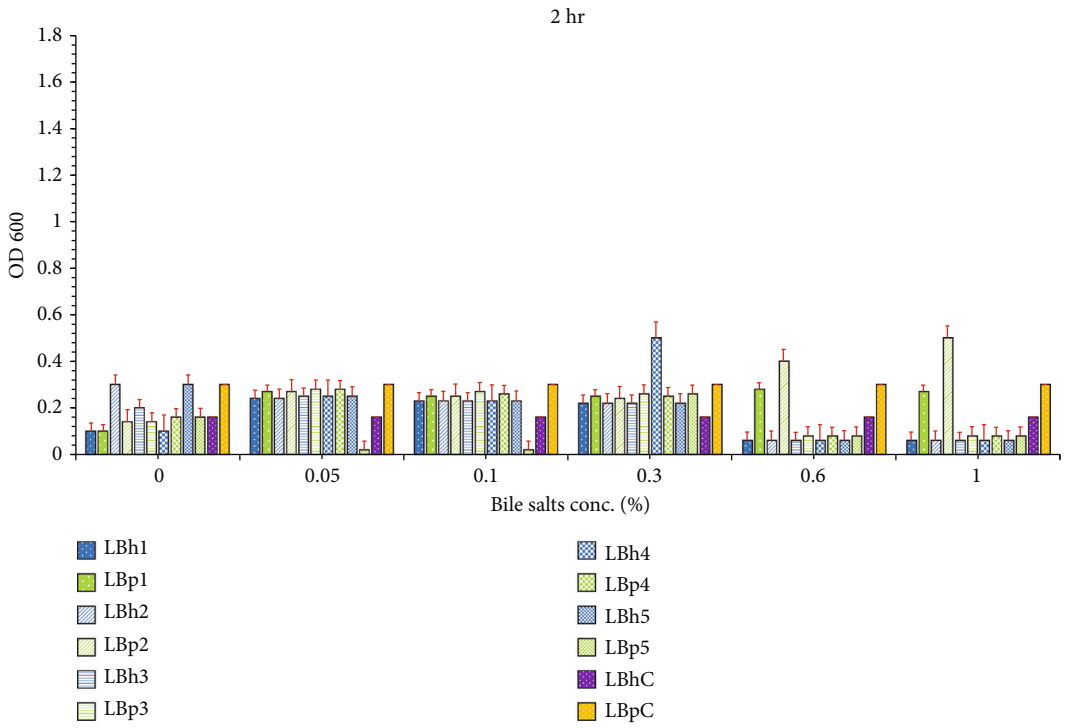

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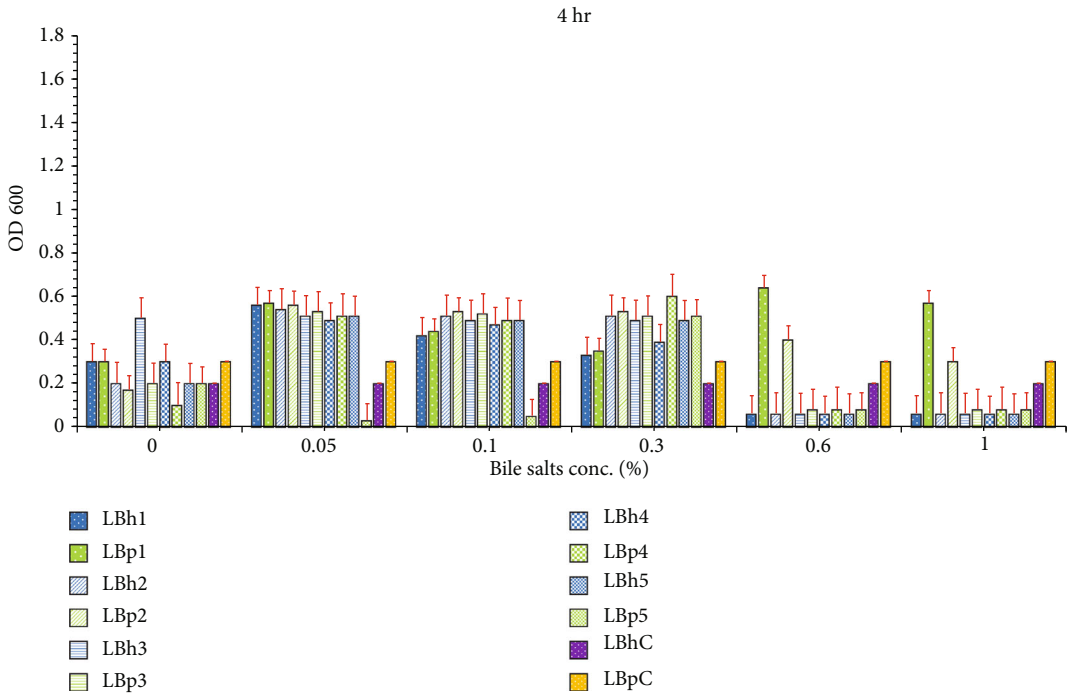

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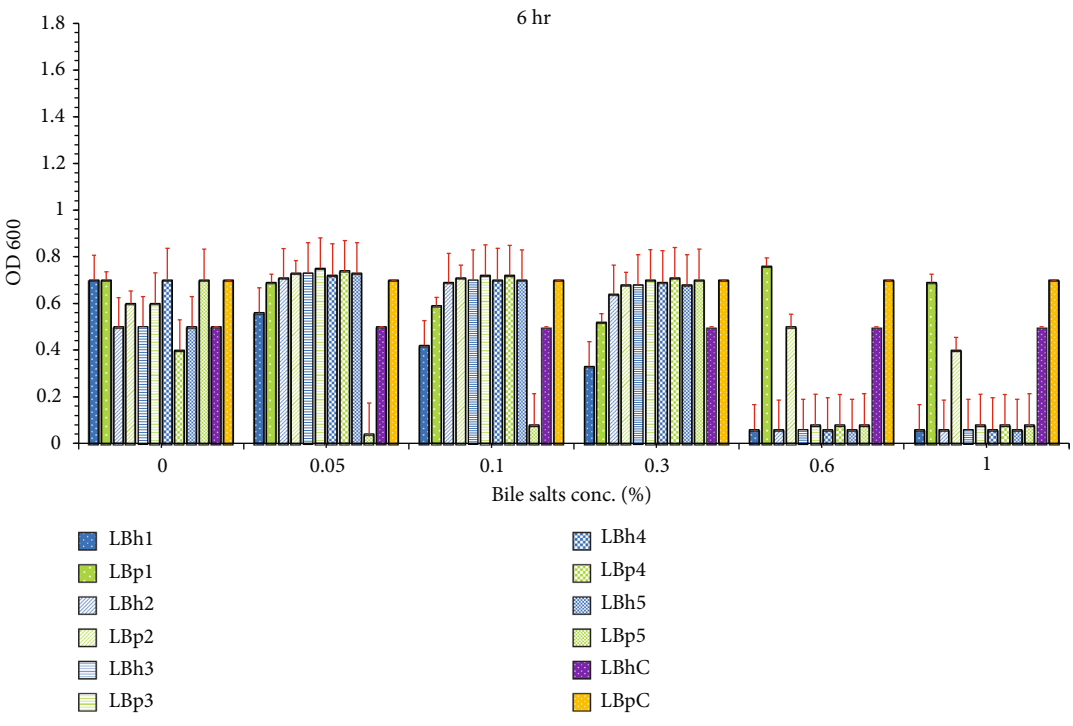

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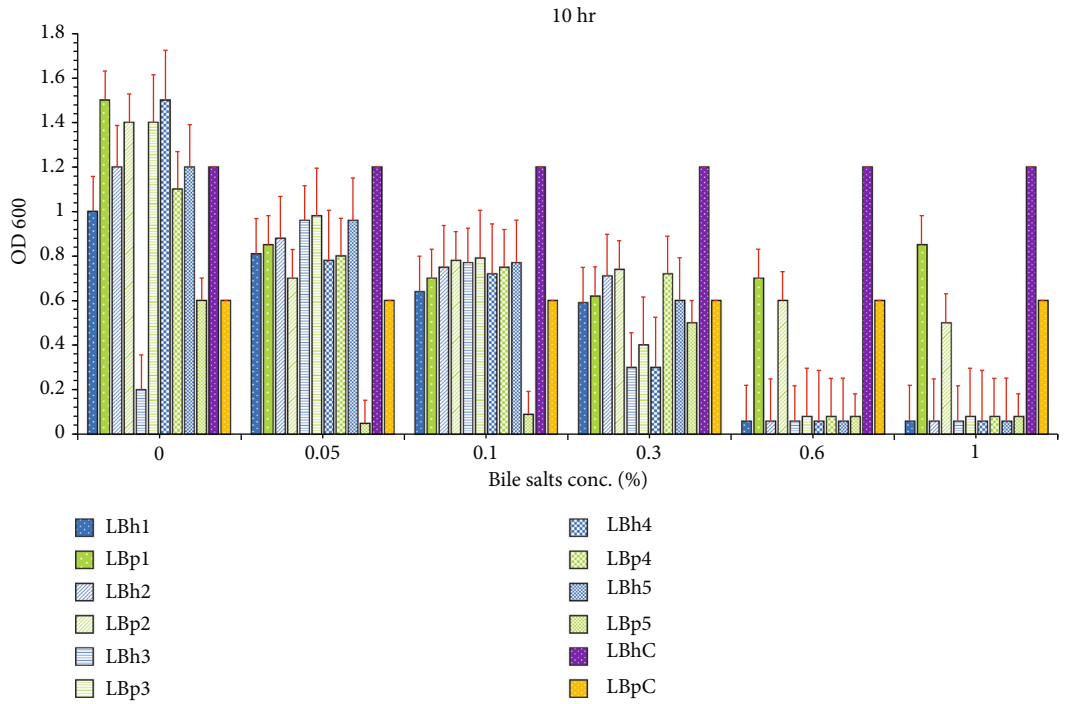

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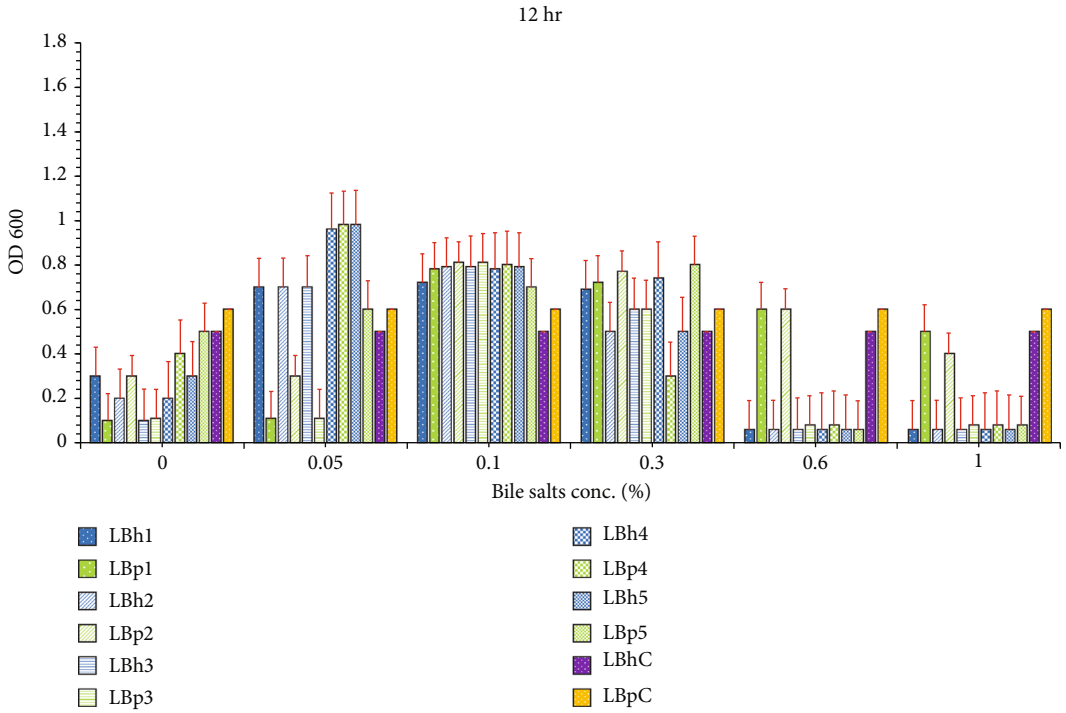

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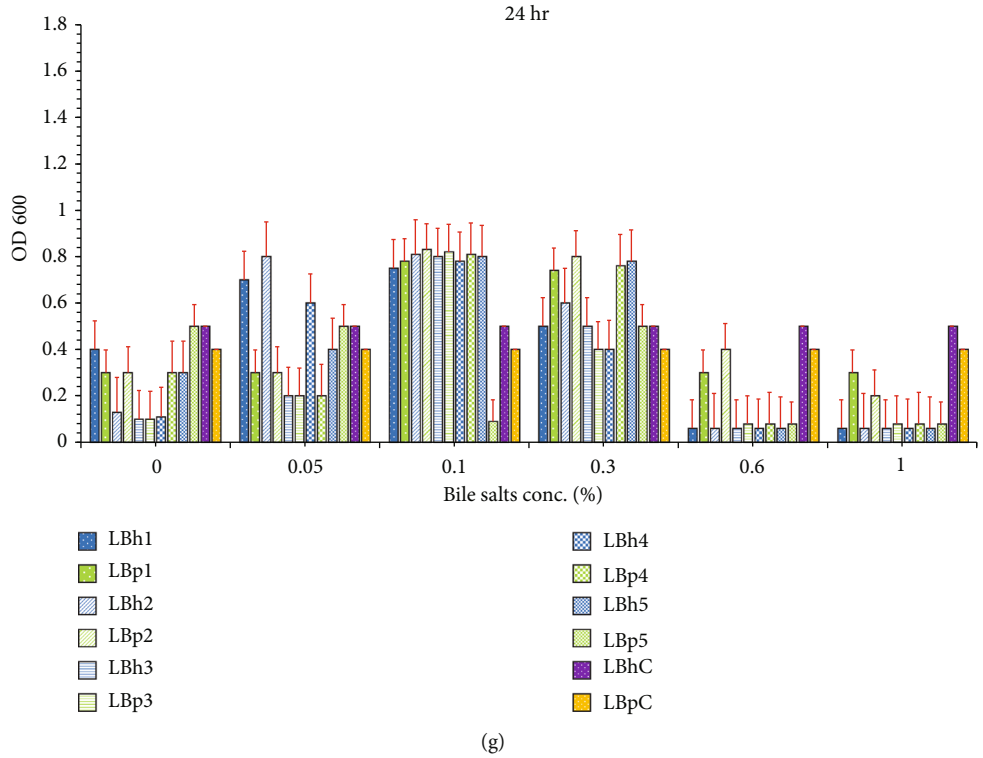

(g)

Figure 4: Bile salt tolerance of *L. plantarum and L. helveticus* isolates at different times: (a) 0 hours, (b) 2 hours, (c) 4 hours, (d) 6 hours, (e) 10 hr, (f) 12 hr, and (g) 24 hr. The red bars show the standard error. LBp1-LBp5 are the *L. plantarum* isolates, LBh1-LBh5 are the *L. helveticus* isolates, CLBp was the control of *L. plantarum*, and CLBh was control of *L. helveticus*.

The isolate LBh5 showed higher tolerance against higher pH (up to pH 8) as well as a lower pH as compared to other isolates used in this study. LBh1 showed the most significant results, among all other isolates. The statistical analysis is as given in Tables S3 and S4.

3.3.2. NaCl Tolerance. Tolerance to a high concentration of NaCl is necessary for a probiotic to be effective in the human gut. NaCl tolerance was considered to be an important parameter because NaCl concentration is a scale to measure how much bacteria are able to tolerate toxic and osmotic shock. For both the *Lactobacillus* spp., optical densities decrease gradually with an increase in NaCl concentrations, as shown in Figure 3. *Lactobacillus plantarum* isolates comparatively showed higher OD values than *L. helveticus* isolates. Both lactobacilli showed growth at 1-4% NaCl concentration, while at a higher concentration of NaCl, from 6 to 7%, bacterial growth was decreased. Balamurugan et al. [38] also reported that *L. helveticus* showed tolerance to NaCl concentration from 1 to 7%, and Chowdhury et al. [41] observed that *L. plantarum* was able to tolerate NaCl concentration from 1 to 9%: both of these studies matched our findings. The isolates LBh5 showed the most substantial tolerance to NaCl concentration as compared to other isolates of *L. helveticus*. In *L. plantarum* isolates, LBp3 showed the most favourable results among the others, as shown in Figure 3; it was concluded that LBh5 showed the most positive results as compared to all isolates of *L. plantarum* and *L. helveticus* used in this study. The results were statistically analysed as shown in Tables S5 and S6.

3.3.3. Bile Salt Tolerance. Bile salt tolerance is an essential factor as it determines the survival of probiotics in the intestine. The bile salts present in the intestinal tract disrupt the cell membrane of bacteria entering the stomach. Probiotics have the ability to tolerate 0.05 to 0.3% of bile. The results calculated showed that the maximum growth of isolates was observed in the presence of bile salts up to 0.3%, as shown in Figure 4. The isolates, however, were less tolerant of the higher concentrations, i.e., 0.6% and 1%. Chowdhury et al. [41] observed that *Lactobacillus* subsp. isolated from yoghurt tolerated around 0.3% of bile concentrations. *Lactobacillus helveticus* isolates showed tolerance up to 0.3%, and maximum tolerance was observed at 0.1%. Barua et al. [42] and Rong et al. [43] observed that *Lactobacillus* spp. showed maximum growth at 0.1%, and maximum tolerance was shown at 0.3% concentration, which correlated to this study. Similar findings were reported in a work done by Baick and Kim [44]. The isolates LBp1 and LBp2 were able to withstand a higher bile salt concentration up to 1% as compared to other isolates of *L. helveticus* and *L. plantarum*. On the contrary, the findings of Succi et al. [45] showed the survival of lactobacillus bacteria at high bile concentrations up to 1%. The isolate LBh4 showed maximum tolerance, i.e., up to 0.3% as compared to other isolates of *L. helveticus*. It was concluded that *L. plantarum* and *L. helveticus* isolates can be considered

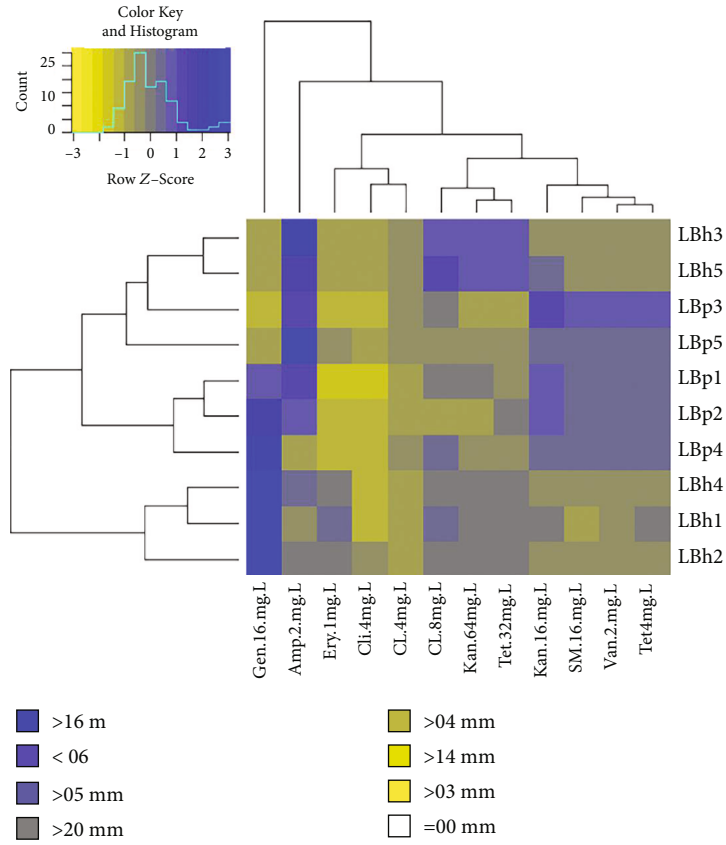

Figure 5: Antibiotic susceptibility of the isolates of *L. plantarum* (LBp) and *L. helveticus* (LBh): ampicillin (Amp.), vancomycin (Van), gentamicin (Gen), kanamycin, (Kan), streptomycin (SM), erythromycin (Ery), clindamycin (Cli), tetracycline (Tet), and chloramphenicol (CL). The tree is a dendrogram, which shows the hierarchy (similarity) between isolates and their activity against the different antibiotics. The graph was plotted using the R program.

to be suitable probiotic isolates as they tolerated 0.3% of bile concentration, which is typical of healthy men [9]. The statistical analysis of the result is shown in Tables S7 and S8.

3.3.4. Resistance to Antibiotics. Potential probiotic bacteria show a natural resistance to antibiotics. All *L. plantarum* isolates exhibited resistance towards clindamycin. Anas et al. [46] reported similar results in their study. The *L. plantarum* isolates showed sensitivity against ampicillin, gentamycin, and other antibiotics, and among all isolates, LBp1 showed the maximum zone of inhibition against kanamycin (38.1 mm), as shown in Figure S1. The isolates LBp3 and LBp5 showed resistance against gentamycin, kanamycin, erythromycin, clindamycin, and tetracycline. It was concluded that among these five isolates, LBp3 and LBp5 were resistant against most of the drugs. Similar observations were reported by Somashekaraiah et al. [47]. LBh3 and Lbh5 showed resistance against vancomycin, gentamycin, streptomycin, erythromycin, and clindamycin, as shown in Figure 5. All the isolates of *L. helveticus* showed sensitivity towards kanamycin and ampicillin. In the case of clindamycin, all the four isolates of *L. helveticus* showed resistance except LBh2 isolate, which was sensitive to this drug. The isolate LBh2 was sensitive to gentamycin with a maximum zone of inhibition of 25.7 mm. The antibiotic susceptibility tests showed that *L. helveticus* isolates had better probiotic properties as compared to *L. plantarum* isolates. Most of the *L. helveticus* isolates were found to be sensitive to all antibiotics used in this study as compared to *L. plantarum* isolates. Resistance to various antibiotics would help the isolates settle in gut microflora for longer; therefore, may alleviate gastrointestinal tract-related side effects due to antibiotics. Tables S9 and S10 show the statistical analysis of the results.

3.3.5. Antibacterial Activity. The antibacterial activity of *L. plantarum* and *L. helveticus* was measured against foodborne pathogens. All *L. plantarum* and *L. helveticus* isolates showed a maximum zone of inhibition against *St. aureus* (22.9 mm and 19.5 mm, respectively). In contrast, minimum zones were found against the gram-positive bacterium *S. pyogenes* (6.8 mm and 4.8 mm), as shown in Figure 6 and Figure S2. Al-Madboly and Abdullah [48] reported that *L. plantarum* showed weaker zones of inhibition against *St. aureus* and stronger zones of inhibition against *E. coli*, *Bacillus cereus*, and *Salmonella typhii*, which contradicts the results of this study. Succi et al. [45] found *L. plantarum* had more potent antagonistic activity against *St. aureus*, which correlates with our studies. In the case of gram-negative bacteria, the *L. plantarum* and *L. helveticus* isolates showed maximum zones of inhibition against *A. baumannii* (22.9 mm and 19.1 mm). The *L. plantarum* isolates showed the minimum

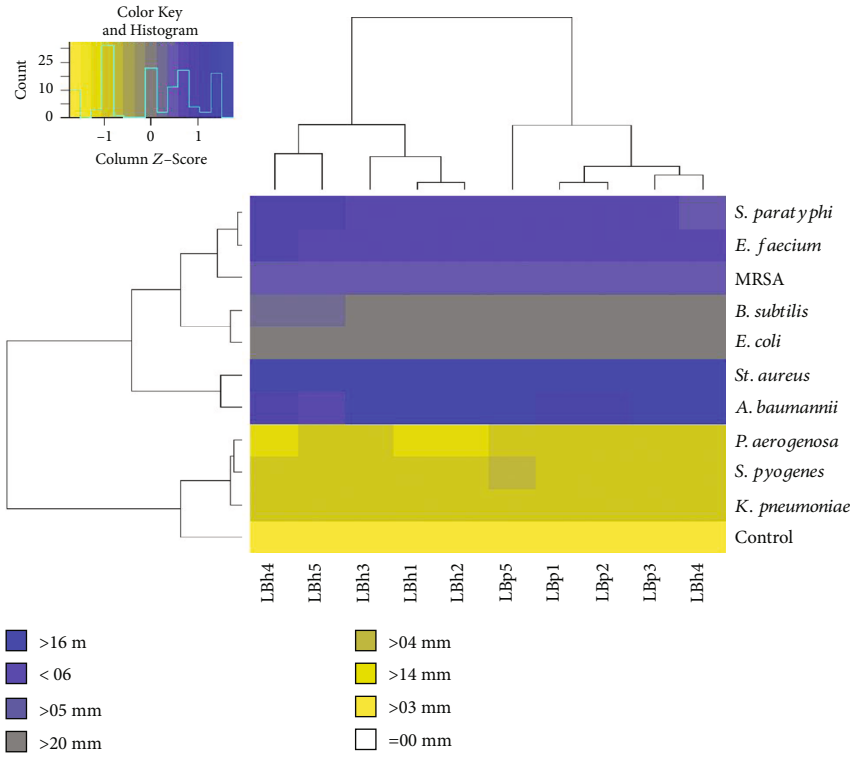

Figure 6: Antibacterial activity of different *L. plantarum* and *L. helveticus* isolates against foodborne pathogens. The tree is a dendrogram, which shows the hierarchy (similarity) between isolates and their activity against the different foodborne pathogens. The graph was plotted using R program.

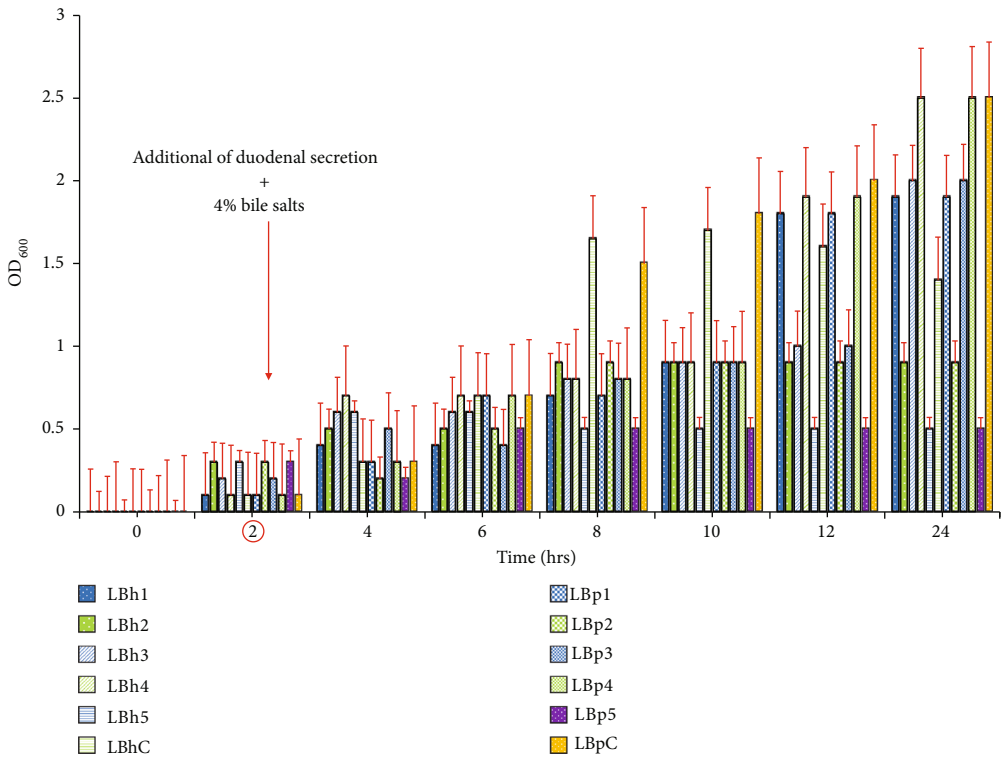

Figure 7: Response to stomach-duodenal stimulus of *L. plantarum* and *L. helveticus* isolates at different times. At 2 hours, duodenal secretion + 4 percent bile salts were added.

zone of inhibition against *K. pneumoniae* (5 mm), and *L. helveticus* isolates showed a minimum zone of inhibition against *P. aeruginosa* (5.6 mm). The isolates LBh5 showed significant results against gram-positive bacteria, while LBh3 isolates showed the most promising results against gram-negative bacteria as compared to other *L. helveticus* isolates. Gupta et al. [49] observed the same results in the case of *L. helveticus* antibacterial activity. The isolate LBp5 showed the most promising results against both gram-positive and gram-negative bacteria in comparison to all other isolates used in this study. Tables S11 and S12 present detailed results of the Pearson correlations.

3.3.6. Response to Stomach-Duodenal Stimulus. The response of lactic acid bacteria isolates to stomach-duodenal stimulus is shown in Figure 7. These results indicated that there was a resistance of most isolates to adverse conditions imposed by the composition of this medium. All the tested isolates were resistant to pH 3 after 2 hrs of incubation. Similar results were reported by Vizoso Pinto et al. [34], although their isolates showed resistance after 1 hour of incubation.

3.3.7. Arginine Hydrolysis Test. Both the isolates were shown to be arginine-positive as on a white background they displayed a bright orange colour.

4. Conclusion

Bacteriocin-producing lactic acid bacteria were successfully isolated from traditional Pakistani yoghurt. The experimental results showed that both *L. plantarum* and *L. helveticus* isolates were able to tolerate low pH, high bile salt, and NaCl concentrations. They also showed significant antimicrobial activity against test foodborne microorganisms and were resistant to many antibiotics. Thus, our isolates proved that they bear probiotic potential and could be used in different food items as probiotics. The *L. helveticus* isolates (LBh5) showed good probiotic properties as compared to other isolates investigated in this study. A further study on these isolates is needed to explore more about their role in increasing the shelf life of food or preventing or treating any gastrointestinal infections. The data was authenticated by replication. The Pearson correlation was applied which showed most of the results with significant values.

4.1. Limitations of the Study. Despite the promising results and uniqueness of our study, these isolates should be further studied to prove their importance in the dairy industry. It would be valuable to test the following characteristics: (i) molecular identification of isolates; (ii) adhesion to mucosal surfaces; (iii) clinical studies for human health; and (iv) technological properties (strain stability, viability into products, and bacteriophage resistance).

Data Availability

The authors will provide the data if needed.

Conflicts of Interest

The authors report no conflict of interest.

Acknowledgments

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Supplementary Materials

The supplementary files consist of antibiotic susceptibility test tables of *L. plantarum* and *L. helveticus* species and their pictorial representation used in this study. It also contains the tables of growth rate curve, tolerance against NaCl, pH, bile salts, response to stomach duodenal stimulus, antibacterial activity, and antibiotic resistance of *L. plantarum* and *L. helveticus* isolates against gram-positive and gram-negative foodborne bacteria and a pictorial representation of antibacterial activity for *L. plantarum* and *L. helveticus* species against *Staphylococcus aureus* species. *(Supplementary Materials)*

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Research Article

Effect of *Bacillus megaterium*-Coated Diets on the Growth, Digestive Enzyme Activity, and Intestinal Microbial Diversity of Songpu Mirror Carp *Cyprinus specularis* Songpu

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The present study was conducted to evaluate the effect of a *Bacillus megaterium*-coated diet on growth performance, digestive enzymes, and intestinal microbial diversity in Songpu mirror carp (*Cyprinus specularis* Songpu). The fish were manually fed two diets (a control diet and a *B. megaterium*-coated diet) three times daily until apparent satiation for 56 days. Compared with the control group, supplementation with the *B. megaterium*-coated diet enhanced the fish growth and significantly reduced the feed conversion ratio ($P < 0.05$). The activities of foregut amylase and lipase in the treatment group were significantly higher than those in the control group ($P < 0.05$). The activities of foregut, midgut, and hindgut proteases in the treatment group were all higher than those in the control group ($P > 0.05$). The results of sequencing the 16S rDNA genes of the microbiota through high-throughput sequencing showed that the diversity and abundance of the intestinal microflora increased along with Songpu mirror carp growth. The Songpu mirror carp fed a diet coated with *B. megaterium* displayed increased proportions of intestinal *Bacillus* and *Lactococcus* at the genus level, and both were significantly higher than those of the control group ($P < 0.05$). These results therefore suggest that dietary *B. megaterium* application can improve the growth and digestive enzyme activity of Songpu mirror carp and enrich the beneficial genus composition of its main intestinal microflora.

1. Introduction

Fish, as one of the main dietary sources of animal protein, play a very important role in the human food structure. However, in recent years, aquatic product safety has become one of the biggest factors hindering the development of aquatic products due to concerns about the safety of hormones, antibiotics, and excessive microorganisms [1]. With the rapid development of high-density intensive farming, the aquaculture water environment is deteriorating daily, resulting in disease outbreaks and even mass deaths of farmed animals. There is an urgent need to find environmentally friendly and safe alternatives to ensure the healthy growth of farmed animals [2].

As a new feed additive after the era of antibiotics, probiotics are considered to be important substitutes for feed antibiotics [3–5]. Studies have shown that the use of probiotics not only can promote the growth of aquatic animals and improve their survival rate but also can reduce the incidence of aquatic animal diseases [6, 7]. *Bacillus* is a saprophytic gram-positive bacterium commonly found in the breeding environment [8]. The research and application of *Bacilli* in the field of aquaculture have attracted much attention. The first application of probiotics in aquaculture was carried out by balancing the bacterial population in the water, and it achieved good results [9]. Some studies have shown that adding *Bacillus subtilis* to feed not only can promote the growth of aquatic animals and improve the activities of their digestive enzymes and their nonspecific immunity but also can improve the structure of their intestinal microflora [10–12]. In the middle of the 20th century, Chinese scholars began to study *Bacillus megaterium*, mainly focusing on screening

of strains, particularly their ability to break down organic matter, their nitrogen metabolism, and their application as probiotics in aquaculture wastewater treatment [13–17].

The common carp (*Cyprinus carpio*) is the most extensively cultured freshwater fish species in China, and the production of common carp was 2,962,218 t in 2018 [18]. Songpu mirror carp (*Cyprinus specularis* Songpu), a variety of common carp, accounts for an increasingly larger proportion of production due to its relatively faster growth, better disease resistance, higher meat conversion rate, and near absence of scales on the body surface [19, 20]. However, with the expansion and promotion of high-density farming, increasing feeding frequency and water pollution, the problems of food safety and quality have become increasingly serious.

Nutrients are among the most important and easily regulated factors affecting the resistance of aquatic animals. Food microbiology includes microorganisms that have both beneficial and deleterious effects on food quality and safety and may therefore be of concern for the public health. In this study, we aimed to evaluate the effect of *B. megaterium*-coated diets on the growth, digestive enzyme activity, and intestinal microbial diversity in Songpu mirror carp and to provide a theoretical basis for the practice of healthy and ecological fish farming.

2. Materials and Methods

2.1. Diet Preparation. B. megaterium was prepared in our laboratory [17]. The content of *B. megaterium* was 1×10^9 CFU/ml. The commercial feed was purchased from Zhejiang AIPHA Feed Co., Ltd., China. The guaranteed values of the feed product composition analysis are shown in Table 1. The fermentation broth of *B. megaterium* was uniformly sprayed on the surface of the commercial material at a rate of 100 ml/kg, and then, the sprayed feed was placed in a cool place for 1 h, after which it was used as the experimental group feed. The control group was fed the unaltered commercial feed.

2.2. Feeding Experiment. Songpu mirror carp (*Cyprinus specularis* Songpu) (body weight 77.75 ± 3.15 g) were obtained from the Hulan Experimental Station of the Heilongjiang River Fisheries Research Institute in Heilongjiang Province, China (45.97°N, 126.63°E). The fish were acclimatized to the laboratory conditions for 14 days and adapted to the experimental control feed prior to the experiment. Then, 90 healthy Songpu mirror carp were selected and randomly distributed into two groups (control group and experimental group). Each treatment was performed in triplicate, and each replicate had 15 fish. The control group was fed commercial feed. The treatment group was fed commercial feed coated with *B. megaterium*. The daily feeding amount was 3% of the body weight of the Songpu mirror carp, three times per day at 08 : 00, 12 : 00, and 19 : 00. The entire experimental period was 56 days. During the experimental period, the laboratory water was replaced by 1/3 of the water volume per week. The water quality was measured (using YSI professional plus, Ohio State, USA) daily during the experimental period, the water temperature ranged from 18 to 25°C, and an air compressor was used to add oxygen 24 h per day. The fish were weighed both at the start and at the end of the feeding trial.

Table 1: Product composition analysis guaranteed value of commercial feed (%).

Item	Contents
Nutrient levels	
Crude protein	≥32.0
Crude lipid	≥3.00
Crude fiber	≤10.0
Crude ash	≤16.0
Total phosphorus	≥0.50
Lysine	≥2.00
Moisture	≤12.0

2.3. Sample Collection. Before the feeding experiment, ten fish were randomly taken from the temporary culture tank, and their initial body weights were measured. Then, the intestinal tract was collected for the measurement of the initial intestinal microflora (initial group). At the end of the 56-day feeding trial, approximately 24 h after the last feeding, all fish were anesthetized with MS-222 diluted in the water at a concentration of 75 mg/l. These fish were counted and weighed to determine the weight gain rate (WGR), specific growth rate (SGR), and feed conversion rate (FCR, Table 2) [21].

After obtaining the final weight of all fish, five fish from each tank were randomly selected and placed in an ice plate for rapid dissection. Tissue samples, including the foregut, midgut, and hindgut, were collected with aseptic scissors, washed with aseptic physiological saline, weighed, and then prepared into a homogenate with aseptic physiological saline (1 : 4). All samples were immediately stored at −80°C in a freezer for digestive enzyme determination. Another five fish were also sampled randomly from each tank. The surfaces of the fish were disinfected with 75% alcohol before the fish were taken into a bioclean room. After further disinfection, the abdominal cavity was opened, the exterior of the intestine was wiped with 75% alcohol and washed four times with sterile water, and then the intestinal tract was collected for evaluation of the final intestinal microflora.

2.4. Digestive Enzyme Determination. Before measuring the immunity indexes, the pooled foregut, midgut, and hindgut were manually homogenized in a glass homogenizer with 0.86% NaCl (*w*/*v*) to obtain a 10% homogenate. After centrifugation (4000 rpm, 10 min) at 4°C, the supernatant, consisting primarily of crude enzyme liquid, was obtained. The activities of amylase, protease, and lipase in the foregut, midgut, and hindgut were analyzed spectrophotometrically using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China).

2.5. 16S rRNA Gene Amplification and Illumina Sequencing. Microbial DNA was extracted from the intestinal samples

Table 2: Growth of Songpu mirror carp (*Cyprinus specularis* Songpu) fed diets coated with *B. megaterium* for 56 days ($n = 3$).

Treatment	Control	*B. megaterium*
Initial body weight (g)	77.75 ± 3.15	77.75 ± 3.15
Final body weight (g)	118.78 ± 5.52	129.44 ± 10.78
WGR (%)	52.75 ± 1.04^{a}	71.03 ± 4.11^{b}
SGR (% day^{-1})	0.33 ± 0.01^{a}	0.42 ± 0.02^{b}
FCR	2.23 ± 0.17^{a}	1.28 ± 0.10^{b}

Note: in the same row, values with different small letter superscripts mean significant difference ($P < 0.05$), while those with the same or no letter superscripts mean no significant difference ($P > 0.05$). Weight gain rate (WGR, %) = 100 × (final body weight − initial body weight)/initial body weight. Specific growth rate (SGR, %day^{-1}) = 100 × (ln final body weight − ln initial weight)/days. Feed conversion ratio (FCR) = dry feed consumed (g)/(final body weight − initial body weight).

using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocols. The final DNA concentration and purification were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was determined by 1% agarose gel electrophoresis. The V4-V5 hypervariable regions of the bacterial 16S rRNA genes were amplified with primers 515F (5′-GTGCCAGC MGCCGCGG-3′) and 907R (5′-CCGTCAATT CMTTTRAGTTT-3′) by using a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCRs were conducted using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. The resulting PCR products were extracted from a 2% agarose gel and further purified using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) [22].

2.6. Statistical Analysis. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse/) with a novel "greedy" algorithm that simultaneously performs chimera filtering and OTU clustering. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the SILVA 16S rRNA database using a confidence threshold of 70%. The Chao and ACE estimator indexes were selected to identify the community richness, and the Shannon and Simpson indexes were used to identify the community diversity [23]. All of these indexes in our samples were calculated with QIIME (version 1.7.0) and prepared for display with R software (version 2.15.3). The statistical analyses were performed with the statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA). The data are expressed as the mean ± SD of three replicates. The data were subjected to one-way ANOVA, and when differences were found, the means were ranked using Duncan's multiple comparison test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of the B. megaterium-Coated Diet on Fish Growth. As shown in Table 2, the weight gain rate and specific growth rate of the treatment group (71.03%, 0.42% day^{-1}) were significantly increased compared with those of the control group (52.75%, 0.33% day^{-1}, $P < 0.05$), and the feed conversion ratio of the treatment group (1.28) was significantly lower than that of the control group (2.23, $P < 0.05$). No mortality was observed during the 56 days of the feeding trial.

3.2. Effect of the B. megaterium-Coated Diet on Digestive Enzyme Activity. The effects of the *B. megaterium*-coated diet on digestive enzyme activity are shown in Table 3. The foregut amylase activity in the treatment group was significantly higher than that in the control group ($P < 0.05$). The midgut and hindgut amylase activities in the treatment group were significantly higher than those in the control group, but there were no significant differences ($P > 0.05$). The foregut, midgut, and hindgut protease activities of the treatment group were higher than those of the control group, but there were no significant differences ($P > 0.05$). The foregut lipase activity in the treatment group was significantly higher than that in the control group ($P < 0.05$). The midgut and hindgut lipase activities in the treatment group were basically the same as those in the control group, and there were no significant differences ($P > 0.05$).

3.3. Effect of the B. megaterium-Coated Diet on Intestinal Microbial Diversity and Richness. The intestinal microbial diversity of the Songpu mirror carp was determined by high-throughput sequencing. The average number of OTUs detected from the initial group sample was 63. After 56 days, the average number of OTUs detected in the control and treatment groups was 114 and 196, respectively. The coverage index of all samples was above 0.97, indicating that there was a high detection rate. As shown in Table 4, the Chao and ACE indexes, which reflect the richness of the intestinal microflora of Songpu mirror carp, were significantly higher in the treatment group after 56 days than in the control and initial groups ($P < 0.05$). The Shannon index of intestinal community diversity in the treatment group was significantly higher than that in the control group ($P < 0.05$). The Simpson index of intestinal community diversity in the treatment group was significantly lower than that in the control group ($P < 0.05$). There were no significant differences for the above four indexes among the control and initial groups ($P > 0.05$).

3.4. Effect of the B. megaterium-Coated Diet on the Composition and Changes in the Main Microbiota in the Intestine of the Songpu Mirror Carp. An intestinal microbial richness of more than 1% at the phylum level was taken as the main microflora for the statistics presented in Figure 1. The dominant phylum in the intestine of the initial group was predominantly Fusobacteria (86.36%) and Bacteroidetes

Table 3: Activities of digestive enzymes in the intestine (foregut, midgut, and hindgut) of Songpu mirror carp fed diets coated with *B. megaterium* (U/g protein).

Treatment	Foregut		Midgut		Hindgut	
	Control	*B. megaterium*	Control	*B. megaterium*	Control	*B. megaterium*
Amylase	13.86 ± 6.24^{a}	64.64 ± 7.93^{b}	67.46 ± 17.73	39.57 ± 17.80	55.37 ± 4.63	56.03 ± 5.69
Protease	2.02 ± 0.04	2.02 ± 0.24	2.26 ± 0.05	2.38 ± 0.14	1.52 ± 0.04^{a}	1.87 ± 0.19^{b}
Lipase	520.13 ± 90.28^{a}	973.29 ± 50.12^{b}	925.27 ± 110.81	941.74 ± 170.45	706.31 ± 46.15	671.14 ± 150.31

Note: in the same column, values with different small letter superscripts mean significant difference ($P < 0.05$), while those with the same or no letter superscripts mean no significant difference ($P > 0.05$).

Table 4: Effects of *B. megaterium*-coated diets on abundance and diversity of the intestinal microflora of Songpu mirror carp.

Groups	Enrichment index		Diversity index	
	Chao	ACE	Simpson	Shannon
Initial	123.52 ± 9.79^{a}	120.63 ± 11.78^{a}	0.42 ± 0.13^{a}	0.87 ± 0.04^{a}
Control	137.66 ± 20.65^{a}	140.93 ± 19.10^{a}	0.59 ± 0.01^{a}	0.99 ± 0.01^{a}
B. megaterium	204.49 ± 8.81^{b}	205.24 ± 7.90^{b}	0.11 ± 0.01^{b}	2.66 ± 0.01^{b}

Note: in the same column, values with different small letter superscripts mean significant difference ($P < 0.05$), while those with the same or no letter superscripts mean no significant difference ($P > 0.05$).

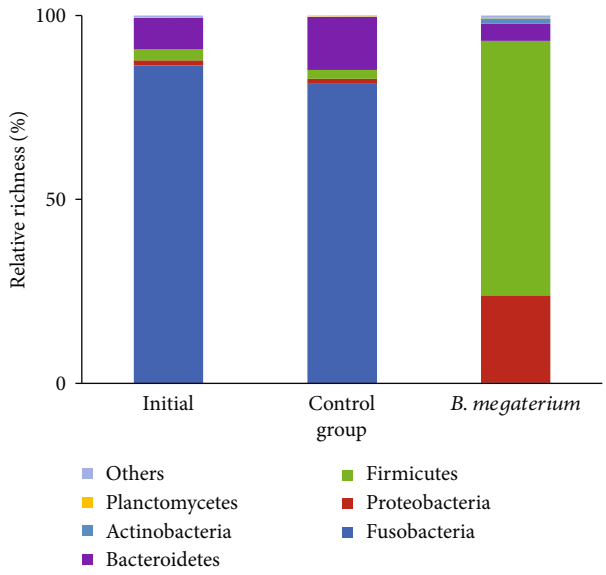

Figure 1: Intestinal microbial compositions at the phylum level in Songpu mirror carp fed a diet coated with *B. megaterium* for 56 days.

(8.60%) before the experiment began. After the 56-day feeding trial, the number of the main intestinal microflora increased in each group. In the control group, the dominant phyla were Fusobacteria (86.36%), Bacteroidetes (14.4%), Firmicutes (2.42%), and Proteobacteria (1.37%). In the treatment group, the dominant phyla were Firmicutes (69.15%), Proteobacteria (23.8%), Bacteroidetes (4.65%), and Actinobacteria (1.43%). An intestinal microbial richness of more than 0.5% at the genus level was taken as the main microflora for the statistical calculations, shown in Table 5. There were only 5 genera of the main intestinal microorganisms in the initial group. After the 56-day feeding trial, the main intestinal microorganisms increased to 11 genera in the treatment group. The most abundant microorganism in the intestine of Songpu mirror carp in the initial group was *Cetobacterium*. After the 56-day feeding trial, the most abundant microorganism in the control group was still *Cetobacterium*. In the control group, the 11 dominant genera were *Bacillus*, *Lactococcus*, *Pseudomonas*, *Stenotrophomonas*, *Psychrobacter*, *Brochothrix*, *Myroides*, *Arthrobacter*, *Flavobacterium*, *Comamonadaceae*_unclassified, and *Yersinia*. The Songpu mirror carp fed a diet coated with *B. megaterium* displayed increased proportions of intestinal *Bacillus* and *Lactococcus* at the genus level, which were both significantly higher than those of the control group ($P < 0.05$).

4. Discussion

Probiotics such as *Bacilli* have been widely used in aquaculture. Some studies have shown that the addition of various strains of *Bacillus* spp. to larva feed has achieved good results and has a good promoting effect on the growth of fish larvae [24, 25]. The addition of bacteria (strain CA2) as a food supplement to xenic larval cultures of the oyster *Crassostrea gigas* consistently enhanced the growth of the larvae during different seasons of the year [26]. Manipulation of microbiota using probiotics has been reported as a worthy practice for aquaculture to control or inhibit pathogenic bacteria and to improve growth performance and the activity of digestive enzymes [27]. In comparison to the untreated control group, the final weight and weight gain were significantly greater in shrimp fed a mixture of two probiotic strain diets [28]. In this study, it was found that a *B. megaterium*-coated diet increased the weight gain rate and specific growth rate and reduced the feed conversion ratio, indicating that *B. megaterium*-coated diets can promote the growth of Songpu mirror carp. Similar results were found in catfish *Clarias* sp., where the addition of *B. megaterium* PTB 1.4 to their feed

Table 5: Percentages of the main genus of the intestinal microflora in Songpu mirror carp fed a diet coated with *B. megaterium* for 56 days.

Genus	Treatments		
	Initial	Control	*B. megaterium*
Cetobacterium	46.33 ± 11.25	75.75 ± 15.28	—
Lactococcus	—	—	27.46 ± 11.32
Bacillus	—	—	34.83 ± 9.48
*Enterobacteriaceae*_unclassified	—	—	—
Bacteroides	9.46 ± 6.45	11.89 ± 8.46	—
*Comamonadaceae*_unclassified		—	0.78 ± 0.43
Yersinia	0.32 ± 0.21	—	0.63 ± 0.53
Pseudomonas	—	—	11.33 ± 8.73
Pseudoxanthomonas	—	—	—
*Fusobacteriales*_unclassified	3.84 ± 2.98	5.66 ± 4.12	—
Stenotrophomonas		—	5.66 ± 4.23
Psychrobacter	—	—	3.94 ± 2.84
Leucobacter	—	—	—
Brochothrix	—	—	3.03 ± 2.13
Myroides		—	2.97 ± 1.96
Barnesiella	1.12 ± 1.01	2.46 ± 1.32	—
Rhodobacter		—	—
Arenimonas		—	—
Clostridium		—	—
Arthrobacter		—	1.36 ± 0.96
Flavobacterium		—	1.07 ± 0.83
Others	2.94 ± 1.46	2.85 ± 1.56	7.31 ± 4.23

Notes: — means that the percentage of the genus accounting for the total intestinal microflora is less than 0.5%.

significantly improved their growth rate [29]. Similar results were also found in *Penaeus monodon*, for which higher FCR and SGR values were obtained after the addition of *Bacillus cereus* [30].

Probiotic bacteria are capable of producing digestive enzymes that help fish use feed nutrients and digest them [31]. The addition of *B. megaterium* to plant protein meals can promote intestinal morphology development and increase digestive enzyme activity [8]. The study of probiotics for the common carp *Cyprinus carpio* based on growth performance and digestive enzyme activities showed that the mean digestive enzyme activities of all probiotic treatment groups were significantly different from that of the control [27]. In the present study, a *B. megaterium*-coated diet had a positive and important effect on digestive enzyme activities, especially those of amylase and protease in the foregut, midgut, and hindgut. Similar results were also found in tilapia, which showed improvement in food digestion and growth after *Bacillus* NP5 was added to their feed [32]. A higher level of enzyme activity obtained with diets containing probiotics improved the digestion of protein, starch, fat, and cellulose, which might, in turn, explain the better growth observed with the probiotic-supplemented diets [24]. Digestive enzymes help fish break down and digest nutrients in feed, making it easier for the fish to absorb the nutrients in the feed [29].

Advances in high-throughput sequencing have enabled an extensive catalog of metagenomic samples, providing insight into the diversity of microbial species from a wide variety of sources, including the ocean, soil, and human body. These studies use both 16S rRNA gene sequencing to determine phylogenetic relationships and more comprehensive shotgun sequencing to predict the detailed species and gene composition [33]. The richness index and diversity index are important indexes to detect the diversity and complexity of microorganisms in samples [2, 34]. In this study, with the growth of Songpu mirror carp, the intestinal microbial diversity and richness were increased, and the Chao and ACE indexes, which reflect the richness of the intestinal microflora of Songpu mirror carp, were significantly higher in the treatment group than in the control group. In addition, the Shannon index of intestinal community diversity in the treatment group was significantly higher than that in the control group. Similarly, the results also showed that the intestinal microflora structure was changed when juvenile blunt snout bream were fed diets supplemented with different levels of *Bacillus subtilis* [3, 35]. The addition of probiotics to feed can also change the number and structure of the original microflora in the intestinal tract of *Litopenaeus vannamei* and promote the complex interactions between the microbial communities in the intestinal tract of *Litopenaeus vannamei* [36].

In this study, the Songpu mirror carp fed a diet coated with *B. megaterium* displayed increased proportions of intestinal *Bacillus* and *Lactococcus* at the genus level, which were both significantly higher than those of the control group. The results also indicated that the composition and proportions of the main microbiota in the intestine of Songpu mirror carp can be changed with *B. megaterium*-coated diets. In conclusion, the use of *B. megaterium*-coated diets can significantly enhance fish growth and reduce the feed conversion ratio, improve the activities of digestive enzymes, and enrich the beneficial genus composition of the main intestinal microflora.

Data Availability

All data was provided in the article, and there are no more data to be uploaded.

Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

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Research Article

Peptide Extracts from Native Lactic Acid Bacteria Generate Ghost Cells and Spheroplasts upon Interaction with *Salmonella enterica*, as Promising Food Antimicrobials

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Protecting foods from contamination applying peptides produced by lactic acid bacteria is a promising strategy to increase the food quality and safety. Interacting with the pathogen membranes might produce visible changes in shape or cell wall damage. Previously, we showed that the peptides produced by *Lactobacillus plantarum* UTNGt2, *Lactobacillus plantarum* UTNCys5-4, and *Lactococcus lactis* subsp. *lactis* UTNGt28 exhibit a broad spectrum of antibacterial activity against several foodborne pathogens *in vitro*. In this study, their possible mode of action against the commensal microorganism *Salmonella enterica* subsp. *enterica* ATCC51741 was investigated. The target membrane permeability was determined by detection of beta-galactosidase release from ONPG (o-nitro-phenyl-L-D-galactoside) substrate and changes in the whole protein profile indicating that the peptide extracts destroy the membrane integrity and may induce breaks in membrane proteins to some extent. The release of aromatic molecules such as DNA/RNA was detected after the interaction of *Salmonella* with the peptide extract. Transmission electronic microscopy (TEM) micrographs depicted at least four simultaneous secondary events after the peptide extract treatment underlying their antimicrobial actions, including morphological alterations of the membrane. Spheroplast and filament formation, vacuolation, and DNA relaxation were identified as the principal events from the Gt2 and Cys5-4 peptide extracts, while Gt28 induced the formation of ghost cells by release of cytoplasmic content, filaments, and separation of cell envelope layers. Gel retarding assays indicate that the Gt2 and Gt28 peptide extracts are clearly binding the *Salmonella* DNA, while Cys5-4 partially interacted with *Salmonella* genomic DNA. These results increased our knowledge about the inhibitory mechanism employed by several peptide extracts from native lactic acid bacteria against *Salmonella*. Further, we shall develop peptide-based formulation and evaluate their biocontrol effect in the food chains.

1. Introduction

The contamination of food by microorganisms is an overarching problem of the food industry. Salmonellosis causes great harm to the livestock and poultry industries; thus, its effective prevention and control are of great importance to animal husbandry and public health [1]. Efforts to reduce the bacterial growth and the associated diseases along with the expanding bacterial resistance have stimulated research to search for novel antimicrobial agents and related technologies. Several antimicrobial peptides (AMPs) have been isolated from a wide range of species, including single-celled microbes, as they have small molecular weights with good solubility, strong thermal stability, and a broad spectrum of antibacterial activity and are available from a wide range of source materials [2, 3]. To be effective against Gram-negative bacteria, AMPs must be able to disrupt either or both inner and outer membranes and induce apoptosis [4]. AMPs from lactic acid bacteria are promising candidates for the treatment of infectious diseases and food preservation. They usually have a relatively narrow spectrum of inhibition, and their effectiveness towards Gram-negative bacteria depends on the bacterial producer [5, 6]. These peptides are classified according to their structure and properties

as follows: (a) class I, called lantibiotics, are very small (>5 kDa) with nisin and lactocin as representatives; (b) class II, called nonlantibiotics, are small (<10 kDa), heat-stable, nonmodified, cationic, hydrophobic peptides containing a double glycine leader peptide, with pediocin PA1, leucocin A, lactococcin G, and plantaricin A as representative of class IIa and class IIb, respectively; (c) class III, larger in size, >30 kDa, are heat-stable peptides with enterolysin A and helveticin J as representative [7]. Within class II, plantaricin EF (PlnEF), plantaricin W, plantaricin JK, and lactacin F are produced by some *Lactobacillus plantarum* species and display antimicrobial activity against both Gram-positive and Gram-negative bacteria [8–10]. Previous research has indicated that some two-peptide bacteriocins induce cell membrane permeabilization or leakage of cellular materials across the cell membrane [10]. Although it was recognized that two-peptide bacteriocins target cellular membranes, there is limited evidence of this mechanism so far [11, 12]; thus, the elucidation of the molecular mechanism of action remains complex [13].

Recently, we showed that the antimicrobial peptide extracts from Gt2, Cys5-4, and Gt28 strains exhibited a broad spectrum of antibacterial activity against several foodborne pathogens; particularly, the mode of action against the commensal microorganisms *Salmonella enterica* subsp. *enterica* ATCC51741, *Escherichia coli* ATCC25922, and *Shigella sonnei* ATCC25931 was investigated. The strains Gt2, Cys5-4, and Gt28 produce two-peptide bacteriocins, such as plantaricin W of Gt2 and Cys5-4 and lacticin 3147, lactococcin M, and lactococcin A of Gt28. Preliminary data indicated that the peptide extract, defined as a mixture of larger size peptides or protein-like components from each individual strain, exerted a bacteriolytic mode of action [14–16]. The efficacy of each peptide extract against *Salmonella* depends on the dose applied, the developmental stage of the target cells, and the incubation time. The Gt28 peptide extract is more effective in inhibition (all bacterial cells were killed upon 3 hours of incubation) than Cys5-4 and Gt2 (marginal inhibitory effect detected at 6 hours of incubation). As the antimicrobial peptide extracts act at the cell membrane, several morphological and ultrastructural changes might occur as the secondary reactions of a live cell to the damage done to its membrane; however, this study was designed to understand the direct injury induced by each peptide extract when individually applied to *Salmonella enterica* on the bacterial envelope or the effect on the intracellular DNA that might explain the microbicidal activity, being a promising approach for biological control of microorganisms in food industry.

2. Materials and Methods

2.1. Preparation of Partially Purified Peptide Extract and Determination of Minimum Inhibitory Concentration (MIC). Peptide extracts of Gt2, Cys5-4, and Gt28 from the producer cells of *Lactobacillus plantarum* UTNGt2 (GenBank accession no. KY041688.1), *Lactobacillus plantarum* UTNCys5-4 (GenBank no. KY041686.1), and *Lactococcus lactis* subsp. *lactis* UTNGt28 (GenBank accession no. MG675576.1), respectively, were obtained as previously described [14–16]. Briefly, the overnight bacterial culture was used to extract cell-free supernatant (CFS) by centrifugation at 13,000 × *g* for 30 min (4°C). The CFS was

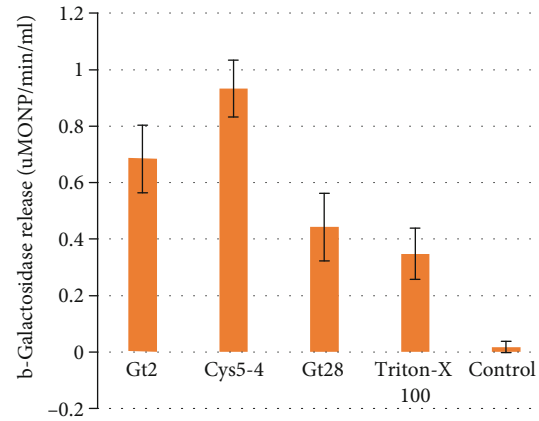


Figure 1: The cytoplasmic membrane permeation of *Salmonella enterica* subsp. *enterica* ATCC51741. Bacteria (after 120 min incubation) were removed by centrifugation, and enzyme release was assayed in the cell-free supernatant. Legend: 1 X MIC of Gt2, 1 X MIC of Cys5-4, 1 X MIC of Gt28 peptide extracts; Triton X-100 (0.1%); control: untreated cells. Results are representative of three independent experiments each made in triplicate. The release of o-nitrophenol (ONP) per minute per milliliter and calculated as described: [A415 × 1000/sample volume (μl)]/reaction time (min) × 4.86, where A415 was the absorbance at 415 nm and 4.86 was the coefficient of extinction (mM^{-1} cm^{-1}) of ONP, respectively.

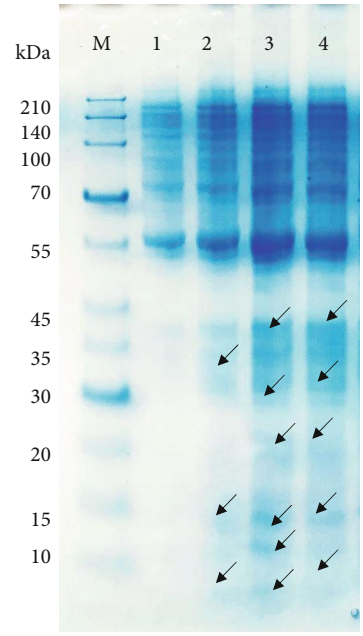
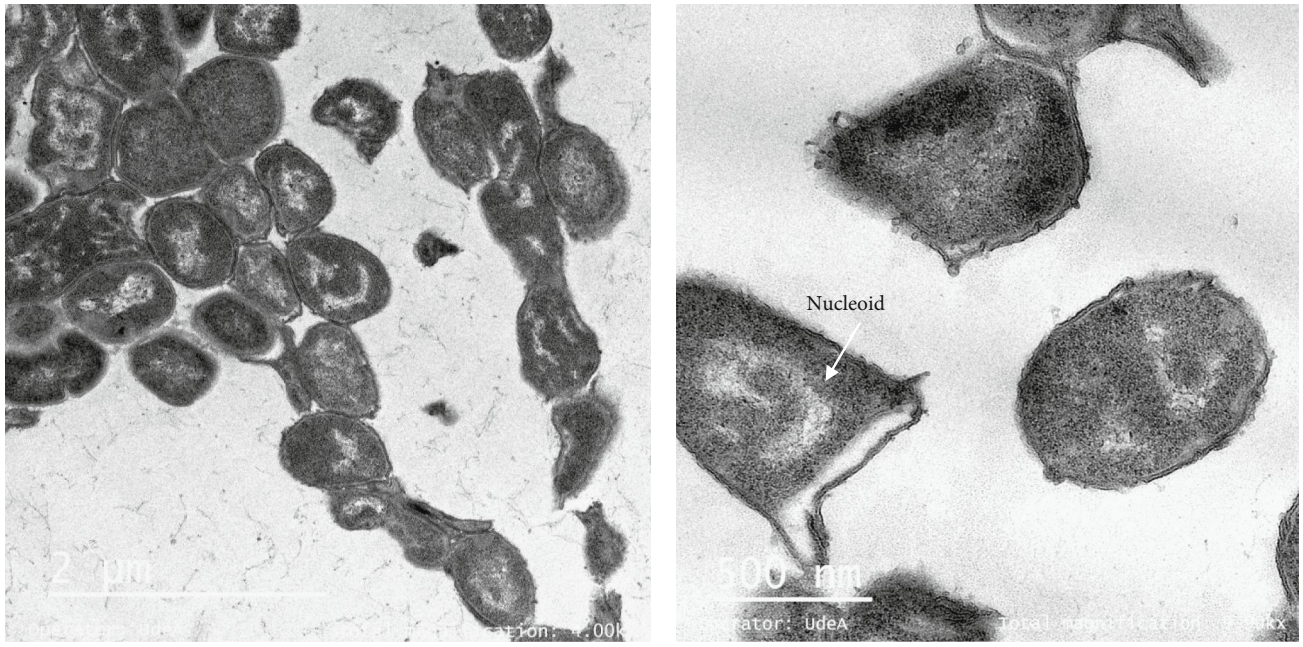


Figure 2: Different expression profiles of *Salmonella enterica* subsp. *enterica* ATCC51741 treated with peptide extract. Legend: lane 1: untreated *Salmonella* (control), lane 2: Gt2, lane 3: Cys5-4, and lane 4: Gt28 at 1 X MIC and 24 h of incubation. Arrows indicate different bands. M: molecular marker (Takara, Clearly Protein Ladder); arrows indicate different bands.



(a) (b)

Figure 3: Transmission electron microscopic image of *Salmonella enterica* subsp. *enterica* ATCC51741 cells (exponential phase). Ten different images were taken, and representative images are shown.

filtered using a 0.22 μm porosity syringe filter (no. STF020025H, ChemLab Group, USA). Then, 60-80% ammonium sulfate was added to precipitate the peptides that were recovered in 25 mM ammonium acetate (pH 6.5), desalted by using a Midi Dialysis Kit (cat no. PURD10005-1KT, Sigma-Aldrich Co. LLC, Saint Louis, MO, USA), preequilibrated with phosphate buffer (pH 7.0), and stored at -20°C before use. Titer, estimated as AU/ml, is defined as the highest dilution that inhibited the growth of the indicator strain. Antimicrobial activity of each peptide was performed using the agar-well diffusion method [17]. The minimum concentration that inhibits 50% of the target was determined as previously described [14]. The MIC was determined as 6400 AU/ml for all peptide extracts.

2.2. The Effect of Peptide Extract on Target Cytoplasmic Membrane Permeabilization. *Salmonella enterica* subsp. *enterica* ATCC51741 was maintained as frozen stock cultures in nutrient broth (Difco, Detroit, MI, USA) containing 20% (*v*/*v*) glycerol. The peptide extracts of each strain at concentration 1 X MIC was added to the indicator strain culture (100 ml) at the midexponential phase followed by incubation at 37°C for 24 h. Similarly, the bacterial cells were treated with 0.1% Triton X-100 and used as positive control. To investigate the effect of each peptide extract on membrane permeabilization, the ONPG (o-nitro-phenyl-L-D-galactoside, no. N1127, Sigma-Aldrich Co. LLC, Saint Louis, MO, USA) substrate was used as previously described [16]. The hydrolysis of ONPG to o-nitrophenol (ONP) was monitored at 415 nm at 120 min of incubation. To distinguish between the cytoplasmic enzyme release and peptide uptake to the cells, β-galactosidase release was measured from the supernatant [16].

2.3. Cell Membrane Integrity Assay. The overnight bacterial suspension of *Salmonella enterica* subsp. *enterica* ATCC51741 washed twice with 1X PBS (phosphate-buffered saline, pH 7.5) were treated individually with 1 X MIC of each peptide extract and incubated for 24 hours at 37°C as described [16]. The cell culture without any treatment was used as control. The DNA/RNA molecules were detected by electrophoresis in 1% agarose gel with ethidium bromide, running in 1X TBE (Tris-borate EDTA, pH 8.0) buffer (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA) after extraction with chloroform (1 : 1, *v*/*v*), and precipitated with isopropanol and ammonium acetate (3 M).

2.4. SDS-PAGE of the Target Whole-Cell Proteins after the Treatment with the Peptide Extract. The effect of each individual peptide extract was analyzed using the SDS-PAGE method as previously described [16]. Samples containing *Salmonella* in nutrient broth were incubated independently with 1 X MIC of Gt2, Cys5-4, and Gt28 peptide extract at 37°C for 24 h. The cell pellet was suspended in 1X SDS-PAGE loading buffer, boiled for 5 min at 100°C, and centrifuged at 300 rpm. The supernatants of treated and untreated cells with each peptide extract were used in SDS-PAGE electrophoresis. The tricine-SDS-PAGE method using RunBlue Bis-Tris protein gels (12%) and Dual Cool Mini Vertical PAGE/Blotting Systems (Expedeon, Abcam, Cambridge, MA, USA) was used. The gel was stained with InstantBlue ready-to-use stain (Expedeon, Abcam, Cambridge, MA, USA) using a protocol recommended by the manufacturer.

2.5. The Effects of Peptide Extract on the Bacterial Cells under TEM. The test bacteria were treated independently with the peptide extracts at 1 X and 2 X MIC and incubated for 24 h

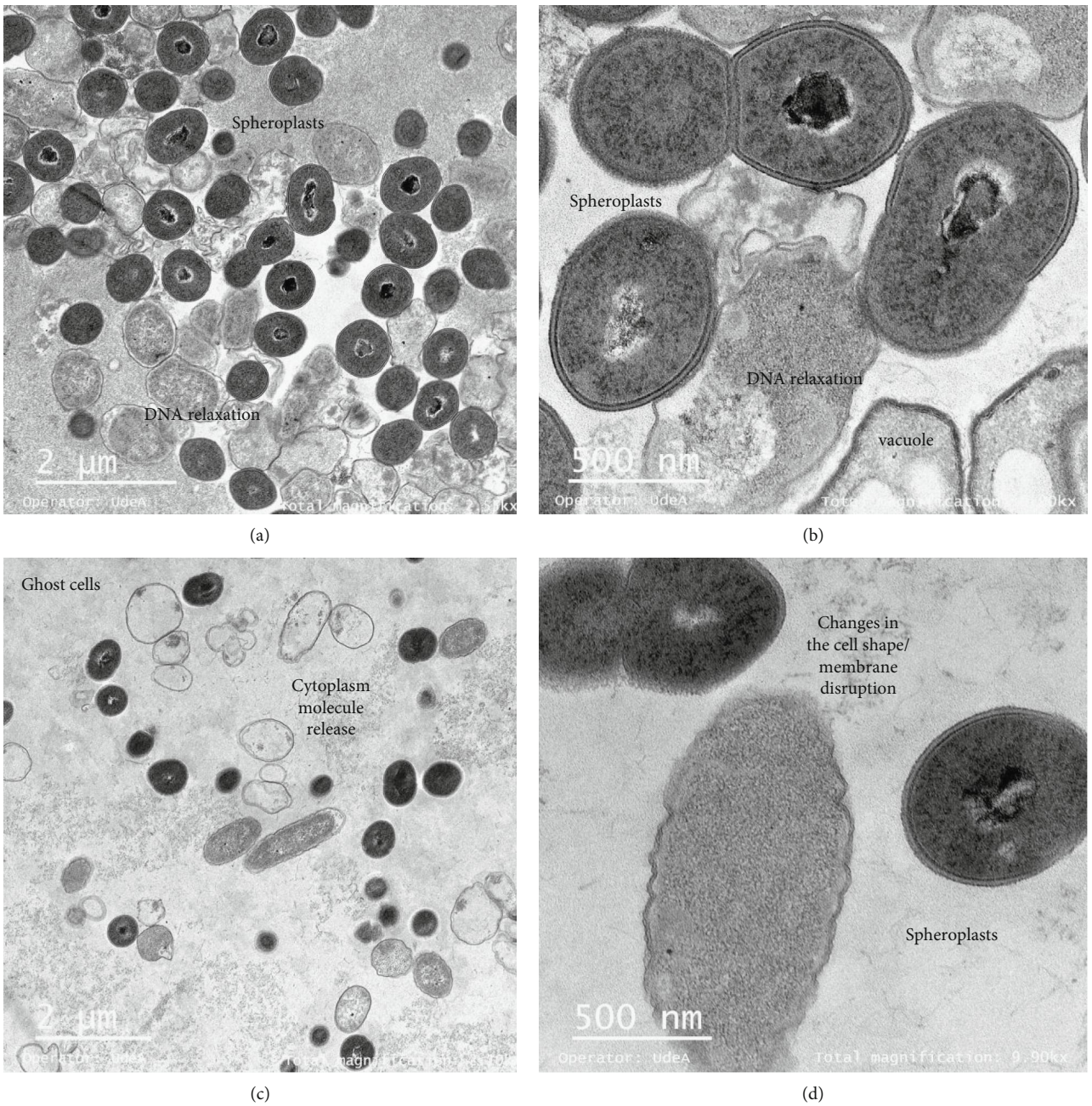


Figure 4: Micrographs of *Salmonella enterica* subsp. *enterica* ATCC51741 treated with Gt2 peptide extract: (a, b) 1 X MIC; (c, d) 2 X MIC. Ten different images were taken for each peptide extract concentration, and representative images are shown.

at 37°C. The peptide extract was washed away thrice by using sodium phosphate buffer by centrifuging at 10,000 × g for 15 min. The cells were fixed with 2.5% glutaraldehyde and stored overnight at 4°C. The buffers and dehydration protocol used were developed by the Laboratory of Electronic Microscopy, University of Antioquia (Medellin, Colombia). Briefly, the samples were washed thrice with cacodylate buffer and postfixed for 1 h with osmium tetroxide 1% and cacodylate buffer in 1 : 1 ratio. Then, they were washed thrice in cacodylate buffer (10 min) and incubated overnight in the same buffer. The samples were then washed thrice with water, once with uranyl acetate (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA), and again thrice with water. The samples were dehydrated in a graded ethanol series and embedded in Epon (resin). Ultrathin sections were prepared and coated on copper grids and stained with uranyl acetate (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA) and lead citrate (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA). The grids (10 random sections per treatment) were examined using the Tecnai G2 F20 transmission electron microscope (FEI Company, USA). Untreated cells of *Salmonella* were used as control.

2.6. Gel Retardation Assay. Genomic DNA of *Salmonella enterica* subsp. *enterica* ATCC51741 was isolated using

Table 1: Summary of the various changes that are observed in antibacterial-treated *Salmonella* cells with Gt2, Cys5-4, and Gt28 peptide extracts.

Peptide/concentration applied (MIC)	Alteration	Brief description
Gt2/1 X MIC	Spheroplasts	Spherical bacteria; changed shape, the inner and outer membranes were intact, but they lost of peptidoglycans.
Gt2/2 X MIC	Spheroplasts	Spherical bacteria; changed shape, the inner and outer membranes were intact, but they lost of peptidoglycans.
	"Ghost's cells"	The bacterial cells devoid or near-devoid of cytoplasm.
	Filamentation	Bacteria become much larger than normal.
	Intracellular vacuolation	Round-shaped transparent areas present in the bacterial cytoplasm.
Cys5-4/1 X MIC	Spheroplasts	Spherical bacteria; changed shape, the inner and outer membranes were intact, but they lost of peptidoglycans.
Cys5-4/2 X MIC	Filamentation	Bacteria become much larger than normal.
	Intracellular vacuolation	Round-shaped transparent areas present in the bacterial cytoplasm.
Gt28/1 X MIC	Filamentation	Bacteria become much larger than normal.
	Cell envelope layer separation	An increase in the distance between the cytoplasmic membrane and outer membrane of the gram-negative cell envelope.
	"Ghost's cells"	The bacterial cells devoid or near-devoid of cytoplasm.
	Cell lysis	The cell membrane was damaged leading to leakage.
Gt28/2 X MIC	"Ghost's cells"	The bacterial cells devoid or near-devoid of cytoplasm.
	Cell envelope layer separation	An increase in the distance between the cytoplasmic membrane and outer membrane of the gram-negative cell envelope.

PureLink Genomic DNA Mini Kit (Invitrogen USA) according to the manufacturer's instruction. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at the final concentration 3 μg/μl. The DNA was individually mixed with each Gt2, Cys5-4, and Gt28 peptide extract at the final concentration 1 X MIC in phosphate buffer (pH 7.0) and incubated at room temperature for 60 min. The ratio between the peptide mixture and DNA was 200/3 (v/v). After incubation, 5 μl DNA mixed with 1 μl 10X loading buffer was analyzed on 1% agarose gel.

3. Results and Discussion

3.1. The Peptide Extracts of Gt2, Cys5-4, and Gt28 Induced Leakage of Cytoplasmic Content from Salmonella Cells. The ability of Gt2, Cys5-4, and Gt28 to permeate *Salmonella* cells was evaluated as a function of cytoplasmic beta-galactosidase release, with bacteria grown in lactose-containing medium. We previously showed that Gt2 has the capacity to increase the *E. coli* cytoplasmic membrane permeability [16]. In this study, the results indicated that the peptide extract of Cys5-4 and Gt2 caused considerable release of the enzyme into the medium at 120 min of incubation (Figure 1). Less membrane permeability was observed with Gt28 and Triton X-100, while no activity was detected in the untreated *Salmonella* cells. Although Triton X-100 is one of the most widely used nonionic surfactants to permeabilize the living cell membrane [18], in this study, it showed lower permeability than Cys5-4 and Gt2 peptide extracts. An early study indicated that a C-type lectin, RegIIIβ (regeneration gene family protein III) protein, enhanced antimicrobial effect towards *Salmonella* typhimurium when the outer membrane integrity was compromised by Triton X-100 [18].

The *Salmonella* cell membrane was compromised after exposure to the peptide extract; thus, a smear DNA with many bands that cannot be easily distinguished along with the RNA molecules was detected in the electrophoresis gel (Figure S1). No DNA/RNA was detected in the untreated sample. Previously, the genomic instability of Gram-negative bacteria induced by antibiotics was observed, but the molecular mechanism was partially understood [19]. In this study, we suggest that the peptide extract possibly might induce double-stranded breaks in the DNA or may interact with target proteins in a manner that might induce partial DNA damage.

3.2. Whole Protein Profile of Salmonella enterica ATCC51741 Treated with Peptide Extract. The protein profile depends on the bacterial species, and the interaction with the antimicrobial agents might induce changes or blocking of the protein to expressed [20]. To evaluate the effect of the peptide on the protein profile of *Salmonella*, the cell culture was incubated overnight with the peptides independently. To confirm the membrane permeabilization action of the peptides, *Salmonella* cells were analyzed for soluble proteins by SDS-PAGE. As shown in Figure 2 (lane 1), higher molecular mass proteins were expressed in the lack of peptide extract sample, while in the peptide extract-treated cells, both high- and lower-mass proteins were detected (Figure 2, lanes 2, 3,

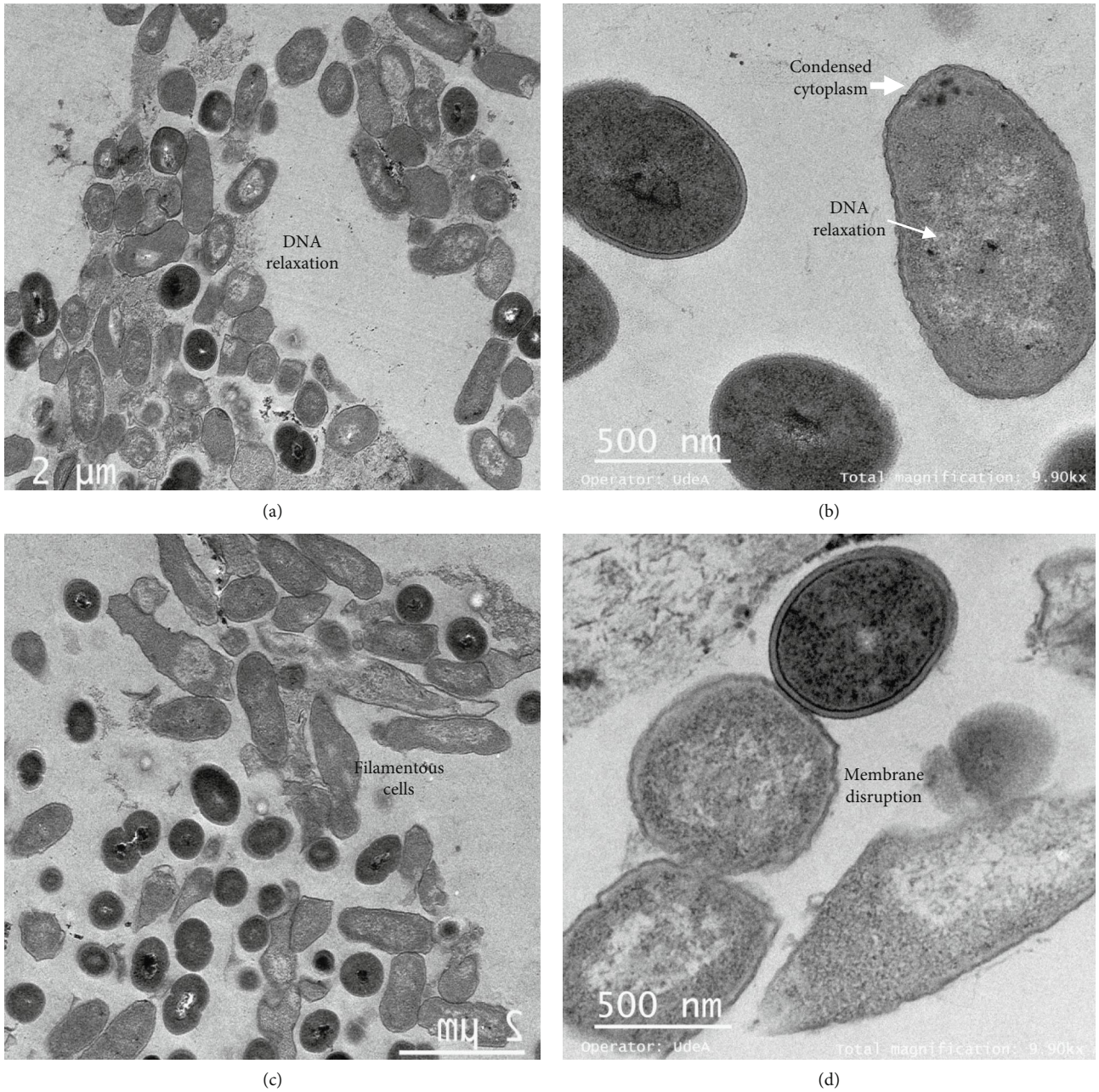


Figure 5: Micrographs of *Salmonella enterica* subsp. *enterica* ATCC51741 treated with Cys5-4 peptide extract: (a, b) 1 X MIC; (c, d) 2 X MIC. Ten different images were taken for each peptide extract concentration, and representative images are shown.

and 4). The protein profile was distinct, but the identity of the proteins was not further investigated in this study. At this point, we do not know if some bands belong to the peptide itself as they might interfere with the bacterial protein having the same size. An early study indicated that by treating *E. coli* cells with sericin, the bacterial proteins gradually disappeared after 12 h of incubation as shown in SDS-PAGE analysis, which indicated that their expression was blocked [20]. In this study, the peptide extract of Gt2, Cys5-4, and Gt28 may induce breaks of membrane proteins to some extent, this being in concordance with the bactericidal mode of action.

3.3. Peptide Extract of Gt2 and Cys5-4 Induced Spheroplast and Filamentous Formation as a Principal Killing Event against Salmonella. TEM was used to observe the effect of peptide extracts on *Salmonella enterica* after 6 h of incubation. Untreated *Salmonella enterica* cells showed a normal cell shape with an undamaged and intact structure of the inner and outer membrane (Figure 3). By treating *Salmonella* cells with Gt2 peptide extract at the final concentration of 1 X MIC, spheroplast formation was observed (Figures 4(a) and 4(b)). The cells showed changed shape, and the inner and outer membranes were intact, but they lost the peptidoglycan layer. The precise role of peptidoglycans with respect to

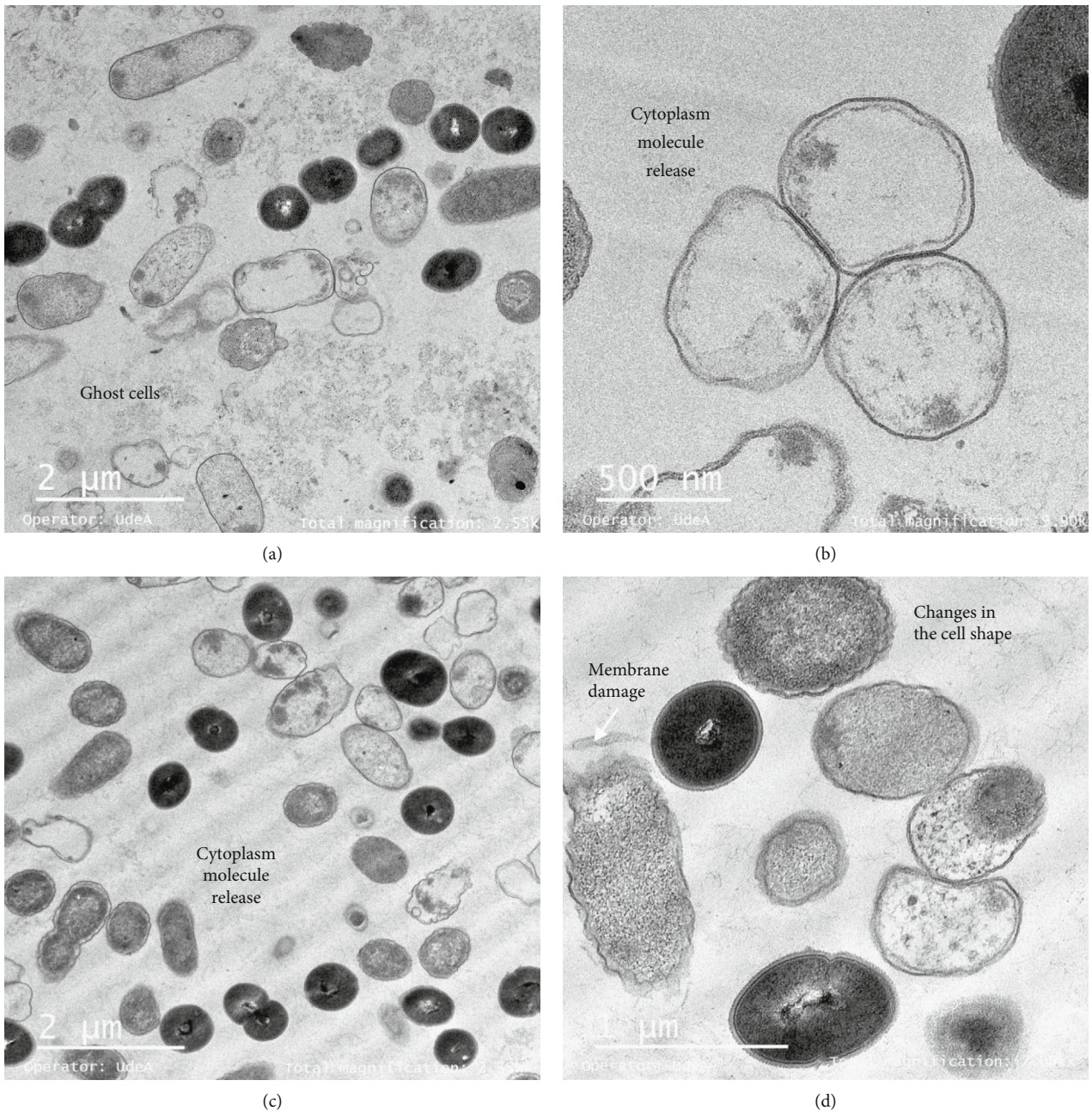


Figure 6: Ghost's cell formation (a–c) and membrane damage (d) after the treatment of *Salmonella enterica* with Gt28 peptide extract at 1 X MIC. Ten different images were taken, and representative images are shown.

interaction with antimicrobial peptides is not well understood; they seem to be targets for pathogen recognition [21]. When the concentration of the peptide Gt2 was increased to 2 X MIC, along with the spheroplast, the appearance of some "ghost cells" was noted, indicating that target bacteria were devoid or near-devoid of cytoplasm (Figure 4(c)). The cells presented intact membranes, but the cytoplasm content was released, implying that the Gt2 peptide extract might induce cell death by more than one mechanism. Table 1 shows a summary of the alteration of the bacterial cell upon the treatment with the peptide extract. Some cells were abnormally longer due to cell elongation or filamentation, the cell membrane changed shape, and intracellular vacuoles were noted (Figure 4(d)). A recent study indicated that cell-free supernatant containing peptides produced by *Lactobacillus taiwanensis* induced ghost cells in *Salmonella gallinarum* [22]. Similarly, vacuole formation increases when the cells are treated with antimicrobials [23]. Treating of *Salmonella* cells with Cys5-4 resulted in spheroplast formation at 1 X MIC (Figures 5(a) and 5(b)), and larger cells with cytoplasmic vacuoles were detected upon the treatment with 1 X MIC (Figures 5(c) and 5(d)). Membrane disruption and DNA relaxation were observed (Figure 5(d)). No ghost cells were detected, indicating that the morphological and ultrastructural changes may rely not only on the concentration and exposure time but also on

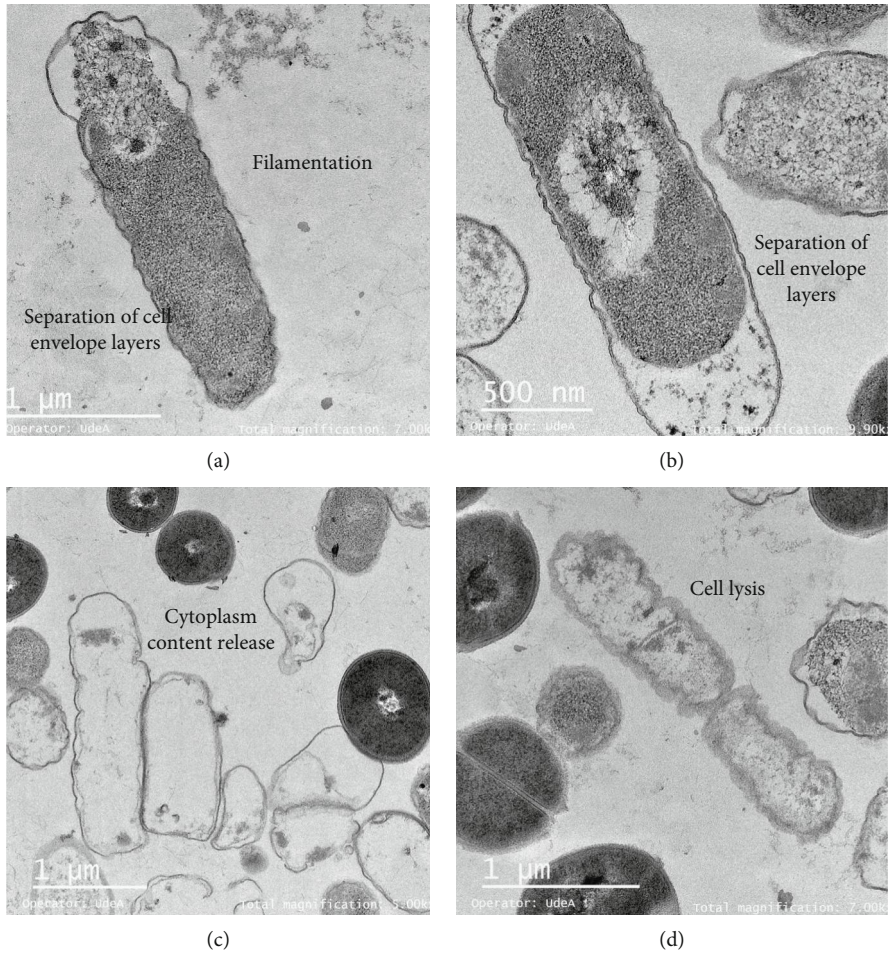


Figure 7: Filamentation and separation of the cell envelope layers (a–c) and ghosts' cells and cell lysis (c, d) after treatment of *Salmonella enterica* with Gt28 peptide extract at 2 X MIC. Ten different images were taken, and representative images are shown.

the identity (origin) of the antimicrobial agent. This mechanism might be explained by the differences in the peptide mixture produced by the Gt2 and Cys5-4 strains; Gt2 has four products of approximately 22, 32, 35, and 55 kDa, while Cys5-4 produces peptides of 10, 15, 20, and 30 kDa [15, 16]. However, more than one peptide or protein-like agent is responsible for the overall antimicrobial activity. Earlier research indicated that antimicrobial agents such as penicillin G, chloramphenicol, oxytetracycline, and kanamycin convert bacteria in spheroplasts [24–26]. In other studies, the beta-lactam induced formation of the spheroplasts in *E. coli* and in many species of Gram-negative bacteria [27]. Most LAB peptides of class II are inhibitory when applied in small concentrations and cause membrane permeabilization and leakage of intracellular components in a sensitive cell [28]. The inhibitory spectrum is limited to Gram-positive bacteria, inducing ion leakage, loss of proton-motive force, and ATP depletion [10]. For example, plantaricin IIA-1A5 produced by *L. plantarum* IIA-1A5 displayed remarkable antimicrobial effects against *S. aureus* by adsorption and attachment onto the cell membrane promoting leakage of the cell membrane with release of organic (proteinaceous and genetic materials) and inorganic (Ca2 +, Mg2+, and K+ ions) compounds [29]. Thus far, no spheroplast formation was observed in *Salmonella* cells treated with two-peptide bacteriocins.

3.4. Gt28 Peptide Extract Induced Lysis and Ghost Cell Formation. The UTNGt28 strain peptide extract was more effective against *Salmonella*, and its action was dose-dependent. TEM micrographs of *Salmonella* treated with Gt28 at 1 X MIC caused filamentation, separation of cell envelope layers, and ghost cell formation (Figure 6). Early research indicated that the treatment of *Salmonella enterica* ATCC51741 at both the vegetative and exponential phases of growth with the cell-free supernatant of Gt28 resulted in complete inactivation upon 3 h, suggesting its bactericidal mode of action [14]. Contrary to Gt2 and Cys5-4 peptide extracts containing a mixture of low- and larger weight peptides [15, 16], peptide extract from Gt28 contained one peptide of 15 kDa and some extra larger peptides or protein-like products as deducted in SDS-PAGE, but further analysis is required to detect the extract composition (Figure S2). The cell membrane showed interrupted stretches, and electron-dense material accumulated in the periplasmic space (Figure 6). After the treatment with 2 X MIC, the cell membrane was damaged and the cytoplasmic cell content was released, as many ghost cells were detected (Figure 7).

The distance between the cytoplasmic membrane and the outer membrane increased, giving the appearance that the layers of the Gram-negative cell envelope had separated (Figures 7(a) and 7(b)). This effect was shown earlier when studying the action mechanism of several antibiotics such as ciprofloxacin, rifampicin, and vancomycin [23, 30]; this might occur due to the outer membrane detaching from the peptidoglycan. The increase of cytoplasmic membrane release caused by membrane disruption led to leakage of cell cytoplasmic content and cell death. The ghost cell formation indicated lysed bacteria devoid or near-devoid of cytoplasm (Figures 7(c) and 7(d)). Early reports indicated this phenomenon when cells were treated with inhibitors of DNA synthesis [31], RNA synthesis [32], or protein synthesis [33]. The results indicated that a lower dosage of Gt28 peptide extract was sufficient to disrupt the membrane and induce filamentation and separation of the cell envelope layers and cytoplasm leakage. Nonetheless, the extent of killing of bacterial cells was enhanced due to the suprasaturation of cell membrane with peptides. Filamentation can occur following inhibition or disruption of peptidoglycan synthesis, DNA synthesis inhibition, or damage by a SOS response process [34]. This result might explain our recent findings showing the effectiveness of the peptide-based formulations containing Gt28 and Cys5-4 peptide extracts in diminishing the cell viability of a pathogenic cocktail consisting of *Salmonella* sp., *Shigella* sp., and *E. coli* cells at the exponential growth phase [35]. In addition, we demonstrated the grate potential of these peptide-based formulations in the control and protection of pathogenic growth in pineapple fresh-cut chunks.

3.5. Gel Retardation Assays Reveal That Gt2 and Gt28 but Not Cys5-4 Peptide Extract Interact with Salmonella Genomic DNA. To identify whether the genomic DNA of *Salmonella* was targeted by the Gt2, Cys5-4, and Gt28 peptide extracts, the genomic DNA was incubated with each peptide extract for 60 min. Figure 8 shows the agarose gel with the DNA migration. As observed, the Gt2 and Gt28 peptide extracts bind the genomic DNA of *Salmonella*, impeding its migration, while the Cys5-4 peptide extract partially binds the genomic DNA, suggesting that the mode of action of Gt2 and Gt28 but not Cys5-4 might involve binding of negatively charged DNA. Previous investigations indicated that peptides produced by *L. paracasei* subsp. *tolerans* FX-6 do progressively interact with the DNA of *Staphylococcus aureus*, with the complete DNA band retained when the peptides bind DNA [36]. Based on our results, the Gt2 and Gt28 peptide extracts disrupt the target cell, causing damage in the membrane and increasing membrane permeabilization as a secondary effect due to the activation of some autodigestive enzymes; the peptide extracts then enter the cell, might bind negatively charged DNA, and finally cause cell death.

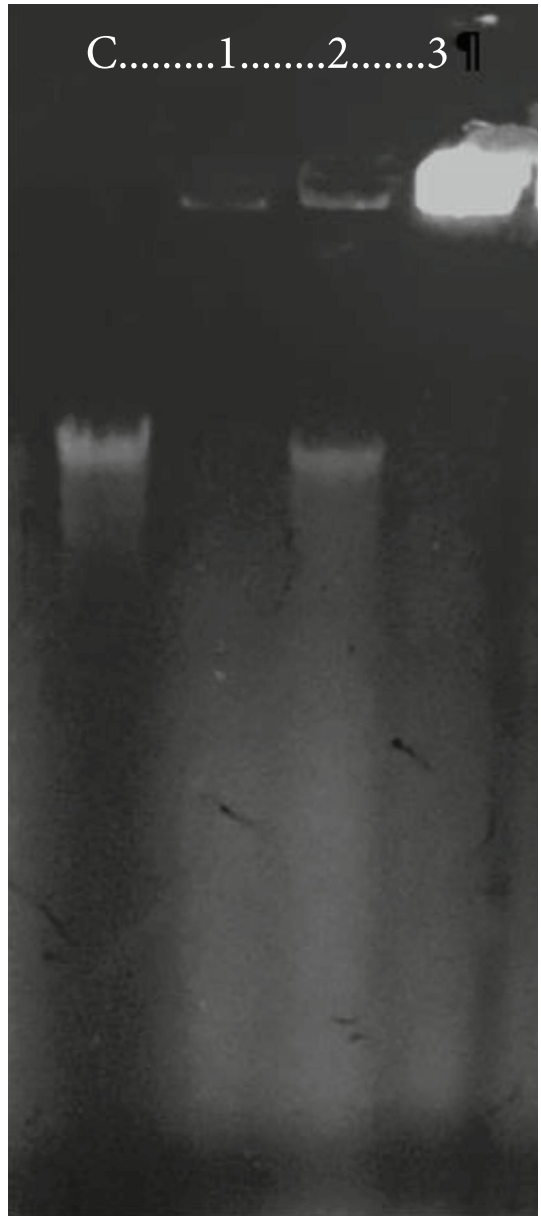


Figure 8: DNA binding analysis of Gt2, Cys5-4, and Gt28 peptide extracts towards *Salmonella enterica* subsp. *enterica* ATCC51741 DNA. C: *Salmonella* genomic DNA; 1-3: genomic DNA treated with Gt2, Cys5-4, and Gt28, peptide extracts.

4. Conclusions

Taken together, our research showed that the cell membrane of *Salmonella* was permeabilized, peptide extracts possibly induced breaks in membrane proteins to some extent, the cell integrity was lost, and DNA/RNA molecules were released, as well as a direct interaction between DNA and peptide extract occurring, leading to cell death. There were at least four key secondary simultaneous membrane shape changes of *Salmonella* cells induced by the peptide extracts including spheroplast formation, ghost cell formation, cell lysis, and filamentation with separation of cell envelope layers, causing membrane disruption leading to cell death. To our knowledge, this is the first evidence of spheroplasts and ghost cell formation observed as a secondary death event of *Salmonella enterica* subsp. *enterica* ATCC51741 by peptide extracts produced by lactic acid bacteria. Nonetheless, further experimental work will focus on the whole-genome sequencing of

these promising antimicrobial strains, allowing to retrieve the gene variants encoding for peptide or protein-like substances responsible for the overall antimicrobial activity, considering the nondairy origin of the producer strains. Finally, these peptide extracts are promising new antimicrobials to enhance the food safety and quality.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Figure S1: detection of DNA/RNA molecules released when *Salmonella* was treated with the G2, Cys5-4, and Gt28 peptide extract. Figure S2: the molecular weight of Gt28 precipitated peptide deducted from SDS-PAGE analysis. *(Supplementary Materials)*

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Research Article

Antimicrobial Resistance Phenotype of *Staphylococcus aureus* and *Escherichia coli* Isolates Obtained from Meat in the Formal and Informal Sectors in South Africa

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Background. Foodborne diseases (FBD) caused by resistant pathogens are a global public health problem. One main driver of the increasing FBD incidence is the transfer of pathogenic organisms from animal guts to carcasses during processing and subsequent transfer from meat products to consumers. *Methods*. In this study, meat samples from abattoirs in the formal meat sector (FMS) ($n = 140$) and slaughter points in the informal meat sector (IMS) ($n = 104$) were collected for microbial detection and phenotypic AMR determination using polymerase chain reaction. *Results*. The antibiogram of *Staphylococcus aureus* isolates revealed that resistance to clindamycin (74.3%) and ampicillin (59.5%) was highest in the FMS, while resistance to penicillin (83.8%) and tetracycline (82.1%) was highest in the IMS. *Escherichia coli* isolates show significant resistance to chloramphenicol (90.7%) and tetracycline (82.3%) in the FMS. Likewise, resistance to tetracycline (92.3%) and sulfamethoxazole/trimethoprim (87.5%) was highest in the IMS. The multiple antibiotic resistance index (MARI) for *S. aureus* and *E. coli* ranged from 0.3 to 0.8 and 0.2 to 0.5, respectively. *Conclusion*. This study suggests high-level contamination of meat with resistant pathogens and highlights the public health consequences associated with consuming such unhygienic products.

1. Introduction

Meat is an essential source of animal protein widely consumed in many parts of the world. In terms of livestock agriculture, statistics show that there about 1.1 million pigs, 7 million goats, 24.6 million sheep, 1.4 million dairy cattle, and 13.6 million beef cattle as well as 1.6 million ostriches, 31.8 million layers, and 113 million broilers in South Africa [1]. South Africa meat consumption per capita per year is said to be 41 kg and is second only to Ghana in Africa [2]. In the Southern African region, meat consumption is four times higher than any other region in Africa and South Africa plays a major role regarding livestock production and meat supply in the continent [1, 3].

Even though meat plays a crucial role in human nutrition, a significant proportion of foodborne diseases have been linked to its consumption. Epidemiological data suggest an escalating incidence of foodborne diseases. A good number

of these diseases occur due to poor animal husbandry systems and failure to maintain proper hygiene during food processing [2, 4]. Poor hygiene management and other faulty abattoir processes such as improper evisceration increase the chances of cross-contamination of gut pathogens (*Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, and enteric bacteria) to meat [5].

Escherichia coli is part of the normal flora of the gastrointestinal tract of humans and animals. It becomes pathogenic to the immunocompromised person (children, pregnant mothers, and people with a chronic debilitating illness such as diabetes) through contaminated water and food [6, 7]. Many *E. coli* strains have emerged as leading zoonotic foodborne pathogens. Diarrheagenic pathotypes frequently implicated foodborne for diseases include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic, diffusely adherent *E. coli* (DAEC), and *E. coli* (EHEC); a subclass of enteroinvasive *E. coli* (EIEC), neonatal meningitis *E. coli* (NMEC), *E. coli*, and uropathogenic *E. coli* (UPEC)[8–10]. Due to their ability to cause numerous foodborne disease outbreaks in humans, they have become a significant public health threat [7, 11–13].

Staphylococcus aureus is among the leading causes of foodborne diseases in humans. It is a Gram-positive, nonspore forming, nonmotile, catalase-positive coccus which is ubiquitous in humans and the environment [14]. *S. aureus* is found commonly on the skin, hair, noses, and respiratory tract of humans and animals. It multiplies rapidly at room temperature producing toxins which cause illnesses when it enters the body. The main route of transmission of *S. aureus* is through a cut, infected wound, and ingestion of contaminated food [15].

Staphylococcus aureus is commonly associated with intoxications due to its ability to produce a variety of potent staphylococcal enterotoxins (SEs) [7]. The SEs are resistant to inactivation by GIT proteases such as pepsin and display strong thermoresistance, an essential property of SEs for food safety considerations and a potential problem for public health [16]. *Staphylococcus aureus* produces three types of hemolysins, known as alpha, beta, and delta toxins. The beta-hemolysin gene encodes the beta toxins that inhibit the ciliary movement of human lungs and corneas [17]. Due to its transient nature, many staphylococcal food poisonings (SFP) go unreported; this is in addition to the fact that the symptoms of SFP are similar to those of food poisoning caused by *Bacillus cereus* [18].

In humans, gastroenteritis attributable to staphyloenterotoxicosis or staphyloenterotoxemia can occur within 1 to 7 hours after consumption of contaminated food [19]. Dehydration due to frequent diarrhea and vomiting; infections of the skin; and soft tissue, joint, bone, respiratory, and endovascular disorders are other common clinical pictures in infected humans. Furthermore, diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, and toxic shock syndrome are commonly associated with staphylococcal infection [15]. Further compounding the challenges posed by staphylococcus infection is the increasing spate of methicillin-resistant *S. aureus* (MRSA), which have been reported in pork, chicken, beef, and other meat in many countries [20–23].

In many developing countries, the incidence of foodborne diseases (FBD) is often associated with resistant bacteria [6, 12, 24]. Food-associated microbes harboring transferable antibiotic resistance genes are of significant public health concern. This is because they can cause FBD and also act as a reservoir for spreading antibiotic resistance genes to enteric and commensal bacteria by horizontal gene transfer of mobile genetic elements [25]. The problem of antibiotic resistance could even be more prominent in South Africa given that farmers under the Stock Remedies Act (Act 36 of 1947) could buy and use the veterinary drug without a prescription [26–28]. Hence, this study is aimed at determining the antimicrobial resistance profile of *Staphylococcus aureus* and *Escherichia coli* isolates from raw meat, slaughtered carcasses in the informal and informal meat sectors in the Eastern Cape Province of South Africa.

2. Material and Method

2.1. Ethical Approval. Approval for this research was obtained from the University of Fort Hare Research and Ethics Committee (UREC). The certificate of approval was issued with reference number MUC351SJAJ01.

2.2. Study Area. The study was conducted at two high-throughput abattoirs (HT1 and HT2). The East London abattoir (HT1) is situated at 32.97°S and 27.87°E in the Buffalo City Metropolitan Municipality, while the Queenstown abattoir (HT2) is located 31°54′S and 26°53′E in the Chris Hani District of the Eastern Cape Province [5, 29]. The informal slaughter point was Alice (32.47°S and 26.50°E), King William's Town (32°53′S and 27°24′E), Queenstown (31°54′S and 26°53′E), and East London (32.97°S and 27.87°). The places receive approximately 480-850 mm of rainfall per year most of which is during the summer months and are situated about 586-2371 meters above sea level. The ambient temperatures in the Eastern Cape during the period of study ranged from 18°C to 39°C with mean temperatures of 20.5°C. The vegetation in this area is composed of bushveld with *Acacia karroo*, *Themeda triandra*, and *Digitaria eriantha*, grasslands, and forests. The predominant farming system is extensive with some commercial farms using a semi-intensive system of management [5, 29].

2.3. Sample Collection and Sampling Design. Swab samples from carcasses of slaughtered animals were collected from November 2016 to October 2017 at the formal and informal meat sectors. The formal refers to livestock producers who are registered with the Provincial Departments of Agriculture and whose activities are governed by relevant acts of the national parliament. The informal livestock producers, on the other hand, are a subset of unincorporated enterprise, with less than a specialized size in terms of the number of persons employed, and may or may not be registered under specific forms of national legislation [30]. Enterprises in the informal sector do not pay tax and/or obey employment regulations and are rarely monitored for health and safety

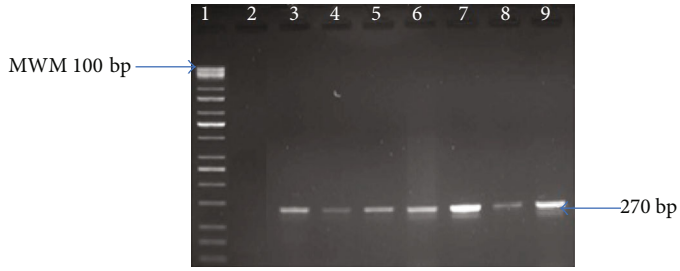

Figure 1: Gel image of amplified PCR products from study isolates with primers designed for the *Nuc* gene. Lane 1 is the MWM (100 bp); lane 2 is the negative control (PCR mix without DNA) with lane 3 as the positive control (ATCC® 25923), while lanes 4 to 9 are *Nuc* (270 bp) gene amplified from *S. aureus* isolates.

standards [31, 32]. In this regard, the abattoir represents the formal meat sector, while the backyard and unapproved slaughter points were included as the informal meat sector.

Carcasses were sampled according to the United States Department of Agriculture Food Safety and Inspection Service (FSIS) protocol on livestock carcass examination. This protocol has been outlined elsewhere [33]. For HT1 and HT2, a simple random sampling technique was adopted for the survey; this sampling method allowed for the convenient swabbing of animal carcasses. In the informal meat sector, a snowball technique was used to identify informal slaughter points for sample collection and carcasses were purposively sampled based on the available number of slaughtered animals. A total of 244 carcasses were sampled from two high-throughput abattoirs represented as HT1 (168 cattle) and HT2 (36 sheep and 40 pigs). In the informal meat sector, a total of 136 swab samples (52 cattle and 84 sheep) were collected. Samples were aseptically collected using cotton throat sponges (CTS) hydrated with 10 ml of buffered peptone water (BPW) (Inqaba® Laboratories, South Africa). All the carcasses were sampled using the same swabbing technique at the end of the slaughter line after dressing but before chilling. The technique entails a horizontally and vertically directed swabbing across the sampling site (neck, brisket, flack, and ramp) on a total of 100 cm^2 quadrant forming a pooled sample for each carcass [34]. Each of the areas on the 4 quadrants of the carcass was firmly swabbed repetitively and abrasively ensuring that most if not all bacteria on the meat surface were removed onto the CTS. Samples were labeled and carefully packed in a cooler box containing ice packs and transported to the laboratory on the same day for bacterial analysis.

2.4. Isolation of Staphylococcus aureus and Escherichia coli. Each polled sample was inoculated into tryptone soy broth (TSB) (Merck, SA) and incubated for 24 h at 37°C. A loop of liquid was removed from the cultures and streaked onto mannitol salt agar (MSA) (Biolab, Midrand, South Africa) plates for *Staphylococcus aureus* isolation and eosin methylene blue agar (EMB) (Oxoid, Basingstoke, UK) for *Escherichia coli* isolation. *S. aureus* was presumed to be positive if yellow or off-white colonies were found on MSA, indicating mannitol fermentation (i.e., presumptive coagulase-positive staphylococci). Salt tolerance and mannitol fermentation properties of *S. aureus* produced the typical yellow colonies

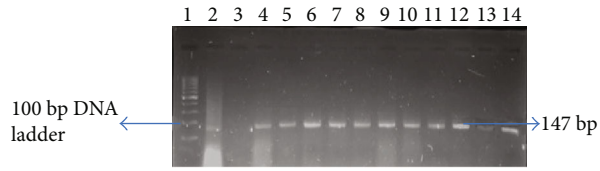

Figure 2: Gel image of amplified PCR products from study isolates with primers designed for the *uidA* gene. Lane 1 is the 100 bp ladder; lane 2 is the negative control (PCR mix without DNA) with lane 3 as the positive control (ATCC® 25922), while lanes 4 to 14 are positive *E. coli* isolates with amplified gene (147 bp).

because of the change in pH [35]. Further confirmation was done by Gram staining and standard biochemical assays such as catalase, oxidase, and coagulase testing [36]. After incubation, colonies with a distinct green metallic sheen on EMB were regarded as *E. coli* [35]. All identified presumptive colonies were kept in glycerol stock and then stored at −80°C for further analyses.

2.5. DNA Extraction. Bacterial deoxyribonucleic acid was extracted from presumptive isolates using the boiling method as described elsewhere [6, 37]. Briefly, the bacteria stored in glycerol stocks were first resuscitated by inoculation into TSB (Merck, SA) and incubated at 37°C for 24 h. Finally, a loop of liquid was removed from TSB and streaked onto nutrient agar (Merck, SA) and incubated at 37°C for 24 h. DNA extraction was performed using a boiling method. The method entails selecting 3–5 colonies using a sterile wire loop into sterile DNAse/RNAse-free Eppendorf tubes (Biologix, USA) containing 200 μl nuclease-free water (Thermo Scientific, USA). Each suspension was vortexed using a mini-shaker (Digisystem Laboratory Instruments Inc., Taiwan), and the cells were lysed using a Dri-Block DB-2A (Techne, South Africa) for 15 min at 100°C. The Eppendorf tubes were then incubated in a heat block at 100°C for 15 min and then kept on ice before the final centrifugation at13,000 rpm for 5 min for removal of cell debris. The supernatant was collected into a sterile Eppendorf tube and preserved at −20°C until further tests.

2.6. Molecular Identification Using Polymerase Chain Reaction. Molecular confirmation of presumptive S. aureus and E. coli isolates was done by PCR using a primer pair to target the thermonuclease (Nuc) gene for S. aureus [14, 38] and uidA gene for E. coli [39, 40] (Figures 1 and 2). Quality

Table 1: Primers used in PCR detection of *S. aureus* and *E. coli*.

Gene			Reference
Nuc	Primer sequence 5′-3′	F: GCGATTGATGGTGATACGGTT	[35]
		R: AGCCAAGCCTTGAACGAACTAAAGC	
	Product size (bp)	270	
	PCR conditions	Initial denaturation at 95°C for 5 min was followed by 37 cycles of amplification (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s) and ending with a final extension at 72°C for 10 min	
uidA	Primer sequence 5′-3′	F: AAAACGGCAAGAAAAAGCAG	[35]
		R: ACGCGTGGTTAACAGTCTTGCG	
	Product size (bp)	147	
	PCR conditions	Initial denaturation at 94°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min and ended with a final extension at 72°C for 2 min. Holding was at 4°C	

control strains S. aureus ATCC 25923 and E. coli ATCC 25922 served as positive controls. Negative controls were used in all reactions containing the reaction mixture except the DNA template, which was replaced by nuclease-free water. The reaction mixture for running PCR contained 12.5 μl of 2x DreamTaq PCR master mixes (Thermo Scientific, SA), 5.5 μl nuclease-free water, 1 μl of both the primers, and 5.0 μl of the DNA template. PCR assay was carried out in a 25 μl reaction volume. The thermocycling program for PCR can be found in Table 1. The amplified products were visualized by standard gel electrophoresis using 5 μl of the amplified product on 2% agarose gels immersed in 0.5x TBE buffer. The TBE buffer contained 0.1 M Tris, 0.1 M boric acid, and 0.002 M NaEDTA. Agarose gels were stained using 1 mg/ml ethidium bromide and photographed under UV light with a transilluminator (Alliance 4.7).

2.7. Antimicrobial Susceptibility Testing. Antibiotic susceptibility testing was performed by the Kirby-Bauer disc diffusion test method, following the guidelines of the Clinical and Laboratory Standards Institute [41]. An inoculum of each pure bacterial isolate was emulsified in 5 ml of sterile normal saline, and the density was adjusted to 0.5 McFarland standards. A sterile cotton swab was dipped into the standardized suspension of bacterial cultures and used to inoculate Mueller Hinton agar (MHA) plates, and the plates were allowed to dry. Antibiotic discs with the following drug contents ampicillin (10 μg), erythromycin (15 μg), rifampicin (5 μg), clindamycin (2 μg), ciprofloxacin (5 μg), penicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), gentamycin (10 μg), trimethoprim-sulfamethoxazole (25 μg), amikacin (30 μg), and ofloxacin (5 μg) were placed onto Mueller Hinton agar (MHA) plates using a disc diffuser (DMM063, Thermo Fisher Scientific, South Africa). The plates were incubated at 37°C for 24 hours. The zone diameter was measured using a ruler, and results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [41].

2.8. Statistical Analysis. All data analysis was performed using Microsoft® Excel (2007) mathematical functions and Statistical Package for the Social Sciences (SPSS) version 22 (SPSS Inc., Chicago, IL). Exploratory data analysis was used to validate the data and calculate crude associations by using2 × 2cross-tabulation tables in which descriptive statistics and summary measures were calculated. Multiple antibiotic resistance phenotypes (MARPs) for *S. aureus* isolates from the formal and informal meat sectors were then generated for isolates that were resistant to five or more antimicrobials [39]. The frequencies, percentages, and number of antimicrobials to which the isolates were resistant and resistance patterns were obtained from the antimicrobial susceptibility testing (AST). The multiple antibiotic resistance indexes (MARI) for bacterial isolates from both meat sectors were mathematically calculated using $MAR_{index} = a/b$, where a stands for the number of antibiotics to which the isolate was resistant and "b" represents the total number of antibiotics used for antimicrobial susceptibility testing [42].

3. Results

3.1. Prevalence and Antibiogram of Staphylococcus aureus and Escherichia coli in the Formal and Informal Meat Sectors. The prevalence of molecularly confirmed *Staphylococcus aureus* in the formal and informal meat sectors was 30.3% (74/244) and 50% (68/244), respectively (Table 2). The molecularly confirmed *E. coli* in the formal and informal meat sectors was 57.4% (140/244) and 76.5% (104/244) (Table 2). For *S. aureus* isolates, resistance to clindamycin 74.3% (55/74) was highest in the FMS (Figure 3), followed by ampicillin 59.5% (44/74), penicillin 52.7% (32/74), and erythromycin 50% (37.74), whereas resistance to penicillin 83.8% (67/68) was highest for the IMS, followed by tetracycline 82.4% (56/68), clindamycin 77.9% (52/68), ampicillin 76.5% (52/68), and rifampicin 69.1% (47/68) (Table 3). Two isolates each showed multiple drug resistance to 9 and 10 antibiotics, respectively. The multiple antibiotic resistance indexes (MARI) for the formal and informal meat sectors ranged from 0.3 to 0.8 (Table 4). For *E. coli* isolates, resistance to chloramphenicol 90.7% (127/140), tetracycline 82.1% (115/140), streptomycin 77.9% (109/140), sulfamethoxazole/trimethoprim 66.4% (93/140), kanamycin 65% (91/140),

Table 2: Percentage isolation of *S. aureus* and *E. coli* in the formal and informal meat sectors.

Meat sector	Abattoirs/slaughter points	Animal	No. of carcasses sampled	*S. aureus*		*E. coli*	
				Presumptive isolates (%)	Confirmed with PCR (%)	Presumptive isolates (%)	Confirmed with PCR (%)
Formal	HT1	Cattle	168	143 (58.6)	51 (20.9)	109 (44.7)	104 (42.6)
Formal	HT2	Sheep	36	23 (9.4)	7 (2.9)	27 (11.1)	24 (9.8)
		Pig	40	31 (12.7)	16 (6.6)	21 (8.6)	12 (4.9)
Total			244	197 (80.7)	74 (30.3)	157 (64.3)	140 (57.4)
Informal	Alice	Cattle	16	16 (11.8)	10 (7.4)	16 (11.8)	14 (10.3)
		Sheep	32	31 (22.8)	9 (6.6)	21 (15.4)	20 (14.7)
Informal	East London	Cattle	20	20 (14.7)	11 (8.1)	16 (11.8)	16 (11.8)
Informal	King William's Town	Cattle	16	15 (11)	9 (6.6)	11 (8.1)	11 (8.1)
Informal	Queenstown	Sheep	52	46 (33.8)	29 (21.3)	44 (32.4)	43 (31.6)
Total			136	128 (94.1)	68 (50)	108 (79.4)	104 (76.5)

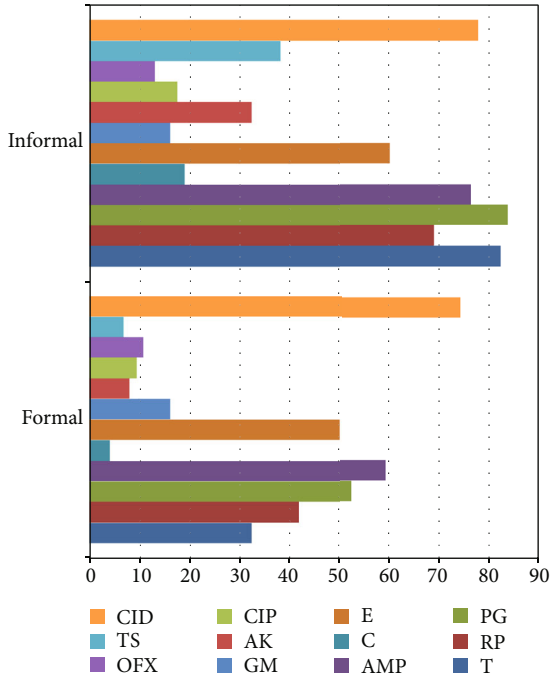

Figure 3: Percentage phenotypic resistance profile of *S. aureus* isolates from the formal and informal meat sectors. T: tetracycline; RP: rifampicin; PG: penicillin; AMP: ampicillin; C: chloramphenicol; E: erythromycin; GM: gentamycin; AK: amikacin; CIP: ciprofloxacin; OFX: ofloxacin; TS: sulfamethoxazole/trimethoprim; CID: clindamycin.

and amoxicillin 58.6% (82/140) was highest in the formal meat sector (Figure 4). All isolates were susceptible to imipenem (Table 5). *E. coli* isolates obtained from the informal meat sector were mostly resistant to tetracycline 92.3% (96/104), sulfamethoxazole/trimethoprim 87.5% (91/104), amoxicillin 85.6% (89/104), chloramphenicol 74% (77/104), streptomycin 67.3% (70/104), and ampicillin 66.3% (69/104) (Table 5). Four and 14 *E. coli* isolates were resistant to 10 antibiotics, and the MARI for these isolates was 0.5 (Table 6).

4. Discussion

Meat consumers in low- and middle-income countries obtain meat from the informal outlets because the meat is cheap, and the market is often situated close to rural communities [31, 43]. However, in the absence of proper meat safety and hygiene management systems, the chemical constituent of meat enhances microbial growth to unacceptable levels. Hence, microbially compromised meat poses the risk of foodborne disease (FBD) transmission to consumers. The prevalence of FBD is a growing public health problem especially in low- and middle-income countries where food safety systems are poorly implemented [23, 44–46].

Foodborne pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* are excreted from the gastrointestinal tract of food-producing animals, and cross-contamination is often as a result of poor slaughter technique and hygiene standard at abattoirs [2, 11, 47–49]. The occurrence of food-related disease is further compounded by the development of antimicrobial resistance (AMR) by bacteria, which limits the efficiency of antibiotic therapeusis. The present study investigated the level of microbial contamination of slaughtered carcasses in the formal and informal meat sectors.

The present study also investigated the antimicrobial resistance (AMR) profile of *S. aureus* and *E. coli* isolates obtained from the formal and informal meat sectors (Figures 3 and 4). *Staphylococcus aureus* isolates from the formal meat sector were mostly resistant to rifampicin (41.9%), penicillin (52.7%), ampicillin (59.5%), erythromycin (50%), and clindamycin (74.3%) (Figure 3). In the informal meat sector, *S. aureus* isolates were mainly resistant to tetracycline (82.4%), penicillin (83.8%), and ampicillin (76.5%) which demonstrates the growing problem of AMR in bacteria from food-producing animals. The resistance to important antibiotics such as rifampicin (69.1%), erythromycin (60.3%), and clindamycin (77.9%) is even more worrisome. One study of poultry meat in South Africa found high resistance to tetracycline in all *S. aureus* [50]. In another study, the resistance to clindamycin was 11.8% for beef cuts and 21.7% for pork. The same study found the

Table 3: Antibiotic susceptibility pattern of *Staphylococcus aureus* isolates in the formal ($n = 74$) and informal ($n = 68$) meat sectors.

Antibiotic class	Antimicrobial agents	Code	Potency (μg)	Formal		Informal	
				S (%)	R (%)	S (%)	R (%)
Tetracycline	Tetracycline	T	30	50 (67.6)	24 (32.4)	12 (17.6)	56 (82.4)
Ansamycins	Rifampicin	RP	5	43 (58.1)	31 (41.9)	21 (30.9)	47 (69.1)
Penicillin	Penicillin	PG	10	35 (47.3)	39 (52.7)	11 (16.2)	57 (83.8)
	Ampicillin	AMP	10	30 (40.50)	44 (59.5)	16 (23.5)	52 (76.5)
Phenicols	Chloramphenicol	C	10	71 (95.9)	3 (4.1)	55 (80.9)	13 (19.1)
Macrolides	Erythromycin	E	15	37 (50)	37 (50)	27 (39.7)	41 (60.3)
Aminoglycosides	Gentamycin	GM	10	62 (83.8)	12 (16.2)	57 (83.8)	11 (16.2)
	Amikacin	AK	30	68 (91.9)	6 (8.1)	46 (67.6)	22 (32.4)
Quinolones	Ciprofloxacin	CIP	5	67 (90.5)	7 (9.5)	56 (82.4)	12 (17.6)
	Ofloxacin	OFX	5	66 (89.2)	8 (10.8)	59 (86.8)	9 (13.2)
Foliate pathway inhibitor	Sulfamethoxazole/trimethoprim	TS	25	69 (93.2)	5 (6.8)	42 (61.8)	26 (38.2)
Lincosamides	Clindamycin	CID	2	19 (25.7)	55 (74.3)	15 (22.1)	53 (77.9)

S: susceptible; R: resistance.

Table 4: Multiple antibiotic resistance patterns (MARPs) and MARI of *Staphylococcus* spp. from the formal and informal meat sectors.

S/no.	Isolate code	Resistance pattern	No. of antibiotics	MARI	S/no.	Isolate code	Resistance pattern	No. of antibiotics	MARI
1	C29C^{INMS}	RP-CD-AP-E-PG	5	0.4	20	C3A	RP-CD-AP-E-PG	5	0.4
2	C18A^{INMS}	CD-GM-CIP-AK	4	0.3	21	C1A^{INMS}	RP-CD-AP-E-T-PG	6	0.5
3	C29B	RP-CD-AP-E-T-PG	6	0.5	22	C24C	RP-C-CD-AP-E-T-GM-TS-PG-AK	10	0.8
4	C3D^{INMS}	AP-E-GM-OFX	4	0.3	23	P9C	CD-AP-E-T	4	0.3
5	C22A^{INMS}	RP-CD-AP-E-T-GM-PG	7	0.6	24	C17C	RP-CD-AP-E-PG	5	0.4
6	C20A	RP-CD-AP-E-T-PG	6	0.5	25	17SD^{INMS}	RP-CD-AP-E-T-GM-PG	7	0.6
7	C22C	RP-CD-AP-E-GM-CIP-OFX-PG-AK	9	0.8	26	C28B	RP-CD-E-PG	4	0.3
8	C5A^{INMS}	CD-AP-E-T-PG	5	0.4	27	C28D	RP-CD-AP-E-OFX-PG	6	0.5
9	C28C	RP-CD-AP-E-PG	5	0.4	28	C7D^{INMS}	CD-AP-GM-TS-OFX-PG	6	0.5
10	17SA	CD-AP-E-T-GM-PG	6	0.5	29	C3D^{INMS}	CD-AP-E-T	4	0.3
11	C16A	RP-CD-AP-E-PG	5	0.4	30	C30D	RP-CD-AP-E-T-PG	6	0.5
12	C3C^{INMS}	RP-CD-AP-T-GM-PG-AK	7	0.6	31	C24A	RP-CD-AP-E-PG	5	0.4
13	C26D	RP-CD-AP-E-PG	5	0.4	32	C27C^{INMS}	RP-CD-AP-E-T-PG	6	0.5
14	14SD	RP-CD-AP-E-GM-CIP-OFX-PG-AK	9	0.8	33	C20A^{INMS}	C-CD-AP-E-T-GM-TS-CIP-OFX-PG	10	0.8
15	DH5	RP-CD-E-T	4	0.3	34	C4C^{INMS}	RP-CD-AP-E	4	0.3
16	12SC^{INMS}	RP-CD-AP-E-T-PG	6	0.5	35	C30A	RP-CD-AP-E-PG	5	0.4
17	26SA	RP-CD-AP-E-PG	5	0.4	36	SM11	RP-CD-AP-E-T-GM	6	0.5
18	C18A^{INMS}	RP-CD-AP-E-TS-CIP-OFX	7	0.6	37	P9B	RP-CD-AP-PG	4	0.3
19	C27D	RP-CD-AP-PG	4	0.3	38	P5A	RP-CD-AP-T-PG	5	0.4

Isolates with superscript ^{INMS} were from the informal meat sector; those without superscript were from the formal sector.

resistance to penicillin to be 63.2% for beef cuts and 88.7% for pork [48].

Approximately 20% and 30% of humans are regarded as persistent and intermittent carriers of *S. aureus* in the nostrils, respectively. Thus, *S. aureus* asymptomatically lives on the skin and nostrils of humans and animals [23, 35]. Cross-contamination from the animal and human skin to the meat during slaughter and processing is inevitable in a situation where the standard hygiene protocol is not strictly implemented. A practical example of the persistence of *S aureus* in human hands and nares was demonstrated in one Brazilian study, where methicillin-resistant *Staphylococcus*

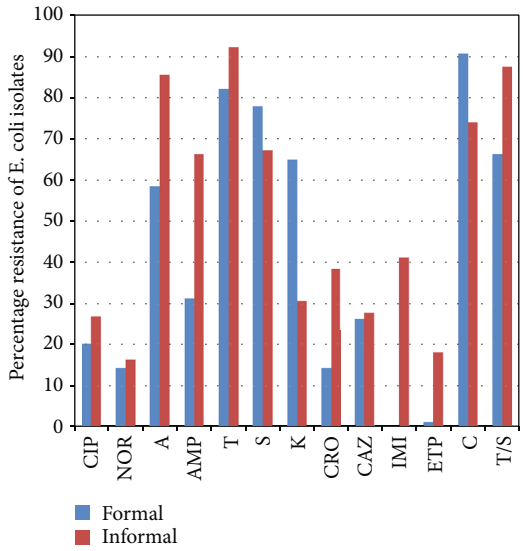

FIGURE 4: Percentage phenotypic resistance profile of *E. coli* isolates from the formal and informal meat sectors. T: tetracycline; PG: penicillin; AMP: ampicillin; C: chloramphenicol; CIP: ciprofloxacin; TS: sulfamethoxazole/trimethoprim; S: streptomycin; CRO: ceftriaxone; CAZ: ceftazidime; IMI: imipenem; ETP: ertapenem; NOR: norfloxacin; A: amoxicillin.

aureus (MRSA) was detected in 28.6% of samples collected from the hands and nares of food handlers in a public hospital. The finding in the Brazilian study reinforces the need for strict sanitary protocols at meat handling points. It further supports our hypotheses that some of the isolates in the present study were a result of cross-contamination from slaughter personnel.

Maintaining hygiene and safety in the informal market is challenging to achieve because many traders are not educated or poorly resourced to implement the standard hygiene protocol. Many of the informal traders wash meat repeatedly in the same water without change, using the same knife the entire day without cleaning or washing in hot water. Temperature violation was common, and meat is usually not efficiently protected from flies and dust that may harbor meatborne pathogens. Poor hygienic behaviors observed in the present study are consistent with previous studies [43, 51]. In these kinds of condition, it is easy for pathogens to be transferred from meat handlers, knives, flies, dust, and tables [35, 52–54]. *Staphylococcus aureus* as a potential pathogen may adversely affect animal and human health by causing abscesses, endocarditis, severe necrotic lesions, and bacteremia [36]. Bacteria harboring resistant determinant and virulence factors could quickly disseminate these factors through mobile genetic element coding for the transfer of resistance horizontally between various bacteria.

Foodborne illnesses caused by *S. aureus* are a result of the ingestion of food contaminated with staphylococcal toxins. Staphylococcal enterotoxins are 23 to 29 kDa single-chain proteins that also possess immunomodulation properties [55] and are mostly carried on mobile genetic elements that aid their horizontal transfer between bacterial populations [19, 56]. The implication of these resistance proportions can be seen in the high MAR index of 0.4-0.8 for isolates from the formal and informal meat sectors. The high levels of *S. aureus* and *E. coli* recovered in this study may pose a public health hazard due to the potential pathogenicity and/or toxigenicity of various strains of these bacteria.

Even though food safety systems and standardization are widely applied in the formal meat sector, unlike the informal sector where there is no regulation governing the safety of meat [57, 58], the microbial quality of meat in the formal meat sector hardly reflects these standards. This is especially true in this instance, given that in this study, 91 and 98 *E. coli* isolates were resistant to either three or more antibiotics (Table 6). Antimicrobial resistance to chloramphenicol (90.7%) and tetracycline (92.3%) was highest in the formal and informal meat sectors, respectively. Although chloramphenicol use in veterinary medicine has been restricted globally [26, 59], its detection in high proportion suggests that carcasses from the study sites were heavily contaminated with pathogens of human or environmental origin. On the other hand, the high resistance to tetracycline is unsurprising given that it is a common over-the-counter (OTC) medication for the treatment of bacteria and tick-borne diseases (TBDs) in South Africa [37, 60]. In many instances, farmers misapply tetracycline to treat unrelated diseases. Such imprudent use of antibiotics exerts selective pressure sustaining the emergence of resistant bacterial strains.

Streptomycin (77.9% and 67.3%), sulfamethoxazole/trimethoprim (66.4% and 87.5%), and amoxicillin (58.6% and 85.6%) were the other antibiotics with high resistant proportion in the formal and informal meat sectors, respectively. The use of antibiotics for prophylaxis, metaphylaxis, and growth promotion in livestock farms is the primary suspect in selecting antibiotic resistance. Antimicrobial agents such as sulfonamides (95.4%); macrolides, lincosamides, and pleuromutilins (61.6%); tetracyclines (14%); quinoxalines (8.2%); lonophores (6.7%); and penicillins (1.8%) have been reportedly sold as in-feed and water antimicrobials medication [60].

The scale of resistant pathogens obtained in this study remains worrisome. Even more worrisome is the growing resistance to third-generation antimicrobial agents such as ceftriaxone, ceftazidime, imipenem, and ertapenem (Table 5). The main driver for cephalosporin remains unclear since it is not commonly used in animal medicine. Hence, we suspect that the extensive use of cephalosporins and carbapenems in clinical practice may play a role in the current resistant profile. Also, antimicrobial resistance to third-generation cephalosporins is frequently related to the production of extended-spectrum β-lactamase (ESBL) enzymes. Aside from ESBLs, antimicrobial resistance to extended-spectrum cephalosporinases (ESCs) in *E. coli* has been associated with plasmid-mediated Ambler class C cephamycinases [61].

The fact that pathogens from animals spread to food products during slaughter and processing has been extensively published [33, 35, 62]. Bacteria with resistance capability can also be transferred from animals and humans during slaughter and processing [37, 63]. More important is the misappropriation of antimicrobial agents by communal farmers who are the primary suppliers of meat in the informal market

Table 5: Percentage antibiotic susceptibility of *E. coli* isolates in the formal ($n = 140$) and informal ($n = 104$) meat sectors.

Antimicrobial class	Antimicrobials	Disc code	Potency (μg)	Meat sector			
				Formal		Informal	
				R (%)	S (%)	R (%)	S (%)
Quinolones	Ciprofloxacin	CIP	5	28 (20)	112 (80)	28 (26.9)	76 (73.1)
	Norfloxacin	NOR	10	20 (14.3)	120 (85.7)	17 (16.3)	87 (83.7)
Beta-lactams	Amoxicillin	A	25	82 (58.6)	58 (41.4)	89 (85.6)	15 (14.4)
	Ampicillin	AMP	25	44 (31.4)	96 (68.6)	69 (66.3)	35 (33.7)
Tetracyclines	Tetracycline	T	30	115 (82.1)	25 (17.9)	96 (92.3)	8 (7.7)
Aminoglycosides	Streptomycin	S	300	109 (77.9)	31 (22.1)	70 (67.3)	34 (32.7)
	Kanamycin	K	30	91 (65)	49 (35)	32 (30.8)	72 (69.2)
Cephalosporins	Ceftriaxone	CRO	30	20 (14.3)	120 (85.7)	40 (38.5)	64 (61.5)
	Ceftazidime	CAZ	10	37 (26.4)	103 (73.6)	29 (27.9)	75 (72.1)
Carbapenems	Imipenem	IMI	10	0 (0)	140 (100)	43 (41.3)	61 (58.7)
	Ertapenem	ETP	10	2 (1.4)	138 (98.6)	19 (18.3)	85 (81.7)
Phenicols	Chloramphenicol	C	30	127 (90.7)	13 (9.3)	77 (74)	27 (26)
Foliate pathway inhibitor	Sulfamethoxazole/trimethoprim	TS	25	93 (66.4)	47 (33.6)	91 (87.5)	13 (12.5)

S: susceptible; R: resistance.

Table 6: Multiple antibiotic resistance patterns (MARPs) and MARI of *E. coli* isolates.

Pattern number	Number of antibiotics	MAR pattern	Meat sector		Total	MARI
			Formal	Informal		
1	3	A-TS-C	4	6	10	0.2
2	3	A-TS-AMP	11	2	13	0.2
3	3	A-AMP-CIP	7	4	11	0.2
4	3	TS-AMP-S	3	0	3	0.2
5	3	S-T-C	8	7	15	0.2
6	4	AMP-A-GM-TS	5	12	17	0.2
7	4	K-CAZ-S-AMP	6	1	7	0.2
8	4	CAZ-CTX-IMI-TS	0	9	9	0.2
9	5	ETP-C-IMI-AMP-CRO	5	5	10	0.3
10	5	TS-AMP-A-C-CAZ	0	3	3	0.3
11	6	AMP-IMI-CAZ-TS-T-C	1	2	3	0.3
12	6	K-AMP-S-A-T-IMI	2	0	2	0.3
13	6	IMI-NOR-S-T-A-TS	5	4	9	0.3
14	6	K-S-CRO-CAZ-AMP-TS	8	2	10	0.3
15	7	A-AMP-TS-K-IMI-NOR-C	0	8	8	0.4
16	7	K-S-IMI-C-T-A-NOR	8	4	12	0.4
17	8	ETP-C-IMI-T-A-AMP-CAZ-S	9	6	15	0.4
18	8	AMP-T-TS-C-K-CRO-ETP-A	0	2	2	0.4
19	8	CRO-ETP-A-S-IMI-K-AMP-T	0	1	1	0.4
20	9	T-CRO-K-AMP-NOR-TS-S-ETP-CAZ	5	3	8	0.5
21	9	CIP-AMP-T-TS-C-S-CAZ-IMI-A	0	8	8	0.5
22	10	C-IMI-TS-T-CRO-ETP-NOR-K-A-AMP	4	9	13	0.5
Total			91	98	189	

[32, 57, 64, 65]. Poorly resourced farmers lack adequate farm infrastructures necessary for modern livestock production [66]. Infrastructures such as crush pans and digital weighing scales are needed to weigh animals to aid proper dosage of medicine for animal prophylaxis and therapy. Thus, during antibiotic administration, animals could be given a suboptimal dose or overdose of antimicrobial agents. Moreover, the acute shortage of veterinary skilled labor further

compounds the problem of AMR as veterinarians are responsible for primary animal health care [67]. Hence, farmers often resort to self-medicating their animals.

The misapplication of antibiotics selects for AMR and the transfer of resistance determinants to other bacteria population. This could fuel the spread of antibiotic-resistant bacteria (ARB), imposing a heavy burden on the health of humans and animals. A high circulating ARB further increases the burden of disease in the community and length of hospitalization of sick human patients.

5. Conclusion

This study demonstrated that multiple antibiotic resistance phenotypes (MARPs) were present in *S. aureus* and *E. coli* isolates obtained from meat in the formal and informal meat sectors. The overall resistance rate was high such as clindamycin, ampicillin, rifampicin, streptomycin, and amoxicillin. The bacterial isolates showed a high MARI of 0.2 to 0.5; however, the informal sector presented a higher number of MARPs than the formal sector, demonstrating a highly compromised hygiene environment for the processing of meat. Even though all samples from the formal meat sector were collected after carcass washing, the prevalence of *E. coli* in meat is disturbing, given that these are export abattoirs with established hygiene management systems. Because complete eradication of bacteria may not be possible, transmission control seems to be an appropriate goal. Some control methods are widely recognized as effective. Of these methods, the first and most effective method is to avoid transmission through hand contamination from slaughter personnel to animal carcass.

There is also an urgent need for policy formulations on the prudent use of antimicrobials in both human and veterinary medicine. Farmers in the formal and informal meat sectors need to be adequately educated about antibiotic stewardship and implication of the persistent indiscriminate use of antimicrobial agents. Likewise, butchers at the abattoir, and other slaughter points in the informal meat sector, should be educated on good slaughter and hygiene techniques. There is still a big gap in understanding the genetic background of antibiotic resistance and virulence of bacteria from food sources. Further study on the genotypic characterization of resistance in bacteria and its pathogenicity is suggested. Furthermore, whole-genome sequencing of isolated bacteria will aid the tracing of the source of contamination.

Data Availability

All data that support the conclusions of this study are described in the article.

Ethical Approval

The research was approved with reference number MUC351SJAJ01.

Conflicts of Interest

The authors report no conflicts of interest associated with this work.

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Research Article

Characterisation of Bacteriocins Produced by *Lactobacillus* spp. Isolated from the Traditional Pakistani Yoghurt and Their Antimicrobial Activity against Common Foodborne Pathogens

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Lactic acid bacteria (LAB) are widely known for their probiotic activities for centuries. These bacteria synthesise some secretory proteinaceous toxins, bacteriocins, which help destroy similar or interrelated bacterial strains. This study was aimed at characterising bacteriocins extracted from *Lactobacillus* spp. found in yoghurt and assessing their bactericidal effect on foodborne bacteria. Twelve isolated *Lactobacillus* spp. were examined to produce bacteriocins by the organic solvent extraction method. Bacteriocins produced by two of these strains, *Lactobacillus helveticus* (BLh) and *Lactobacillus plantarum* (BLp), showed the most significant antimicrobial activity, especially against *Staphylococcus aureus* and *Acinetobacter baumannii*. Analysis of SDS-PAGE showed that *L. plantarum* and *L. helveticus* bacteriocins have a molecular weight of ~10 kDa and ~15 kDa, respectively. *L. plantarum* (BLp) bacteriocin was heat stable while *L. helveticus* (BLh) bacteriocin was heat labile. Both bacteriocins have shown activity at acidic pH. Exposure to a UV light enhances the activity of the BLh; however, it had negligible effects on the BLp. Different proteolytic enzymes confirmed the proteinaceous nature of both the bacteriocins. From this study, it was concluded that bacteriocin extracts from *L. helveticus* (BLh) can be considered a preferable candidate against foodborne pathogens as compared to *L. plantarum* (BLp). These partially purified bacteriocins should be further processed to attain purified product that could be useful for food spoilage and preservation purposes.

1. Introduction

Foodborne diseases (FBDs) have been a global concern. Despite the use of modern food preservation techniques, the rate of food-related illness still increases and is a substantial cause of death, especially in countries where there is a lack of proper food safety monitoring systems. About one-third of the world population is suffering from food-related diseases each year due to the consumption of contaminated or intoxicated food like canned food, meat, poultry, and fermented dairy products [1].

Consumer exposure to a nutritious and balanced diet has encouraged scientific research in the food industry to investigate and introduce natural compounds in food processing and preservation to reduce the use of chemicals to inhibit microbial growth and increase shelf life of food products. A careful and comprehensive work to combat the food-related diseases is a highly complex challenge that involves expertise in the field of science and technology for the safety, control, and management of food [2].

Yoghurt is one of the widely used dairy products that has been traditionally made and used for centuries around the

globe (local name is *Dahi* in Pakistan). It is synthesised by milk fermentation with the help of bacteria. As a general belief for centuries, Dahi is used for curing diarrhoea in many countries including Pakistan. Many research studies have shown that yoghurt contains beneficial diversifying microflora, such as lactic acid bacteria (LAB) [3].

Lactic acid bacteria (LAB) are among the generally recognised as safe (GRAS) microorganisms and widely used as food and feed fermentation and preservatives added under strictly controlled conditions. Lactic acid bacteria, also known as Lactobacillales, are Gram-positive anaerobes that are nonsporulating rods or cocci and show a negative catalase test. During the fermentation of carbohydrates, they produce lactic acid as the primary end product and tolerate extremely low levels of pH. *Lactobacillus* is the largest genus used in the manufacturing of a variety of not only dairy products like milk, yoghurt, and cheese but also pickles, beer, wine, cider, chocolate, and other fermented foods. These bacteria are also used to produce animal feeds, e.g., silage [4].

Many strains of LAB isolated from yoghurt, which are involved in the fermentation of milk, also provide many other antimicrobial compounds such as hydrogen peroxide, diacetyl, fatty acids, reuterin (3-hydroxypropionaldehyde), ethanol, and bacteriocins. These bacteria confer health benefits by showing antagonistic activity against many pathogens. Yoghurt is considered a source of *Lactobacillus* bacteria that combats the pathogens causing certain digestive disorders and gut infections. Yoghurt is regarded as a healthy probiotic diet. Various strains of *Lactobacillus* bacteria have been isolated from yoghurt in the world in search of a novel strain with the highest efficacy [3, 4].

The bacteriocins have attracted many researchers and have been studied extensively. These small proteins or peptides (typically containing less than 60 amino acids) kill or inhibit the growth of some bacterial strains from similar or closely related species and usually have a narrow spectrum of bacterial growth inhibition [5, 6]. Based on their amino acid sequences, molecular weights, posttranslational modifications, and genetic characteristics, Gram-positive bacteriocins have been divided into four classes. Class I bacteriocins (also known as lantibiotics) are extensively posttranslationally modified and heat stable. They typically contain less than 28 amino acids (<5 kDa) and are linear or globular peptides containing the modified amino acids lanthionine and β-methyl lanthionine and dehydrated amino acids [7, 8]. Class II bacteriocins are not modified and typically contain 30-60 amino acids (<10 kDa). They have the unique properties of being heat stable, with the absence of nonlanthionine, and are positively charged. This category has further been divided into five subclasses [9]. Class III bacteriocins (also known as bacteriolysis) are large (>30 kDa) and heat labile. On exposure to high temperature (100°C), these bacteriocins usually get inactivated within 30 minutes. The genus *Lactobacillus* produces Class III bacteriocins such as helveticins J and V and lactacin B. Complex or Class IV bacteriocins contain a protein with lipid and carbohydrate molecules, which help in antimicrobial activity [10].

Bacteriocin-producing strains can be used as probiotics. Bacteriocin can be used as food additives, in the treatment of pathogen-associated diseases and cancer therapy [11, 12]. Indeed, certain infectious diseases caused by pathogenic strains of Gram-positive bacteria (staphylococci, micrococci, and streptococci) can be prevented by bacteriocins [13]. Moreover, many Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Listeria*, and *Vibrio* have also been used as a test organism to investigate the antagonistic activity of newly isolated antimicrobial peptides. Although bacteriocin has been isolated from many strains of *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus lactis*, it has shown a significant activity against a pathogen. Nevertheless, scientists are exploring novel strains from different food products to resolve the issue of most common food-related infections [12].

Increased consumer demand for natural preservatives has also made scientists focus on finding new natural inhibitors. Bacteriocin-producing LAB was used in many starter cultures to prevent pathogenic microbe from colonising many food products. These promising characteristics of bacteriocin and bacteriocin-producing LAB made them important not only for food preservation but also for treating certain drug-resistant pathogens [13, 14].

The purpose of the current study was to achieve partial purification of bacteriocins synthesised by *Lactobacillus* spp. found in locally made yoghurt samples. These partially purified bacteriocins were further compared for their physical and biochemical properties, and their antagonistic activity was tested against foodborne bacterial pathogens. The two isolates chosen for assessing bacteriocin production and activity were *L. helveticus* and *L. plantarum*.

1.1. Contributions. Our contributions in this study are as follows:

(i) In contrast to the aforementioned studies, we did partial purification, characterisation, and antibacterial activity of bacteriocin isolated from *Lactobacillus* species found in traditional yoghurt. The *Lactobacillus* species isolated from yoghurt samples were further assessed, and characterisation has shown that *L. helveticus* and *L. plantarum* strains were involved in the production of bacteriocins. The comparative study analysis on both bacteriocins showed that BLh bacteriocin was the most suitable biocontrol agent against foodborne pathogens.

(ii) Among these two strains, bacteriocin extracted from *L. helveticus* was seldom found anywhere in the world. It was the first time that this strain has been isolated from a yoghurt sample, while *L. plantarum* has been isolated many times from dairy products. Bacteriocin from theses isolates could further be subjected to some in vivo approaches to find the actual target of the extracted bacteriocin.

2. Methodology

2.1. Bacterial Strains, Media, and Cultivation Conditions. Ten clinically identified strains (indicator organism) used in this study were ordered from the Bacteriology Lab, Department

of Microbiology, at the Faculty of Sciences, Punjab University, Pakistan. The indicator strains included in this study are as follows: *Enterococcus faecium* DO, *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Salmonella paratyphi* A.

2.2. Isolation, Screening, and Identification of Bacteriocin-Synthesising Strains. Overall, 50 "yoghurt" samples were purchased from the local shops in loose from several urban areas in Khyber Pakhtunkhwa province of Pakistan, over three months (from January 2019 to April 2019). These traditional yoghurts were made from previously homemade yoghurts and then homogenised aseptically. Ten grams of each yoghurt sample was mixed in 50 ml of 0.9% *w*/*v* of a sterile normal saline and vortexed. Then, this mixture was serially diluted eight times. The diluted samples were plated (0.1 ml suspension) on De Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany) by the spread plate method. The MRS agar plates were incubated anaerobically at 37°C for a maximum of 48 hours using the BBL GasPak system. About twelve different colonies appeared on each plate, which was then subcultured on the MRS medium for further screening of bacteriocin-producing isolates.

All isolates were identified by the method described by Schillinger and Lücke [15]. API 50 CHL test strips (bioMérieux, Marcy l'Etoile, France) were used in sugar fermentation tests.

2.3. Detection of Antagonistic Activity. The bacteriocin-producing isolates (test organisms) were screened by the agar well diffusion method [16]. An inoculum of test organisms was added into MRS broth (50 ml) which was then subjected to 24 hrs incubation at 37°C in a shaking incubator. Following incubation, broth containing the test organisms was centrifuged at 2500 x g for 5 minutes. Following incubation, the indicator organism (MRSA bacteria) lawn was produced on an MRS agar plate by the spread plate method. Clear wells containing a diameter of 6 mm were made in the agar with the help of a sterile Pasteur pipette. An aliquot of the supernatant (~60 μl) was loaded into each well, and these were labelled accordingly. The plates were then incubated at 37°C for 18-24 hrs. The plates were examined for clear zones surrounding the wells, which indicate the bacteriocin activity of the test organisms. The size of the zone of inhibition was measured in millimeters and was duly logged.

2.4. Extraction of Bacteriocins. The solvent extraction method of Westley et al. [17] was employed for the extraction of bacteriocins from the selected strains. To obtain a cell-free extract, the isolated bacteria were inoculated into MRS broth (100 ml) and incubated at 37°C for 24 hours. In a 500 ml separating funnel, culture broth (~50 ml) and an equal volume of ethyl acetate were added. The separating funnel was shaken vigorously for 10 minutes, and then, the content was allowed to settle, making two distinct layers. The upper organic layer that contained the bacteriocin was separated carefully. The same procedure was repeated with the rest of the broth culture, and the upper solvent layer was separated. The solvent was then removed under vacuum using a rotary evaporator. The final dried extract was dissolved in 1 ml of methanol, and the supernatant was adjusted to pH 6 with 1 M NaOH to eliminate inhibitory activity from acid and then treated with 5 mg/ml catalase to remove the antagonistic activity from hydrogen peroxide. The extract was passed through a 0.20 μm pore size membrane filter (Sartorius Stedim). The filtered supernatant was stored in glass vials.

2.5. Activity of Extracted Bacteriocins against Indicator Strains. The antibacterial activity of extracted bacteriocin was performed by agar well diffusion assay—activity against ten indicator strains. Antibacterial activity was measured by the zone of inhibition.

2.6. Physical and Biochemical Characteristics of Bacteriocins. The factors affecting the antimicrobial activity of bacteriocins partially extracted from *L. helveticus* (BLh) and *L. plantarum* (BLp) were as follows.

2.6.1. Temperature. All samples of bacteriocin extracts were exposed to different temperatures for 15 minutes. Then, their activities were tested using the agar well diffusion method against all indicator organisms, and activities were compared to nonexposed bacteriocins as a control.

2.6.2. pH. To test how pH affects the bacteriocin activity, an aliquot of bacteriocin extract (0.5 ml) was added into MRS broth (4.5 ml) at different pH values (3 to 11) and incubated for 30 minutes at 37°C. Bacteriocin samples were exposed to different pH values and were assayed against indicator organisms by the agar well diffusion method, and activities were compared to nonexposed bacteriocins as a control.

2.6.3. Bile Salts. The bile salts affecting bacteriocin activity were tested by adding an aliquot of bacteriocin extract (0.5 ml) into MRS broth (4.5 ml) at different bile salt concentrations (0.1 to 0.6%) and incubated for 30 minutes at 37°C. Bacteriocin samples exposed to different bile salt concentrations were assayed against indicator organisms by the agar well diffusion method, and activities were compared to nonexposed bacteriocins as a control.

2.6.4. UV Light. Bacteriocin extracts (10 ml) were exposed to UV light of different wavelengths for the periods of 15, 30, 45, 60, and 75 minutes. Bacteriocin samples exposed to different UV light conditions were assayed against indicator organisms by the agar well diffusion method, and activities were compared to nonexposed bacteriocins as a control.

2.6.5. Enzymes. The extracted bacteriocin was tested with different enzymes (1 mg/ml) and incubated for 2 h at 37°C: proteinase K, chymotrypsin, trypsin, pepsin, pronase, papain, and α-amylase (Sigma, USA). The control without enzyme was also incubated. The agar well diffusion method was applied to measure the antimicrobial activity.

2.6.6. Size Estimation of Extracted Bacteriocins. The molecular weights of bacteriocins were estimated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

PAGE) using LKB Bromma 2050 Midget electrophoresis units (Pharmacia Amersham Co.). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. A protein ladder (170-10 kDa) was used for estimating the molecular weights of bacteriocins.

2.7. Statistical Analysis. Statistical tools like the Pearson's correlation and ANOVA were applied to analyse the data. R program version 1.3.959, GraphPad Prism version 7.04, and MS Excel 2016 were used to analyse data.

3. Results and Discussion

3.1. Isolation, Screening, and Identification of Lactobacillus spp. from Yoghurt. Around 50 samples of yoghurt were collected from different areas of KPK, Pakistan, out of which twelve *Lactobacillus* strains were isolated and screened for antimicrobial activity against the indicator organisms. Among these 12 strains, *L. helveticus* and *L. plantarum* showed the highest antibacterial activity against MRSA. Both the strains of *Lactobacillus* species (*L. helveticus* and *L. plantarum*) were identified by the first Culture Bank of Pakistan. During the morphological characterisation, *L. helveticus* produced white colonies and *L. plantarum* produced yellow colonies on MRS agar plates. Gram staining confirmed that the *Lactobacillus* spp. were Gram-positive rods which are nonmotile and noncapsulated, with rounded ends, and did not form spores. *L. helveticus* showed a negative pigmentation test, while *L. plantarum* was positive for the test. Biochemical features of the *L. helveticus* and *L. plantarum* isolates included positive results for the citrate utilisation test and negative results for the indole, methyl red, hydrogen sulphide, and oxidase tests as shown in Table S1. The catalase test was negative for *L. helveticus* and positive for *L. plantarum*. The salt tolerance test revealed *L. helveticus* as NaCl tolerant, while *L. plantarum* failed to show the tolerance.

3.2. Screening for Antagonistic Activity against Indicator Organism. Bacteriocin production occurred when *Lactobacillus* spp. were in the log phase of growth. Among twelve supernatants, only two isolates showed antagonistic activity against the indicator. The agar well diffusion method revealed that *L. helveticus* (BLh) showed the most significant activity against MRSA after 18 to 24 hours of incubation at 37°C. In comparison, *L. plantarum* (BLp) showed similar activity against MRSA after 24 to 48 hours of incubation at 37°C under anaerobic conditions, as shown in Figure S1.

3.3. Antimicrobial Activity of Bacteriocin Extracts. The partial purification of bacteriocins from *Lactobacillus* sp. isolates was achieved using the organic solvent extraction method. Here, the upper ethyl acetate layer contains bacteriocins, and the lower aqueous layer contains waste material. Bacteriocin purification was not achievable through the ammonium sulphate method as no pellet was obtained during this method. The antimicrobial activities of bacteriocin extracts from *L. helveticus* (BLh) and *L. plantarum* (BLp) were tested against Gram-positive and Gram-negative indicator organisms using the agar well diffusion method, and they both showed the most significant activity against *S. aureus* (21.2 ± 0.1 and 19.3 ± 0.1 mm, respectively) (Figure 1 and Table S2). There was lower activity against *S. pyogenes* (2.6 ± 0.1 and 3.5 ± 0.1 mm) as shown in Table S3. A similar pattern for the inhibitory zone of bacteriocins against food spoilage bacteria was reported by Abo-Amer [18]. Here, *L. plantarum* released bacteriocin between the late log and stationary growth phases with concomitant antimicrobial activity. Consistent with our study, Abo-Amer [18] isolated *L. plantarum* AA135 from Egyptian homemade yoghurt, and this showed the most significant activity against foodborne Gram-positive pathogens (*S. epidermidis*, *S. aureus*, and *Bacillus cereus*) and weaker activity against *Listeria monocytogenes* and *Bacillus subtilis*. The partially purified bacteriocin isolated from *L. plantarum* and *Lactobacillus helveticus* showed strong activity against Gram-negative bacteria such as *E. coli*, *A. baumannii*, *P. aeruginosa*, and *S. paratyphi* A. Triplicate studies were performed on the zone of inhibition to measure the standard deviation.

3.4. Effects of Environmental Conditions on the Activity of Lactobacillus sp. Bacteriocin Extracts. The effects of temperature, pH, bile salts, and UV light on the stability and activity of bacteriocins extracted from *L. helveticus* (BLh) and *L. plantarum* (BLp) were observed. These conditions affected the antagonistic activity of bacteriocins against the Gram-positive and Gram-negative indicator organisms, which are all foodborne pathogens. Some of the conditions enhanced bacteriocin activity, while other conditions decreased their antimicrobial activity.

3.4.1. Temperature. Temperature plays a crucial role in bacteriocin activity, and this was shown regarding the graph between the zone of inhibition of the indicator organisms and temperature (Tables 1–4). There was a general trend showing a decrease in bacteriocin activity with an increase in temperature with a clear difference between the two *Lactobacillus* species. The bacteriocin extracted from *L. plantarum* (BLp) was quite active even passing through high temperature and pressure during sterilisation, indicating it is a heat-stable protein. Abo-Amer and Fatima and Mebrouk have reported similar observations in different studies [18, 19], emphasising the usefulness of *L. plantarum* bacteriocin in food preservation procedures due to its high-temperature tolerance. The bacteriocin synthesised by *L. helveticus* (BLh) was more heat labile, withstanding temperatures of up to only 50°C for 15 minutes. Exposure to temperatures of 60°C or above for 15 minutes resulted in the loss of activity. Bacteriocins produced by both *L. helveticus* and *L. plantarum* showed the most significant activity following exposure to 30°C for 15 minutes with a broad range of activity against *S. aureus*, MRSA, *E. coli*, *S. paratyphi*, and *A. baumannii* as shown in Figures S2 and S3.

3.4.2. pH. Bacteriocins produced by the isolated *Lactobacillus* spp. were active over a wide-ranging pH, with maximum activity recorded at pH 5 (Tables 5–8). Both retained antimicrobial activity following exposure to acidic conditions at pH 3 to 6 and pH 7. Following exposure to alkaline conditions at pH 8-11, the *L. plantarum* (BLp) bacteriocin showed

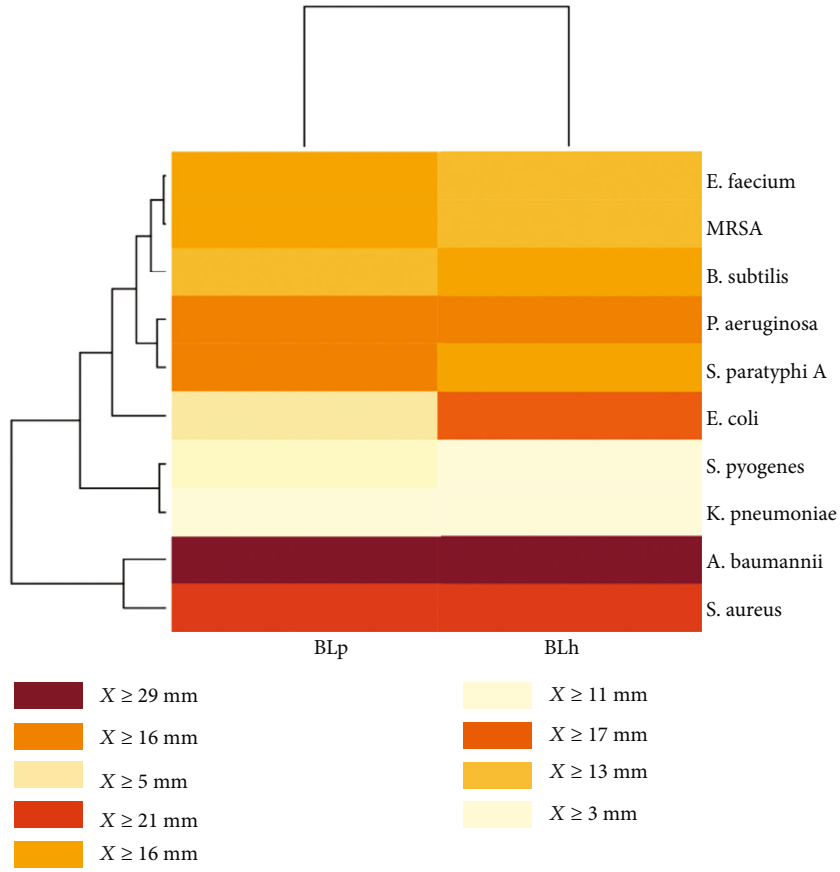

Figure 1: Graphical representation of the antagonistic activity map of partially purified bacteriocins against ten indicator strains. BLh stands for the bacteriocin isolated from *L. helveticus*, and BLp stands for bacteriocin isolated from *L. plantarum*.

Table 1: Effect of temperature on *L. helveticus* (BLh) bacteriocin activity against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. temperature variations (°C)									Pearson's correlation b/w temperature variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		30°C	40°C	50°C	60°C	70°C	80°C	90°C	100°C	121°C		
BLh bacteriocin	*E. faecium*	16.8	14.7	12.6	0.9	0.5	0.3	0.2	0.1	0.0	0.006 (significant)	0.68
	B. subtilis	18.1	13.76	12.0	0.7	0.4	0.2	0.1	0.0	0.0	0.006 (significant)	0.67
	S. pyogenes	16.4	12.80	10.8	0.6	0.3	0.1	0.0	0.0	0.0	0.006 (significant)	0.67
	MRSA	20.5	18.83	16.5	0.4	0.2	0.0	0.0	0.0	0.0	0.007 (significant)	0.65
	S. aureus	20.2	17.8	16.0	0.0	0.0	0.0	0.0	0.0	0.0	0.008 (significant)	0.65

Table 2: Effect of temperature on *L. helveticus* (BLh) bacteriocin activity against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. temperature variations (°C)									Pearson's correlation b/w temperature variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		30°C	40°C	50°C	60°C	70°C	80°C	90°C	100°C	121°C		
BLh bacteriocin	*K. pneumoniae*	10.8	10.3	8.9	0.3	0.2	0.1	0.0	0.0	0.0	0.009 (significant)	0.64
	P. aeruginosa	12.1	11.2	10.7	0.4	0.1	0.0	0.0	0.0	0.0	0.007 (significant)	0.65
	A. baumannii	12.7	12.3	11.6	0.8	0.5	0.0	0.0	0.0	0.0	0.006 (significant)	0.67
	S. paratyphi	20.0	19.6	18.0	0.6	0.4	0.2	0.1	0.0	0.0	0.008 (significant)	0.65
	E. coli	30.2	28.8	27.4	0.9	0.3	0.1	0.0	0.0	0.0	0.008 (significant)	0.65

Table 3: Effect of temperature on the activity of the purified *L. plantarum* (BLp) bacteriocin against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. temperature variations (°C)									Pearson's correlation b/w temperature variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		30°C	40°C	50°C	60°C	70°C	80°C	90°C	100°C	121°C		
BLp bacteriocin	*E. faecium*	16.3	14.2	13.0	11.2	10.5	8.7	6.5	4.7	2.6	$P < 0.00001$ (significant)	0.9914
	S. pyogenes	17.4	13.6	11.6	10.5	7.5	5.7	4.2	3.7	1.7	$P < 0.00001$ (significant)	0.9487
	B. subtilis	16.3	12.5	10.8	8.7	6.5	4.5	3.3	2.6	1.4	$P < 0.000026$ (significant)	0.9326
	MRSA	20.6	18.8	16.4	16.7	14.5	12.7	10.4	8.4	4.3	$P < 0.00001$ (significant)	0.9843
	S. aureus	19.7	17.7	15.4	15.7	13.5	11.6	9.5	7.4	3.3	$P < 0.00001$ (significant)	0.9841

Table 4: Effect of temperature on the activity of the purified *L. plantarum* bacteriocin (BLp) against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. temperature variations (°C)									Pearson's correlation b/w temperature variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		30°C	40°C	50°C	60°C	70°C	80°C	90°C	100°C	121°C		
BLp bacteriocin	*K. pneumoniae*	10.8	10.3	8.9	7.7	7.03	5.8	5.4	2.3	0.0	$P < 0.00001$ (significant)	0.98
	P. aeruginosa	12.1	11.2	10.7	9.0	7.8	6.6	5.8	3.8	0.0	$P < 0.00001$ (significant)	0.97
	E. coli	30.2	28.8	27.4	26.0	24.9	23.7	22.8	10.9	0.0	$P < 0.001$ (significant)	0.77
	A. baumannii	12.7	12.3	11.6	10.56	9.0	8.0	7.1	5.66	0.0	$P < 0.00001$ (significant)	0.94
	S. paratyphi	20.0	19.6	18.06	16.63	15.7	15.3	14.2	8.8	0.0	$P < 0.0004$ (significant)	0.84

Table 5: Effect of pH (scale 3 to 11) on the *Lactobacillus helveticus* (BLh) bacteriocin activity against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. pH variations									Pearson's correlation b/w pH variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		3	4	5	6	7	8	9	10	11		
BLh bacteriocin	*E. faecium*	10.83	15.80	4.0	3.5	0.9	0.08	0.24	0.04	0.30	$P = 0.00799$ (significant)	0.6589
	B. subtilis	10.0	15.13	10	5.0	1.2	0.07	0.04	0.04	0.10	$P = 0.002118$ (significant)	0.7621
	S. pyogenes	9.06	13.83	12.0	10.0	1.5	0.32	0.06	0.02	0.03	$P = 0.002734$ (significant)	0.7451
	S. aureus	8.23	12.90	25.0	15.0	2.0	0.22	0.29	0.02	0.03	$P = 0.038482$ (significant)	0.4809
	MRSA	6.56	11.96	30.0	20.0	3.56	0.28	0.58	0.42	0.42	$P = 0.091685$ (not significant)	0.3535

Table 6: Effect of pH (scale 3 to 11) on the *Lactobacillus helveticus* (BLh) bacteriocin activity against Gram-negative bacteria.

	Gram negative bacterial trains	Zone of inhibition (mm) vs. pH variations									Pearson's correlation (r) b/w pH variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		3	4	5	6	7	8	9	10	11		
BLh bacteriocin	*K. pneumoniae*	10.66	16.00	8.0	5.0	0.9	0.08	0.24	0.04	0.30	$P = 0.00287$ (significant)	0.7413
	P. aeruginosa	9.33	15.00	13.0	9.0	1.2	0.07	0.04	0..040	0.01	$P = 0.003307$ (significant)	0.7321
	A. baumannii	8.66	13.83	17.0	15.0	1.5	0.32	0.06	0.02	0.03	$P = 0.013958$ (significant)	0.6023
	S. paratyphi	7.33	12.50	22.0	18.0	2.0	0.22	0.29	0.02	0.03	$P = 0.044299$ (significant)	0.462
	E. coli	5.53	11.6	30.6	25.0	3.56	0.28	0.58	0.42	0.42	$P = 0.110604$ (not significant)	0.3236

a sharp decline at alkaline pH, while the *L. helveticus* (BLh) bacteriocin did not show any activity beyond pH 8. Similar observations were made by Sankar et al. [20], where bacteriocins maintained only partial antagonistic activity when the pH was changed from acidic to basic. Fatima and Mebrouk [19] also observed that bacteriocin synthesised by *L. plantarum* had maximum activity at acidic pH, and Joerger and Klaenhammer [21] reported that bacteriocin produced by *L. helveticus* had maximum activity at acidic pH. Bacteriocins produced by both *Lactobacillus* spp. showed the most significant activity against MRSA, *S. aureus*, *S. paratyphi*, and *E. coli* than to compared to the rest of the tested organisms, as shown in Figures S4 and S5. The weaker antagonistic activity was observed for *K. pneumoniae* and *P. aeruginosa* (Tables 5 and 7).

3.4.3. Bile Salts. The tolerance to bile salts was assessed by exposing the bacteriocin extracts to different concentrations of bile salts (0.1 to 0.6%) (Tables 9–12). The *L. plantarum* (BLp) bacteriocin had a bile salt tolerance of 0.4%, while

Table 7: Effect of pH (scale 3 to 11) on the *Lactobacillus plantarum* (BLp) bacteriocin activity against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. pH variations									Pearson's correlation (r) b/w pH variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		3	4	5	6	7	8	9	10	11		
BLp bacteriocin	MRSA	10.70	11.00	25.0	20.0	18.0	4.0	3.5	2.5	2.0	$P = 0.065033$ (not significant)	0.4062
	B. subtilis	10.40	10.33	10.0	10.0	9.0	4.06	4.40	3.56	4.00	$P = 0.000659$ (significant)	0.8281
	S. pyogenes	10.36	10.13	16.0	14.0	11.0	2.80	2.50	2.0	0.90	$P = 0.009628$ (not significant)	0.6414
	S. aureus	10.96	11.73	22.0	17.0	16.0	3.5	3.2	3.1	1.5	$P = 0.033546$ (significant)	0.4984
	E. faecium	12.00	11.26	8.0	8.0	7.0	1.5	1.3	1.2	00	$P = 0.000028$ (significant)	0.9296

Table 8: Effect of pH (scale 3 to 11) on the *Lactobacillus plantarum* (BLp) bacteriocin activity against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. pH variations (°C)									Pearson's correlation (r) b/w pH variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		3	4	5	6	7	8	9	10	11		
BLp bacteriocin	*K. pneumoniae*	10.66	16.00	8.0	5.00	0.9	0.08	0.24	0.04	0.30	$P = 0.00287$ (significant)	0.7413
	P. aeruginosa	9.33	15.00	13.0	9.00	1.2	0.07	0.04	0.04	0.01	$P = 0.003307$ (significant)	0.7321
	A. baumannii	8.66	13.83	17.0	15.0	1.5	0.32	0.06	0.02	0.03	$P = 0.013958$ (significant)	0.6023
	S. paratyphi	7.33	12.50	22.0	18.0	2.0	0.22	0.29	0.02	0.03	$P = 0.044299$ (not significant)	0.4620
	E. coli	5.53	11.60	30.6	25.0	3.56	0.28	0.58	0.42	0.42	$P = 0.110604$ (not significant)	0.3236

Table 9: Effect of bile salts (concentration 0.1 to 0.6) on *Lactobacillus helveticus* (BLh) activity against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. bile salt (solution variations in %)					Pearson's correlation b/w solution variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		0.1%	0.2%	0.3%	0.4%	0.6%		
BLh bacteriocin	*E. faecium*	2.20	0.86	0.06	0.07	0.02	$P = 0.104853$ (not significant)	0.6394
	B. subtilis	2.46	1.06	0.17	0.08	0.03	$P = 0.089157$ (not significant)	0.6729
	S. pyogenes	2.80	1.36	0.30	0.04	0.07	$P = 0.081962$ (not significant)	0.7012
	S. aureus	2.96	1.56	0.42	0.09	0.06	$P = 0.062848$ (not significant)	0.7265
	MRSA	3.20	1.90	0.73	0.09	0.06	$P = 0.043065$ (significant)	0.7932

Table 10: Effect of bile salts (concentration 0.1 to 0.6) on *Lactobacillus helveticus* (BLh) bacteriocin activity against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. bile salt (solution variations in %)					Pearson's correlation b/w solution variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		0.1%	0.2%	0.3%	0.4%	0.6%		
BLh bacteriocin	*K. pneumoniae*	1.73	1.00	0.06	0.06	0.08	$P = 0.093562$ (not significant)	0.6641
	P. aeruginosa	2.66	1.26	0.08	0.07	0.06	$P = 0.095045$ (not significant)	0.6593
	A. baumannii	2.83	2.02	0.90	0.08	0.08	$P = 0.03092$ (significant)	0.833
	S. paratyphi	3.02	2.03	0.47	0.04	0.05	$P = 0.052663$ (not significant)	0.7639
	E. coli	3.29	2.93	1.06	0.08	0.07	$P = 0.035733$ (significant)	0.816

the *L. helveticus* (BLh) bacteriocin did not show any tolerance to 0.4% of bile salts. The *L. helveticus* bacteriocin was found to be highly active against Gram-positive and Gram-negative bacteria in the presence of 0.1 and 0.2% bile salts, while the bacteriocin synthesised by *L. plantarum* was found significantly active against indicator strains in the presence of 0.1 and 0.3% bile salts as shown in Figures S6 and S7. Previous studies have reported that *Lactobacillus* spp. showed tolerance to 0.3% bile salts, especially those that release thermally stable bacteriocins [22]. In agreement with this, the heat-labile *L. helveticus* bacteriocin had a bile salt tolerance of up to only 0.1%. Bile salt tolerance allows such bacteria to survive, grow, and perform useful functions in the gastrointestinal tract [23].

3.4.4. UV Light. Exposure to UV light could change the nature, structure, and function of a protein. It was noticed that the bacteriocin synthesised by *L. helveticus* (BLh) had increased activity following UV light exposure for 30 and 15 minutes, while the bacteriocin synthesised by *L. plantarum* (BLp) was not affected under the same conditions as shown in Figure S8. Similar observations were described by Fatima and Mebrouk [19] and Ogunbanwo et al. [24]. The bacteriocin synthesised by *L. helveticus* had the highest

Table 11: Effect of bile salts (concentration 0.1 to 0.6) on *L. plantarum* (BLp) bacteriocin activity against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. bile salt (solution variations in %)					Pearson's correlation b/w solution variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		0.1%	0.2%	0.3%	0.4%	0.6%		
BLp bacteriocin	*K. pneumoniae*	10.10	8.66	10.30	7.30	6.23	$P = 0.074956$ (not significant)	0.7063
	P. aeruginosa	15.46	12.76	14.30	7.63	4.23	$P = 0.020608$ (significant)	0.8707
	E. coli	29.86	10.16	20.66	8.16	7.50	$P = 0.164037$ (not significant)	0.5295
	A. baumannii	5.43	3.66	4.43	2.80	1.80	$P = 0.027335$ (significant)	0.8446
	S. paratyphi	25.16	15.50	23.5	7.36	6.36	$P = 0.092823$ (not significant)	0.6646

Table 12: Effect of bile salts (concentration 0.1 to 0.6) on *L. plantarum* (BLp) bacteriocin activity against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. bile salt (solution variations in %)					Pearson's correlation b/w solution variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		0.1%	0.2%	0.3%	0.4%	0.6%		
BLp bacteriocin	*E. faecium*	13.00	9.53	11.80	8.26	6.10	$P = 0.047788$ (significant)	0.7795
	B. subtilis	18.70	10.23	15.63	7.33	5.23	$P = 0.081962$ (not significant)	0.6897
	S. pyogenes	22.70	5.23	19.86	4.43	3.33	$P = 0.229289$ (not significant)	0.4314
	MRSA	29.16	0.09	25.8	0.07	0.03	$P = 0.287817$ (not significant)	0.3574
	S. aureus	25.80	0.90	24.00	1.30	1.06	$P = 0.302209$ (significant)	0.3404

Table 13: Effect of UV on *Lactobacillus helveticus* (BLh) bacteriocin against Gram-positive bacteria.

	Gram-positive bacterial strains	Zone of inhibition (mm) vs. time variations (minute)					Pearson's correlation b/w time variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		15 min	30 min	45 min	60 min	75 min		
BLh bacteriocin	*E. faecium*	2.9	2.58	2.42	2.37	1.96	0.006 (significant)	0.927
	B. subtilis	2.0	1.56	1.55	1.32	1.20	0.01453 (significant)	0.8981
	S. pyogenes	0.9	0.73	0.61	0.42	0.18	0.000424 (significant)	0.99
	S. aureus	5.0	5.05	4.98	4.98	4.58	0.084817 (not significant)	0.6838
	MRSA	3.97	4.15	4.01	3.78	3.48	0.087703 (not significant)	0.6768

Table 14: Effect of UV on *Lactobacillus helveticus* (BLh) bacteriocin against Gram-negative bacteria.

	Gram-negative bacterial strains	Zone of inhibition (mm) vs. time variations (minute)					P value from Pearson's correlation b/w time variations and activity (P value = 0.05 (two-tailed))	R squared (r^2)
		15 min	30 min	45 min	60 min	75 min		
BLh bacteriocin	*K. pneumoniae*	6.75	5.42	5.71	5.92	6.62	0.916535 (not significant)	0.0043
	P. aeruginosa	5.02	4.50	4.27	3.84	5.26	0.94018 (not significant)	0.0022
	A. baumannii	30.32	10.46	8.23	6.23	18.49	0.451567 (not significant)	0.1997
	S. paratyphi	5.86	4.41	4.44	4.27	5.48	0.833179 (not significant)	0.0173
	E. coli	16.02	15.42	15.33	14.66	14.44	0.003904 (significant)	0.9579

antagonistic activity against *Acinetobacter baumannii*, *E. coli*, *S. aureus*, and MRSA following 30 minutes of UV exposure and weaker antagonistic activity against *S. pyogenes*, *S. paratyphi*, and *P. aeruginosa* (Tables 13 and 14). Similar results were reported by Joerger and Klaenhammer [21] on bacteriocin produced by *L. helveticus*.

3.4.5. Enzymes. To confirm the protein nature of bacteriocins from two species, the extracts were exposed to proteolytic enzymes. The antimicrobial activity of both crude bacteriocins was completely lost after the treatment with proteinase K, chymotrypsin, trypsin, papain, pronase, and pepsin, which confirmed the proteinaceous nature. The crude extracts of both bacteriocins when treated with α-amylase showed no change in antimicrobial activity, indicating that carbohydrate moieties were not required for antimicrobial activity, as shown in Table 15.

3.5. Molecular Weights and Classification of Bacteriocins Extracted from Lactobacillus spp. The molecular size of bacteriocins extracted from *Lactobacillus* spp. was estimated by SDS-PAGE alongside molecular weight marker proteins

Table 15: Effect of enzymes activity on a crude extract of bacteriocin.

Enzyme	Crude bacteriocin from *L. plantarum* (BLp)	Crude bacteriocin from *L. helveticus* (BLh)
Proteinase K	-	-
Pepsin	-	-
Trypsin	-	-
Chymotrypsin	-	-
Papain	-	-
Pronase	-	-
Alpha-amylase	+	+

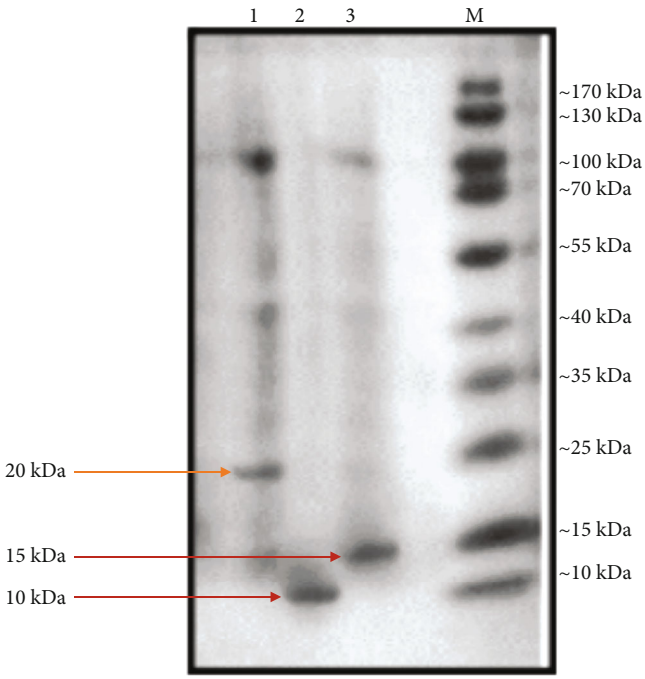

Figure 2: Molecular weight determination of bacteriocin by SDS-PAGE. Lane 1 contains the positive control, Lane 2 contains bacteriocin from *Lactobacillus plantarum*, Lane 3 contains bacteriocin from *Lactobacillus helveticus*, and Lane M contains the molecular weight marker.

(Figure 2). The bacteriocin from *L. plantarum* (BLp) had a molecular weight of approximately 10 kDa. Because this bacteriocin showed thermal stability, it might belong to the Class II bacteriocin family. The same molecular weight for bacteriocin synthesised by an *L. plantarum* strain was reported by Todorov et al. [25]. The bacteriocin from *L. helveticus* (BLh) had a molecular weight of approximately 15 kDa. Because this bacteriocin was thermally labile, it could belong to the Class III bacteriocin family. The same molecular weight for bacteriocin produced by an *L. helveticus* strain was reported by Bonadè et al. [26]. Further confirmation for the classification of bacteriocins extracted from *L. helveticus* and *L. plantarum* could be achieved by performing mass spectrometry and structural analysis of the purified proteins by NMR.

4. Conclusion

Entire analyses were completed in triplicate, following data compilation using R program 1.3.959, MS Excel 2016, and GraphPad Prism version 7.04. Pearson's correlation with a P value 0.05 was applied to the data. The data has strongly supported the alternative hypothesis, which means there was certainly some effect of bacteriocin on the indicator pathogens, especially where it showed significant values for the activity of bacteriocins during temperature variations.

In summary, bacteriocins extracted from *Lactobacillus* spp. had vigorous antimicrobial activity against Gram-positive and Gram-negative pathogenic organisms, including *S. aureus*, MRSA, *E. coli*, *Salmonella paratyphi*, and *Acinetobacter baumannii*. Various levels of activity were retained or enhanced following exposure to a range of conditions of temperature, pH, bile salts, and UV light. Based on its estimated molecular weight and the observed property of thermal stability, the bacteriocin produced by *L. helveticus* likely belongs to the Class II bacteriocin family. The larger and thermally labile bacteriocin produced by *L. helveticus* may belong to the Class III bacteriocin family. It is also concluded from our study that bacteriocin produced by *L. helveticus* was more effective against foodborne pathogens as compared with bacteriocin produced by *L. plantarum*. Further research to study the properties, structure, and mode of action of such bacteriocins is required, especially for assessing their potential use in the biocontrol of bacterial diseases and as a substitute to antibiotics.

Data Availability

The data used to support the study are available from the corresponding author upon request.

Conflicts of Interest

The authors report no conflict of interest.

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Supplementary Materials

Table S1: identification of *Lactobacillus* spp. based on physical and biochemical characteristics and antagonistic activity of bacteriocin extracts against Gram-positive and Gram-negative indicator organisms using the agar well diffusion method. Table S2: antagonistic activity of partially purified bacteriocin against Gram-negative clinical pathogens by agar well diffusion. Table S3: antagonistic activity of partially purified bacteriocin against Gram-positive clinical pathogens by agar well diffusion. Figure S1: effect of bacteriocin against methicillin-resistant *Staphylococcus aureus*. Figure S2: effect of temperature on bacteriocin against methicillin-resistant *Staphylococcus aureus*. Figure S3: effect of temperature on

bacteriocin against *E. coli*. Figure S4: effect of pH on bacteriocin against methicillin-resistant *Staphylococcus aureus*. Figure S5: effect of pH on bacteriocin against *Acinetobacter baumannii*. Figure S6: effect of bile salt on bacteriocin against methicillin-resistant *Staphylococcus aureus*. Figure S7: effect of bile salts on bacteriocin against *Acinetobacter baumannii*. Figure S8: effect of UV on bacteriocin against *Acinetobacter baumannii*. *(Supplementary Materials)*

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Research Article

Immunomodulatory and Antioxidant Effects of Polysaccharides from the Parasitic Fungus *Cordyceps kyushuensis*

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The ascomycete Cordyceps genus has been used as valued traditional Chinese medicine. *Cordyceps kyushuensis* is a unique species of Cordyceps, which parasitizes on the larvae of *Clanis bilineata* Walker, and its major component cordycepin and aqueous extract are known to have many pharmacological effects. However, the physiological function of water-soluble polysaccharides has not been explored in detail. In this study, to resolve these doubts, we extracted and separated Cordyceps-derived polysaccharides and then evaluated the immunomodulatory and antioxidant activities. Four polysaccharide fractions were purified from Cordyceps-cultured stroma by DEAE-cellulose 23 and Sephadex G-150 column chromatography. Basic structural information was elucidated on the basis of physicochemical property and spectroscopic evidences. The antioxidant activities were evaluated by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method and protective effect of DNA damage. The qualified immunologic activities were also determined *in vivo* and *in vitro*. The polysaccharides could stimulate the proliferation of mouse splenocytes whether concanavalin A (ConA) and lipopolysaccharide (LPS) existed or not, strengthen peritoneal macrophages to devour neutral red, and increase the content of interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) in serum. The research provides the corresponding evidence for Cordyceps polysaccharides as a potential candidate for functional foods and therapeutic agents.

1. Introduction

It is reported that about a total of 110,000 fungal species had been recognized [1]. Macrofungi, also known as mushrooms, have been extensively used as foods, nutraceuticals, and medicines since time immemorial [2]. The edible and medicinal mushrooms are recognized as one of the most important food supplements and have been recently studied for bioactive metabolites because of their vital roles in human health, nutrition, and various illnesses. Fungal-derived polysaccharides, a kind of natural biological macromolecules, are recognized as "biological response modifiers," mainly derived from the mycelium, fruiting bodies, and fermentation broth of the Basidiomycetes family and some from the ascomycetes. Depending on their chemical composition, molecular weight, conformation, glycosidic bond, degree of branching, etc. [3], fungal polysaccharides presented good nutritional values and pharmaceutical properties, such as immunomodulatory [4–6], antitumor [7–9], antioxidant [10], hypocholesterolemic [11, 12], hepatoprotective [13], and anti-inflammatory activities [14].

The genus Cordyceps belongs to the entomopathogenic fungi, Clavicipitaceae, and Ascomycotina [15]. *Cordyceps sinensis* and *Cordyceps militaris* have been used as traditional Chinese medicines for the effectiveness in improving lung and kidney functions, restoring health after prolonged sickness, and enhancing physical performance [16, 17]. Continuous attentions had been paid to Cordyceps-derived polysaccharides [18–21]. Polysaccharides from the stroma and mycelia culture of Cordyceps fungus have extensive health effects and pharmacological activities, such as stimulating the innate and adaptive immune responses and

activating macrophage production [22, 23]. *Cordyceps kyushuensis* is a unique species of Cordyceps, and it is worthy of further investigation. As far as we know, the only host of *C. kyushuensis* is the larvae of *Clanis bilineata* Walker [24]. Two polysaccharides purified from the cultured stroma of *C. kyushuensis* were reported previously by our group, showing remarkably antioxidant effects by assays of various antioxidant *in vitro* systems [25].

Up to now, there are only a few research papers on purification of polysaccharides from *C. kyushuensis* and their immunostimulatory activity. Therefore, in the present study, four other water-soluble polysaccharides of *C. kyushuensis* were extracted, purified, and preliminarily characterized. Moreover, immune regulation and antioxidant properties of the fractions were also evaluated *in vitro* and *in vivo*. The research would serve as a good foundation for further investigation, development, and industrial application of Cordyceps-derived polysaccharides in functional food and therapeutic agents.

2. Materials and Methods

2.1. Materials and Reagents

2.1.1. Biological Materials. The anamorph strain JY1A of *C. kyushuensis*, originally isolated from fresh natural specimen, was confirmed by means of both morphological and molecular methods and conserved by our lab. Cultured *C. kyushuensis* grew on solid rice medium and was obtained after about 90 days. The stroma was oven-dried at 60°C to constant weight, homogenized with a microplant crusher, and screened with an 80-mesh sieve for further experiment.

2.1.2. Reagents. DEAE-cellulose 23 and Sephadex G-150 were purchased from Amersham Biosciences (Uppsala, Sweden). The standard monosaccharide (D-mannose, D-galactose, D-arabinose, D-fructose, L-rhamnose, D-glucuronic acid, D-glucosamine, and D-galactosamine), trifluoroacetic acid (TFA), 1-phenyl-3-methyl-5-pyrazolone (PMP), LPS, and ConA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The RPMI 1640 medium and fetal bovine serum was provided by Gibco (Vienna, NY, USA). All other chemicals and solvents used were of analytical grade and obtained from Sinopharm (Shanghai, China).

2.2. Purification of Polysaccharides. 300 g dry stroma powder was extracted with 3 L distilled water at 90°C for 2 hours, and the supernatant was centrifuged. The above operation was repeated three times, and the supernatant was combined. The mixtures were concentrated to one-third volume and precipitated by adding four volumes of 95% ethanol (*v*/*v*) and kept at 4°C overnight. The precipitate was collected and washed with 95% ethanol, acetone, and ethyl ether, respectively. The resulting fraction was dialyzed in cellulose membrane tubing (exclusion limit 3500 Da) against deionized water and lyophilized. Protein was removed by the Sevag method [26]. Crude polysaccharide was preliminarily separated by subfractionation with gradient final concentrations of ethanol (15%, 30%, 50%, and 95%). The fraction at the final concentration of 50% ethanol, named as *Cordyceps kyushuensis* polysaccharide (CKPS), was obtained by lyophilization and selected for further study. CKPS powder (100 mg) was dissolved in 2 mL water, and the supernatant was applied to a DEAE-23 column (50 × 2.0 cm i.d.), which was eluted with water and followed by a 4-step gradient of 0-0.32 M NaCl (0.06, 0.1, 0.16, and 0.32 M). Guided by the phenol-sulfuric acid method, the NaCl-eluted fraction with high content of sugar was collected, dialyzed, lyophilized, and purified with a Sephadex G-150 column (80 × 2.0 cm i.d.).

2.3. Analysis of Physicochemical Properties. Total sugar content was determined by a phenol-sulfuric acid method [27] with glucose as the standard. Protein concentration was measured with a Bradford protein assay kit (Beyotime, Shanghai, China). Sulfate content was evaluated using the barium chloride-gelatin method [28], and the content of uronic acid was assessed by the method of m-hydroxydiphenyl using galacturonic acid as the standard [29].

Identification and quantification of monosaccharide were carried out by the HPLC method [10] with some modification. The polysaccharide (5 mg) was hydrolyzed with 2 M TFA at 120°C for 4 hours in a sealed tube fulfilled with N_2. Then, excessive acid was removed with methanol. The dried sample was dissolved in solution containing 0.3 M aqueous NaOH (0.5 mL) and 0.5 M methanol solution of PMP (0.5 mL) and incubated at 70°C for 100 min and then neutralized with 0.5 mL of HCl (0.3 M). The resulting solution was extracted with chloroform for three times. The aqueous layer was filtered through a 0.22 μm nylon membrane (MSI, Westborough, MA, USA) and injected into a Kromasil 100-C_{18} column (250 × 4.6 mm i.d., 5 μm) at 260 nm and at a column temperature of 25°C. The mobile phase, a solution of 0.02 M phosphate buffer (pH 6.7) : acetonitrile = 80 : 20 (*v*/*v*), was eluted at a flow rate of 0.9 mL/min. Identification of the target compounds was based on the comparison with reference sugars. Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

Homogeneity and absolute measure of molecular weight of the purified fractions were obtained by high-performance size exclusion chromatography (HPSEC) (Agilent Technologies, 1200 series LC system, St. Clara, CA, USA) coupled with multiangle laser light scattering (MALLS) (Wyatt Technology DAWN HELEOS II, St. Barbara, CA, USA) at 690 nm. The sample was dissolved in the mobile phase (5 mg/mL) and filtered through a 0.22 μm MSI nylon syringe filter before injection. A serial column which combined TSK-Gel G-6000 PWXL (300 × 7.8 mm i.d., Tokyo, Japan) with TSK-Gel G-5000 PWXL columns was then employed to separate the samples at 30°C. The predegassed 0.2 M NaCl aqueous solution was applied as the elution buffer at a flow rate of 0.6 mL/min. Three injection operations of the polysaccharide were performed, and molecular mass values were determined by averaging these results.

FTIR spectra were recorded in the region 4000-400 cm^{-1} on a Thermo Nicolet 20sx spectrometer at 4 cm^{-1} resolution. The samples were blended with KBr powder, grounded, and pressed into 1 mm pellets. The ^1H and ^{13}C NMR spectra were

recorded on a Brucker AM-400 MHz NMR spectrometer (Rheinstetten, Germany) at 25°C. The polysaccharide sample (25 mg) was exchanged 3 times with DMSO-*d*6 upon freeze-drying, redissolved in 0.5 mL DMSO-*d*6, and centrifuged prior to analysis.

2.4. Vertebrate Animal Study Methods

2.4.1. Animal Care, Feeding, Housing, and Grouping. A total of 60 male healthy Kunming mice (8 weeks old, 20.0 ± 2.0 g), specific pathogen free (SPF) grade, were purchased from the Laboratory Animal Center of Shandong University. The mice were housed on a 12 h dark/12 h light cycle at 22 ± 1° C and 50-60% relative humidity, free to access to a standard laboratory rodent diet and water during the experiments. All procedures involving an animal study were approved by the Ethics Committee of School of Life Science of Shandong University. After being adapted to the environment for one week, the mice were randomly divided into test and control groups (10 for each).

2.4.2. Assay of Immunity Activity In Vivo. The polysaccharide samples were dissolved in physiological saline and given intraperitoneally to mice at doses of 10 mg/kg/d for 7 consecutive days. The control group was treated with 0.2 mL physiological saline instead of the polysaccharide solution. The mice were sacrificed via cervical vertebra dislocation 24 h after the last administration. The spleen was removed aseptically and then was placed in aseptic PBS buffer. Spleen cells were harvested by gently mincing and grinding the spleen fragment through sterilized meshes (200 meshes) and centrifuged at 3000 rpm/min at 4°C for 5 min. After red blood cells were removed with erythrocyte lysis buffer, the remaining cells were washed twice and suspended to 1×10^6 cells/mL by RPMI 1640 complete medium containing 10% fetal bovine serum. The spleen cells (100 μL/well) were placed in a 96-well plate with a total volume of 200 μL per well, in the presence of mitogen (5.0 μg/mL ConA or 10.0 μg/mL LPS, final concentration) or RPMI 1640 medium, and incubated at 37°C in a humidified 5% CO_2 incubator for 48 h. Cell proliferation was determined by the CCK-8 assay (Dojindo, Kumamoto, Japan).

The blood samples were obtained from the eye orbital sinus under light ether anaesthesia prior to being sacrificed. After centrifugation at 2000 × g for 10 min, the serum samples were collected. The IL-2 and TNF-α concentrations were measured with a mouse IL-2 or TNF-α Sunny ELISA kit (MultiSciences, Hangzhou, China) according to the indication of the manufacturer.

2.4.3. Assay of Immunity Activity In Vitro. 8-12-week old SPF mice were sacrificed by cervical dislocation, spleens were collected under aseptic conditions in RPMI 1640, and spleen cells were prepared and adjusted to 1×10^6 cells/mL. 100 μL/well of splenocyte suspension was seeded into a 96-well culture plate and mixed with 100 μL polysaccharide solutions (62.5, 125, 250, and 500 μg/mL, final concentration, respectively) in triplicate. The RPMI 1640 medium was added as a blank control, and ConA (5.0 μg/mL, final concentration) and LPS (10.0 μg/mL, final concentration) was used as positive controls, respectively. The plate was incubated at 37°C in a humidified 5% CO_2 incubator for 48 h. The viable cells were determined at 450 nm. The cell proliferation rate (%) was calculated as the absorbance of sample-treated cells divided by the absorbance of control cells. Cell viability of the control group was 100%.

The resident macrophages of mice were harvested by peritoneal lavage, and the cells were subsequently cultured in RPMI 1640 complete medium and diluted to a density of 2×10^6 cells/mL. The purity of macrophages was tested by adherence. Macrophage suspension (100 μL/well) was pipetted into a 96-well culture plate and incubated for 3 h (37°C, 5% CO_2). The adherent macrophages were washed twice with complete medium and then incubated with 100 μL various concentrations (125, 250, 500, and 1000 μg/mL) of polysaccharides for 24 h. The stimulated cells were washed twice with PBS, and 100 μL neutral red (0.1%, *w*/*v*) was used to assess the phagocytosis. The plate was incubated for 3 h. After the removal of unphagocytized neutral red with PBS, 200 μL cell lysate (the volume ratio of acetic acid to ethanol was 1 : 1) was added in and kept for 3 h. The OD value of each well was read at 540 nm using the Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). DMEM and LPS (10 μg/mL) were used as the blank and positive controls, respectively.

2.4.4. Dislocation Euthanasia Method. At the end of the experiments, the cervical vertebrae of all surviving animals were dislocated by external force and the spinal cord was severed to make them die painlessly.

2.5. DPPH Radical Scavenging Assay. The radical scavenging effects of the polysaccharides were estimated by using the DPPH free radical method [30]. DPPH solution (50 μL, 0.1 mM) in 50% ethanol was added in a 96-well plate with equivalent aliquot sample solution at different final concentrations (0.5, 1, 1.5, 2, and 2.5 mg/mL). The reaction solution was shaken vigorously and incubated at room temperature for 30 min, and the absorbance at 517 nm was measured. Vitamin C (VC) was used as a positive control. The DPPH scavenging rate (R) was calculated as the following formula: $R\,(\%) = [1 - (A_s - A_i)/\mathrm{A0}] \times 100$, as indicated by the absorbance of sample or VC. Sample reference solution, which contained equivalent 50% ethanol instead of the DPPH solution, was recorded as A_i, while distilled water instead of sample was used for the blank A_0. All tests were performed in triplicate, and the mean of Abs was used in the equation above.

2.6. Determination of DNA Damage Protective Effect. DNA damage protection activities of polysaccharides were determined with pUC19 plasmid DNA, isolated from *Escherichia coli* DH5α with a SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China). pUC19 plasmid was damaged by H_2O_2 and UV treatment using the method of Yang et al. [31]. Rutin was used as a positive control. Different structural or conformational forms of plasmid DNA were separated by electrophoresis. The reaction mixture (10 mL) contained 3 mL of plasmid DNA, 5 mL of 5 mg/mL

polysaccharide or 0.4 mg/mL rutin, 1 mL of 10 mmol/mL H_2O_2, and 1 mL of water. The mixtures were located in a super clean bench with an ultraviolet lamp (20 W). After UV irradiation lasted for 5 min at room temperature, reaction samples along with a 10× gel loading dye were analyzed on a 1% agarose gel in TBE buffer at pH 8.0 for 30 min (100 V).

2.7. Statistical Analysis. For each polysaccharide, three samples were prepared for the determinations of physicochemical properties and assays of every antioxidant attribute. The samples were prepared at least in triplicate to ensure reproducibility. All bioassay results were expressed as means ± standard deviation (SD). The experimental data were fitted by using the Statistical Package of the Social Sciences (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA) and subjected to an analysis of variance (ANOVA) for a completely random design. A probability of $p < 0.05$ and $p < 0.01$ was considered significant.

3. Results

3.1. Isolation and Purification. The crude polysaccharide was obtained by water extraction, ethanol precipitation, deproteinization, and lyophilization. As shown in Figure 1(a), the 50% ethanol portion (CKPS) with a yield of 4.86% was further fractionated on a DE-23 column eluted with deionized water and at different concentrations of stepwise NaCl solution (0.06, 0.10, 0.16, and 0.32 M). Guided by the phenol-sulfuric acid method, the NaCl elutes F1, F2, F3, and F4 were further purified with a Sephadex G-150 column, respectively. Four resulting fractions shown in Figure 1(b) were named as CKPS-1, CKPS-2, CKPS-3, and CKPS-4, collected and treated for follow-up research.

3.2. Physicochemical Characterization. Positive response to the Bradford method and the adsorption detected by UV spectrum at 280 nm indicated the presence of protein. The results proved that CKPS-1, CKPS-2, CKPS-3, and CKPS-4 all contained minor amounts of protein (0.45, 1.07, 1.53, and 4.34%, respectively) and uronic acid (0.51, 0.77, 0.86, and 1.22%, respectively) and did not have any sulfate ester. Uronic acid was found in all four fractions, which suggested that these fractions were starch-like polysaccharides. The total carbohydrate contents of the samples were 84.35, 77.33, 84.29, and 78.22%, respectively. Neutral monosaccharide constitutions of the polysaccharides were analyzed by reversed-phase HPLC. CKPS-1 was mainly composed of Fru, Man, Glu, and Gal with molar ratios of 1 : 0.92 : 1.09 : 0.72. CKPS-2, CKPS-3, and CKPS-4 consisted of Fru, Man, and Gal in a molar percentage of 1 : 0.63 : 0.61, 1 : 1.65 : 1.4, and 1 : 2.06 : 1.97, respectively. Results showed that fructose, mannose, and galactose were the main monosaccharide components in four samples with different molar ratios and glucose was only found in CKPS-1, strongly indicating that the polysaccharides were heterogeneous. Large amounts of fructose components were found in both 50% ethanol and 90% ethanol precipitates of *C. kyushuensis* [24], which is quite different from the reports on polysaccharide of other Cordyceps species. The high-performance size exclusion chromatography (HPSEC) equipped with MALLS was considered to be a powerful, effective, and reliable technique for determining molecular characteristics of macromolecules without any calibration standard. Single and symmetrical peaks indicated that the four fractions were homogeneous polysaccharides. The weight-average molecular weight (Mw) of the purified polysaccharides was estimated to be 7153, 5945, 5643, and 5642 kDa, respectively. The IR spectra of four fractions exhibited the characteristic absorption of polysaccharides. All the fractions had similar infrared absorption bands indicating similarities in their structural features. The strong and broad peak between 3600 cm^{-1} and 3200 cm^{-1} was due to the stretching vibration of O-H. The bands at 2924 and 2854 cm^{-1}, which corresponded to C-H stretching vibration in -CH_2 and -CH_3 groups (usually present in hexoses, like glucose or galactose, or deoxyhexoses like rhamnose or fucose), are further proven that what we are dealing with is polysaccharide containing glucuronic acid [32]. The band at 1645 cm^{-1} corresponds to the stretching vibration of the carbonyl bond that is a part of the amide group, and the band at 1545 cm^{-1} is related to the N-H bending vibration of the same group. Occurrence of these two vibrations due to the amide group indicates the presence of protein. The signal at 1408 cm^{-1} could be assigned to stretch vibration of C-O within COOH [33]. The signal at 1225 cm^{-1} accounted for asymmetric stretching vibration of the sulfate group [34]. The absorptions in the range of 1000-1200 cm^{-1}, attributed to the stretching vibrations of C-O-C and C-O-H, were observed. It indicated the strong absorptions at around 1048 cm^{-1} due to stretching vibration of the pyranose ring. In addition, the absorption band at 811 cm^{-1} and 880 cm^{-1} indicated the presence of *d*-mannopyranose and galactose units [35].

The anomeric protons from each monosaccharide can give recognizable signals depending on α- or β-configurations. Most of α-anomeric protons usually appear in the 5-6 ppm region in ^{1}H NMR while most of the β-anomeric protons in the 4-5 ppm range [36]. The signals at 5.53 and 5.46 ppm of Figure 2(a) were attributed to α-configuration pyranose units of CKPS-1. The resonance at 4.91 ppm may be attributed to glucosyl residues [37], and ^{1}H signals at 4.53 ppm conformed to the β-form of D-galactopyranosyl residues. The chemical shifts from 3.4 to 4.2 ppm were assigned to protons of C-2-C-6 of the hexose glycosidic ring [38]. Thus, there were possibly both α- and β-type glycosidic linkages in CKPS-1. In a ^{13}C spectrum, the signals derived from α-anomeric carbons usually appear in the 95-101 ppm region while most of the β-anomeric carbons will appear in the range 101-105 ppm [36]. The major resonance in the anomeric region occurs at 97-101 ppm rather than at 90 ppm as shown in Figure 2(b), indicating that C-1 of the α-monosaccharide residue is linked [39]. The signal at 172.79 ppm was due to the carboxyl resonance signal of uronic acid, which was consistent with the IR results. As judged by the absence of signals within δ 82-88, all sugar residues were in the pyranose form. The NMR data of other three fractions were similar to those of CKPS-1 (result not shown). The detailed structural features of the four polysaccharides should be further investigated by 2D NMR, periodate oxidation, and methylation analysis.

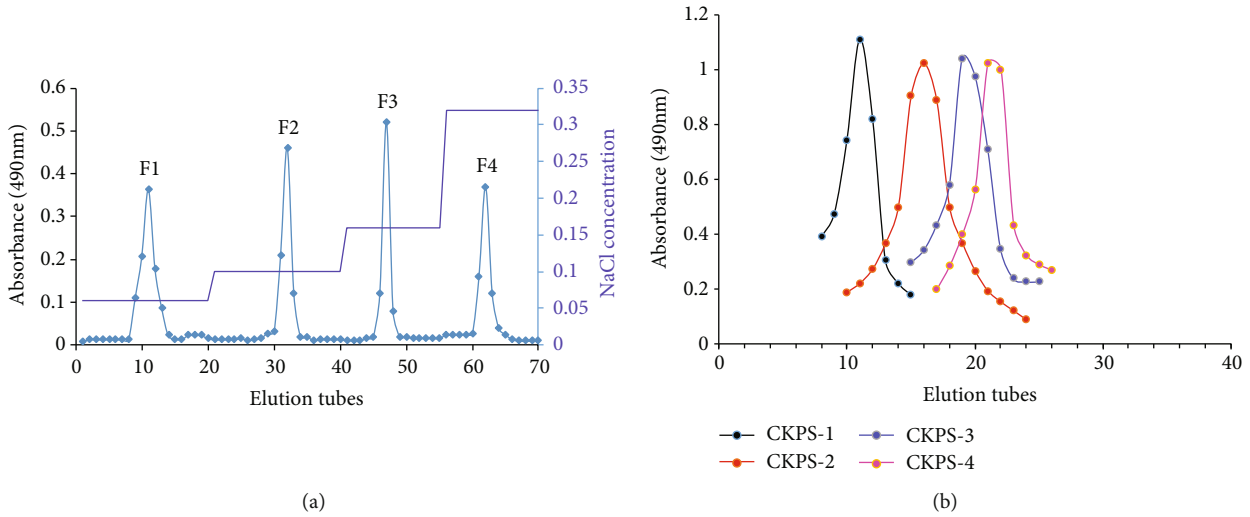

Figure 1: Separation and purification of polysaccharides of *Cordyceps kyushuensis*: (a) DE-23 chromatographic profile for CKPS eluted with different NaCl solutions; (b) Sephadex G-150 chromatographic profile for CKPS eluted with water.

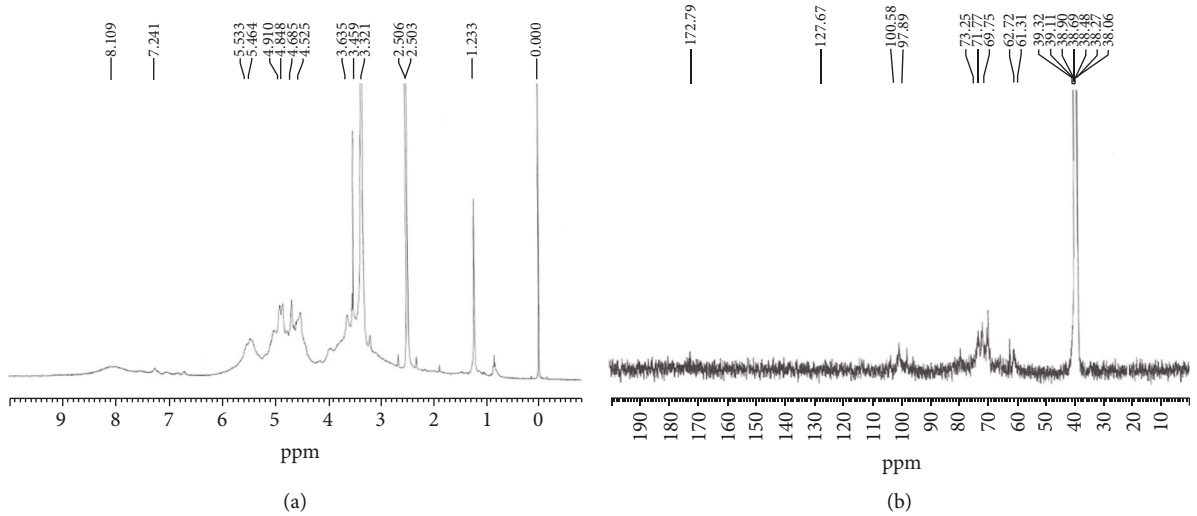

Figure 2: (a) ^{1}H NMR spectra and (b) ^{13}C NMR spectra of the purified CKPS-1 of *Cordyceps kyushuensis*.

3.2.1. Assay of Immunity Activity In Vivo. In this study, Cordyceps kyushuensis is considered to be macrofungi rich in active compounds. Good immunomodulatory and antioxidant effects were evaluated in vivo and in vitro. Lymphocytes are the key effector cells of the mammalian immune system. Proliferation of splenocytes is an indicator of immune activation, being related to immunity improvement of T lymphocyte or B lymphocyte [40]. Effects of CKPS-1, CKPS-2, CKPS-3, and CKPS-4 on splenocyte proliferation with or without mitogen (ConA or LPS) are shown in Figure 3. Spleen lymphocyte proliferation induced by ConA in vivo has been used to evaluate T lymphocyte activity, while that induced by LPS has been used to examine B lymphocyte activity. The data of Figures 3(a) and 3(b) proved that, with the administration of the four polysaccharides at the doses of 10 mg/kg, the splenocyte proliferation induced by ConA or LPS was significantly enhanced ($p < 0.01$), respectively. Figure 3(c) demonstrated that four polysaccharides still stimulated lymphocyte proliferation even without mitogenic stimuli (ConA or LPS), and the experiment results were markedly higher than those of the control medium group ($p < 0.01$). The present data also indicated that the effect of CKPS-2 on the proliferation of mixed lymphocytes (Figure 3(c)) and B lymphocytes induced by LPS (Figure 3(b)) was greater than that of the other three components, while CKPS-4 had a stronger effect on the proliferation of T lymphocytes induced by ConA (Figure 3(a)).

The IL-2 and TNF-α expression levels were measured to determine the stimulation properties of the immune response of the purified polysaccharide fractions. The mouse blood samples were taken from the orbit at the 24th hour after the last administration, and the serum samples were collected and ready to determine IL-2 and TNF-α level by extrapolation from a cytokine standard curve, according to the manufacturer's protocol. As shown in Figure 4(a), compared with the control group, the stimulating effects on the

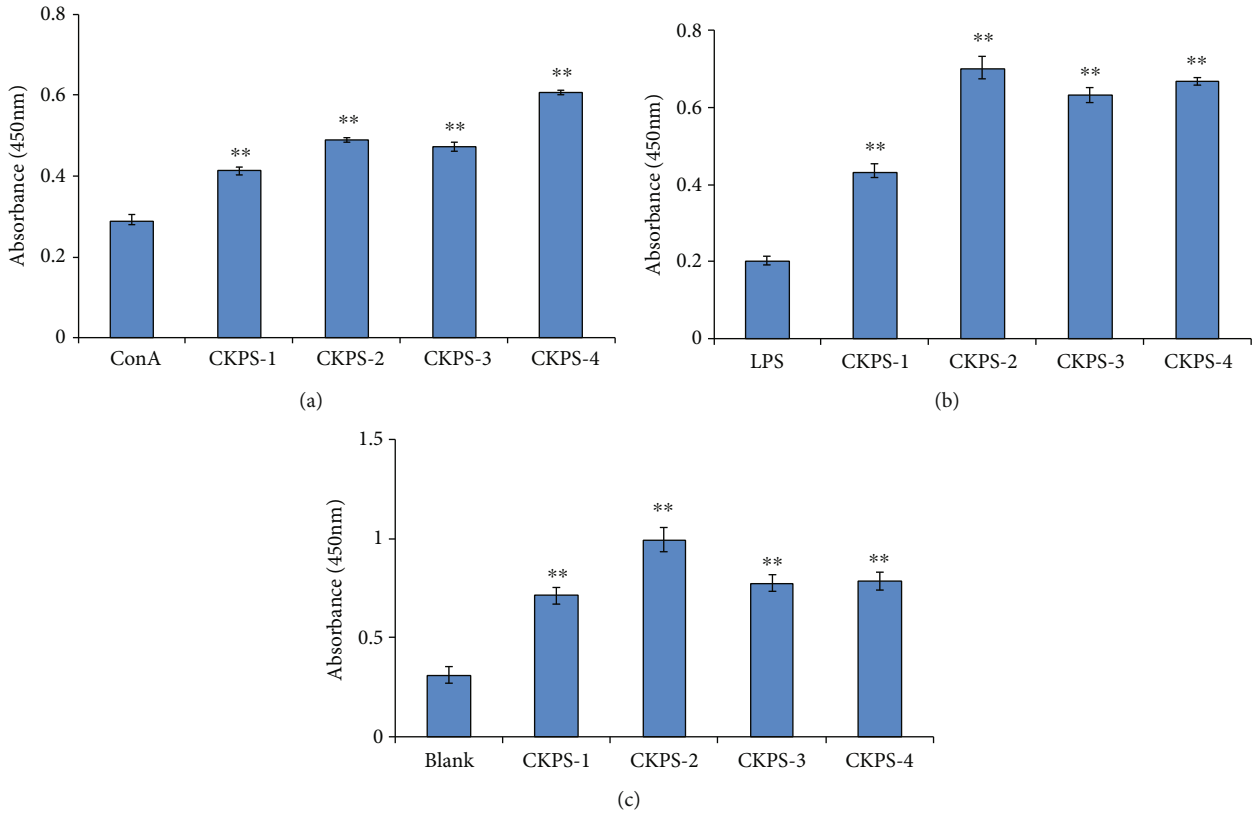

Figure 3: Splenocyte proliferation of polysaccharide fractions (a, b) with mitogens ConA and LPS or (c) without *in vivo*. Values are means ± SD ($n = 3$). $^{**}p < 0.01$ vs. control.

secretion of TNF-α were strongly enhanced by all the four fractions ($p < 0.01$). The IL-2 expression levels shown in Figure 4(b) were found to be elevated by the polysaccharides CKPS-1, CKPS-2, and CKPS-3 ($p < 0.05$). Additionally, CKPS-4 significantly promoted the secretion of IL-2 in serum ($p < 0.01$). IL-2 is essential for the growth, proliferation, and differentiation of T cells and is produced by T cells normally during an immune response [41]. TNF-α is a cytokine with tumor necrosis activity that is secreted mainly by macrophages and has been recognized as an important host regulatory molecule [42]. The experiment data demonstrated that the four polysaccharides could enhance the immune function by promoting cytokine expression levels for both T lymphocytes and peritoneal macrophages *in vivo*.

3.3. Assay of Immunity Activity In Vitro. By performing the CCK-8 assay, the effects of CKPS on normal (without mitogen) and mitogen-induced splenic lymphocyte proliferation were investigated in the final dose range of 62.5-500 μg/mL. As shown in Figure 5, both ConA and LPS could greatly stimulate lymphocyte proliferation compared with the blank. Compared with the mitogen control, CKPS-4 had excellent activities on normal proliferation ($p < 0.01$). CKPS-1, CKPS-3, and CKPS-4 exhibited significant stimulation on normal proliferation at the final concentration of 62.5-500 μg/mL ($p < 0.01$), but the promotion on the proliferation of lymphocytes had not shown a dose-dependent suppressive effect. At the lowest concentration of 62.5 μg/mL, the proliferation rate of CKPS-2 was significantly higher than that of ConA or LPS; however, in the range of 250-500 μg/mL, the high concentration of CKPS-2 did not show a stimulation effect on normal proliferation ($p > 0.05$).

One of the most distinguished features of activated macrophages is an increase in phagocytosis. The CKPS fractions were evaluated with regard to the effect on the phagocytic activity of macrophages using a neutral red uptake assay. As seen in Figure 6, each fraction had various enhancing effects on macrophage phagocytosis in the dose range of 62.5-500 μg/mL. The phagocytic indexes of macrophages under the sample treatments all exceeded 1.0. Compared with the blank control, the fractions could considerably stimulate the phagocytosis of macrophages ($p < 0.05$ or $p < 0.01$) after administration, as well as LPS action (10 μg/mL, $p < 0.01$). Macrophages played an important role in the immune system and could phagocytose aging cells, necrotic tissues, malignant cells, and pathogens invading the body and produce cytokines. The phagocytosis of macrophages was thought as one of the most important indicators of the body's nonspecific immunity [43–45]. Our present results proved that the beneficial effect of the polysaccharides on immune and inflammatory diseases might be partly attributed to the improvement of defective or deficient phagocytosis of macrophages.

3.4. Antioxidant Properties. Natural antioxidants are known to play an important role against various diseases and aging

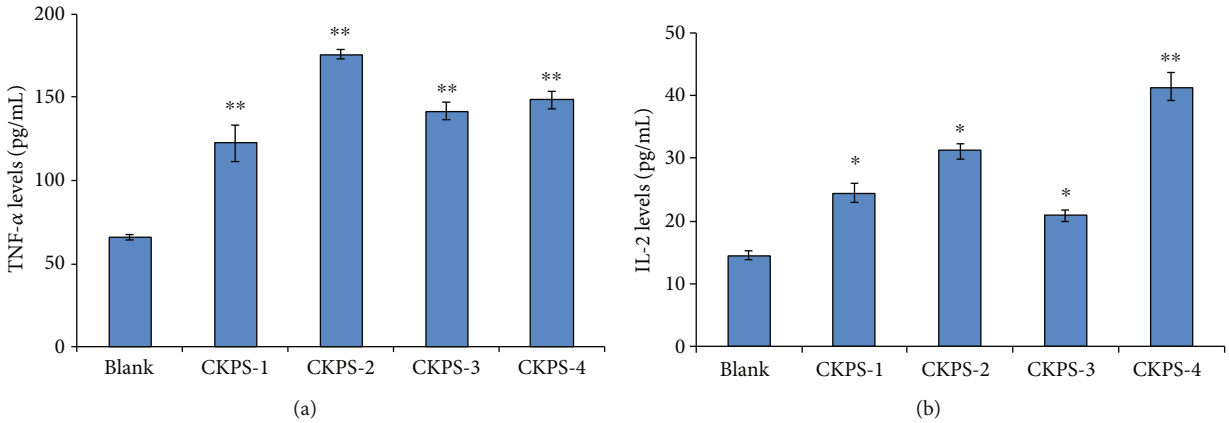

Figure 4: Effects of polysaccharide fractions on levels of serum (a) TNF-α and (b) IL-2 in mice. Values are means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

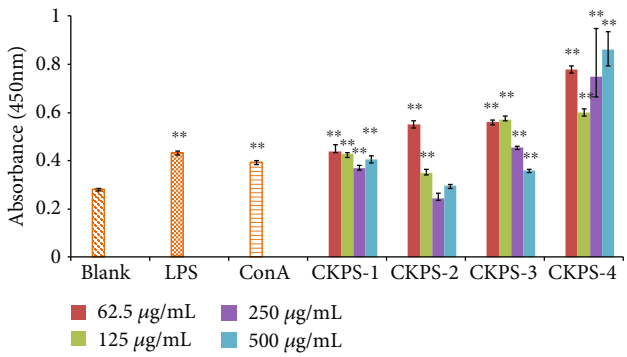

Figure 5: Splenocyte proliferation of polysaccharide fractions *in vitro*. Values are means ± SD ($n = 3$). $^{**}p < 0.01$ vs. control.

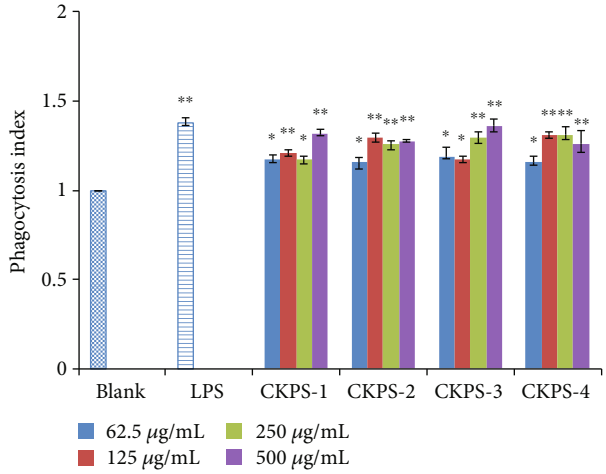

Figure 6: Macrophage phagocytosis of polysaccharide fractions and LPS by the neutral red uptake assay. Values are means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

processes. Polysaccharides were generally considered to have potential antioxidant activity. Thus, it is essential to determine the antioxidant capacities of four fractions from the stroma of *C. kyushuensis*. DPPH radical methods were often conducted to evaluate the free radical scavenging ability of natural compounds [46]. As a stable free radical, DPPH showed the maximum absorption at 517 nm with violet color due to its odd electron. When DPPH encountered antioxidant scavengers, the resulting decolorization was stoichiometric with respect to the ability to bleach the DPPH radical. The scavenging effect was measured and is shown in Figure 7(a). All the polysaccharide fractions showed a good scavenging effect against DPPH radical in a dose-dependent manner at each concentration level. The scavenging ratios at the highest concentration of CKPS-3 and CKPS-4 were 63.5% and 59.7%, respectively. And compared with other samples, especially CKPS-4 had much stronger antioxidant activity even at the low concentration of 1 mg/mL.

The protective effects of the polysaccharides on the damage induced by the coaction of H_2O_2 and UV were studied on pUC19 plasmid. Figure 7(b) demonstrates the electrophoretic pattern of DNA after UV photolysis of H_2O_2 (2.5 mmol/L) in the absence or presence of CKPS-1 to CKPS-4 and rutin. DNA derived from pUC19 plasmid showed the band corresponding to the native form of supercoiled circular DNA (Sc DNA) on agarose gel (lane 7). After the UV irradiation of DNA with H_2O_2, the graph of lane 6 proved the result of the cleavage of Sc DNA to an open circular form (Oc DNA) [47]. With the addition of rutin and CKPS-1 to CKPS-4, lanes 1-5 revealed the protection effect of the polysaccharides to the damage of native Sc DNA. Lanes 2 and 3 of the gel showed clearly the Sc DNA band, which indicated that CKPS-1 and CKPS-2 had a relatively stronger capacity to suppress the formation of Oc DNA than other polysaccharides (lanes 4 and 5). The positive control of rutin (lane 1) had almost the same protective effect. A good foundation was established for the application of Cordyceps in food and pharmaceutical industry.

4. Discussion

Edible and medicinal mushrooms have been well known and widely consumed in far Asia as part of traditional diet and medicine for a long history for their flavor, nutrition, and

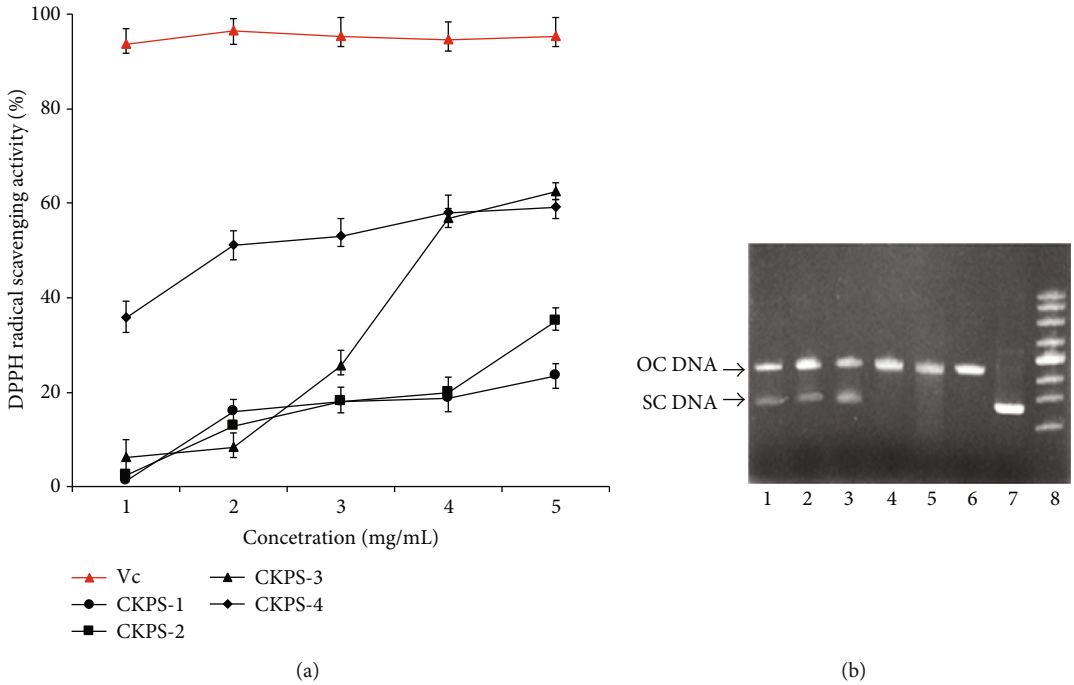

Figure 7: Evaluation of antioxidant activity of polysaccharides extracted from *Cordyceps kyushuensis*. (a) Scavenging activity on DPPH radical of polysaccharide fractions from *Cordyceps kyushuensis*. Values are means ± SD ($n = 3$). (b) Effects of polysaccharide fractions on the protection of supercoiled DNA (plasmid pUC19). Lanes 1-5, H_2O_2+UV treated with rutin, CKPS-1-4, respectively; lane 6, H_2O_2+UV treated without polysaccharide; lane 7, untreated DNA (control); lane 8, supercoiled DNA marker.

biological functions. In the last decades, fungal bioactive polysaccharides have been the core of intense research for the understanding and the utilization of their special properties in naturally produced pharmaceuticals. Many biological activities of fungal polysaccharides, such as immunomodulatory, anticancer, antimicrobial, hypocholesterolemic, hypoglycemic, and health-promoting properties, have been reported. In fact, fungal bioactive polysaccharides produced by edible mushrooms make them also very good candidates for the formulation of novel functional foods and nutraceuticals without any serious safety concerns.

It has been reported that the immune response to fungal polysaccharide mixture may differ from that of purified ones [48], and as we know, the presence of other compounds, such as proteins, polyphenols, and lipids, can affect the biological activity of the fungal components. Purification and structural characterization of fungal polysaccharides are thus very important for their further application as selective and effective immune modulators [49]. The present study was undertaken to elucidate the antioxidant and immune stimulatory activities of the polysaccharides from the stoma of *C. kyushuensis*. Four water-soluble homogeneous polysaccharide fractions were isolated at the final ethanol concentration of 50% and purified by column chromatography. Preliminary structural characterizations were conducted, and DPPH scavenging activity and protection to DNA damage in vitro were carried out to evaluate the antioxidant potential of these fractions. The four polysaccharides could significantly enhance the splenocyte proliferation with or without mitogen (ConA or LPS) *in vivo* and *in vitro*. The effects on the production of cytokines IL-2 and TNF-α were investigated. The results showed that the levels of serum IL-2 and TNF-α were increased significantly by the fraction administration compared with those of the control group, suggesting that the physiological effect of the polysaccharides was implemented by increasing the immune response. Moreover, the tests of macrophage phagocytosis offered demonstrative evidence that these polysaccharides could effectively activate macrophage response. The results indicated that the polysaccharides of *C. kyushuensis* could be applied to the potential health and functional food source. This may provide new strategies for the discovery of effective and safe approaches for cancer treatment from natural resources. Of course, with the deepening of follow-up research, if several prerequisites such as economically feasible production of fungal polysaccharides with stable and standardized quality, composition, purity, and homogeneity, understanding the structure-activity relationship of bioactive polysaccharides, the molecular interaction between polysaccharides and other food ingredients, and the influence of food processing on their functions can be solved smoothly, there will be a bright future waiting for the industrial production of health products and functional food from Cordyceps polysaccharides.

Data Availability

All data was provided in the article, and there are no more data to be uploaded.

Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

Acknowledgments

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Research Article

Isolation and Characterization of Probiotic LAB from Kimchi and Spontaneously Fermented Teff (*Eragrostis tef* (Zucc.) Trotter) Batter: Their Effects on Phenolic Content of Teff during Fermentation

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Microbial fermentation is proven to induce molecular transformations and produce bioactive compounds thereby enhancing sensory and nutritional quality of flour-based fermented foods. In this study, lactic acid bacteria (LAB) were isolated from Korean kimchi and Ethiopian fermented teff (*Eragrostis tef* (Zucc.) Trotter) flour batter. Isolates were identified using 16S rRNA gene sequencing and characterized for various probiotic properties. Few trains were selected for further teff flour batter fermentation and evaluating their effects on phenolic contents and compositions. Out of 200 bacterial isolates, 44 of them showed considerable acid and bile tolerance and 22 were tested positive for protease activity. A large number of the isolates showed antimicrobial activities against *Salmonella gallinarium* indicator strains. Majority of these probiotic strains belonged to *Lactobacillus plantarum* and *Lactobacillus brevis* species. All the strains used for fermentation of teff were able to significantly increase total phenolic contents (TPC). An increase in TPC of up to 7-fold was observed in some strains.

1. Introduction

Lactic acid bacteria (LAB) are industrially useful, Gram-positive bacteria widely used in food fermentation and probiotic formulations. Probiotics are microorganisms with potential beneficial effects on the health of host organisms [1]. The definition also includes the use of such organisms in the preparations of foods. Interestingly, LABs are the most common types of probiotic microorganisms with a variety of beneficial effects for humans and animals [2]. The most common species are *Lactobacillus acidophilus*, *L. fermentum*, *L. casei*, *L. brevis*, and *L. plantarum*. The potential health benefits are improving immune function, protecting against hostile microorganisms, aiding food digestion and absorption, and producing bioactive compounds that have health benefits. Among the variety of mechanisms by which LAB can confer health promoting effects is the production of bioactive peptides through proteolytic activities in popular probiotic delivery systems such as fermented milk. Bioactive peptides are peptide fragments from food proteins that remain inactive until they are released through hydrolysis to promote a particular physiological effect. For instance, α-casein and β-casein, the principal milk proteins, can liberate more than 20,000 peptides each on hydrolysis [3]. Similarly, a variety of other microbial enzymes can cleave bonds that hold phenolic compounds and release them to induce their functional properties in food.

LAB fermentation is known to promote hydrolysis of macromolecules and produce bioactive compounds thereby enhancing nutritional quality of flour-based foods such as bread [4]. The flour type can also affect the technological and nutritional qualities of food products and the effectiveness of microbial fermentation [5]. Therefore, it requires

selecting suitable starter culture strains for optimizing applications in food preparations. In several previous studies, LAB were mostly isolated from spontaneously fermented common dairy foods [6]. However, it might be more advantageous to isolate LAB strains from a wide range of fermented foods that are claimed to have potential nutritional and functional properties such as Korean kimchi and Ethiopian *injera*.

Teff (*Eragrostis tef* (Zucc.) Trotter) is a small seed (about 0.7% of mass of wheat grain) from Ethiopia mainly used in making pancake-like fermented bread called *injera*. *Injera* is a staple food in Ethiopia and Eritrea and is mainly fermented by LAB [7]. It is also used to prepare several other foods such as cookies and soup. There was no interest in teff in the rest of the world until its nutritional and health benefits were elucidated recently leading to its branding as 'the new super food'. The increased global demand of teff is the result of its high levels of essential amino acids, gluten-free property, and high mineral contents [8]. The national research council of the USA suggested that teff seeds contain higher amounts of essential amino acids than other cereals and the balance of amino acids is comparable to that of eggs. It is also proven to have a relatively higher phenolic content compared to other common cereals such as wheat. On the other hand, kimchi is a side dish of salted and fermented cabbage with seasoning such as chili powder, spring onion, garlic, and ginger. Several health benefits have been reported for kimchi including improved heart health and a healthy digestive system. The wealth of antioxidants in it exercise healing effects in medical conditions like cancer, diabetes, obesity, atopic dermatitis, and gastric ulcers. This flavonoid and probiotic-rich food delays aging regulates cholesterol levels and boosts the immune system.

Phenolic compounds are present in soluble and bound forms in cereals like wheat and teff. Therefore, the bioaccessibility of significant biochemical transformations occurs to teff bioactive compounds during fermentation resulting in changes of their compositions that, in turn, affect the product property and bioactivity. Changes in teff phenolic profiles during fermentation are important, not only from sensory point of view but also predict potential effects on chronic disease prevention. The aim of this research was to isolate LAB strains with probiotic properties from Korean kimchi and Ethiopian fermented teff batter, identify them to species level using 16s rRNA gene sequencing, screen them for proteolytic activities, and use the selected strains for teff fermentation and evaluate their effects on phenolic contents and compositions.

2. Materials and Methods

2.1. Chemicals and Reagents. Skim milk, gallic acid, quercetin, and folin-ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Lactobacilli* MRS broth was purchased from Difco Co. (Franklin Lakes, NJ, USA). HPLC grade methanol, acetonitrile, and deionized water were purchased from J.T. Baker Co. (Phillipsburg, NJ, USA). All the other reagents and kits were purchased from different commercial suppliers and were of analytical grade.

2.2. Sample Preparation. Five kimchi (cabbage kimchi) samples and 5 fermented teff batter samples were collected from different households in Korea and Ethiopia, respectively. Two teff grain samples (one white and one brown) were collected from local markets in Ethiopia and brought to South Korea with proper packaging. Whole grains were finely ground using a roll mill (single type stainless roller, Shinpoong Eng. Ltd., Gwangju, Gyeonggi, Korea) with 0 mm gap between the rollers and four smashes. Then, the flour was sieved using a 1 mm mesh sieve and stored in the refrigerator at 4°C until use.

2.3. Bacterial Isolation, Enumeration and Culture Conditions. Ten (10) grams of kimchi and fermented teff batter samples were prepared separately by mixing with 40 and 60 mL PBS, respectively, and homogenized for 2 min in plastic bags with filter using an automatic bag mixer (BagMixer, InterScience Laboratories, Inc., St. Norn, France). 100 μL from the filtrate compartment of each sample was mixed with 900 μL PBS. Subsequently, appropriate dilutions in PBS were plated on MRS agar. After 18 hrs of incubation at 37 C, microbes were enumerated and predominant LAB colonies were picked. Selection of colonies was based on diversification of colony morphology so as to include all different colony types as much as possible. Then, each strain was purified through successive streaking on fresh MRS agar before culturing in MRS broth for further experiments. Finally, isolated strains were stored at –80 C with 30% glycerol at a concentration of above 10^6 cfu/mL.

2.4. Identification of Strains through 16S rRNA Gene Sequencing. Bacterial genome was extracted from 1 mL 18 hrs old culture using GeneAll Exgene Cell SV kit (GeneAll Biotechnology Co. Ltd., Seoul, Korea), and the 16S rRNA gene was amplified by PCR. The universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used for the PCR amplification of the 16S rRNA gene. Then, PCR products were purified using HiYield Gel/PCR DNA Mini kit (Real Biotech Corporation, Minsheng Rd, Banqiao City, Taiwan) after separating them electrophoreticaly at 50 V on 0.8% (w/v) agarose gel. Molecular sizes of the PCR products were estimated by comparison with 1 kb DNA marker (Bioneer, Munpyeongseo Rd., Daejeon, Korea). Nucleotide sequences were determined at Macrogene Korea (Beotkkot Rd, Seoul, Korea) through cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing results of each primer were imported to the BioEdit software program for assembly and alignment to generate reliable contigs after editing and removing ambiguous sequences. Finally, sequence similarities of each contig were examined by searching their homologies in the GeneBank database using BLAST. The hits after analyses were used to identify each isolate at a species level.

2.5. Determination of Proteolysis Activity of Isolates. Candidate strains were screened for proteolytic activity using casein as a substrate. Overnight cultures (18 hrs old) of strains were centrifuged at 13,000 rcf for 10 min at 4°C. Then,

the supernatant was used for the enzymatic assay described as follows. A 20 μL of culture supernatant was dotted on a 6 mm nitrocellulose disc previously placed on skimmed milk agar (final composition of 1% skim milk, 1.5% agar) and air dried for 30 min. Finally, plates were inverted and incubated at 37°C overnight (18 hrs). The diameters of clear zones around the discs were measured to estimate proteolytic activity.

2.6. Screening for Survival under Gastrointestinal Tract (GIT) Conditions. Each isolated strain was tested for acid and bile salt tolerance as follows. For acid tolerance assay, purified bacterial colonies were inoculated in MRS broth (pH 6.5) and incubated at 37°C for 18 hrs. Then, a fresh MRS broth was inoculated with 1% of the overnight culture and reincubated overnight (18 hrs) to balance growth phase (late exponential). To measure their survival in acidity, 1 mL of overnight culture was transferred to 9 mL PBS (pH adjusted to 2.5 with 2 N HCl) and incubated at 37°C shaking incubator. The number of viable bacteria was counted at 0 and 3 hrs of incubation by plating on MRS agar. Experiments were done in triplicate and the mean survival rates were calculated.

For the bile salt tolerance assay, purified colonies were inoculated into MRS broth and incubated at 37°C for 18 hrs. Then, a fresh MRS broth was inoculated with 1% overnight culture and reincubated overnight (18 hrs) to balance growth phase (late exponential). To measure their survival in bile salts, 1 mL of overnight culture was transferred to 9 mL MRS broth containing 0.3% (*w*/*v*) bile salts (cholic acid sodium salt 50%, deoxycholic acid sodium salt 50%), Sigma) and incubated at 37°C shaking incubator. Finally, viable bacteria were enumerated at 0 and 24 hrs of incubation time by plating on MRS agar. Experiments were done in triplicate and the mean survival rates were calculated.

2.7. Antimicrobial Activity Assay. Antimicrobial activity was examined by well diffusion assay (WDA) against eight food born pathogenic indicator strains—four *Salmonella enteritidis* (HJL344, HJL349, HJL377, and HJL385) and four *Salmonella gallinarium* (HJL467, HJL482, HJL510, HJL517—obtained from the Department of Veterinary Science, Chonbuk National University, Iksan campus. Determination of antimicrobial activity was conducted as follows. MRS broth was inoculated with LAB strains and incubated for 24 hrs at 37°C. Extracellular cell free supernatants (ECFS) from these cultures were collected by centrifugation (8,500 rcf for 10 min). The pH of the ECFS were measured and divided in to two equal parts. The first part was left acidic and the second was neutralized with 6 N NaOH to pH 6.0. All the supernatants were filter sterilized. A 100 μL overnight culture of indicator strains was spread on TSB agar and an 8 mm by 5 mm well was made on the agar using cork borer. Then, 10–20 μL supernatants were added to the wells and incubated for 10 hrs. Finally, the diameters of inhibition zones around the wells were measured.

2.8. Fermentation of Teff Batter. Teff fermentation was conducted as described by Fischer et al. [7] with some modifications. For the lab-scale fermentation of teff flour, each LAB strain was grown overnight in MRS broth. Each cell culture was plated to measure and calculate cfu/mL based on a previous standard curve prepared for each isolate. Teff flour was autoclaved by spreading it on aluminum foil. A 9.6 g sterile tap water and 0.4 mL peptone solution containing the respective starter cultures at 10^7–10^8 cfu/g and 6 g flour were mixed in 100 mL bottles. Then, samples were incubated at 37°C. As control, teff flour suspension was held under the same conditions without starter cultures. Three types of samples (0, 24, and 48 hrs of fermentations) were used for analysis. At the end of the fermentation, batter samples were freeze dried and stored at 4°C until use.

2.9. Extraction of Soluble Phenolic Compounds. Extraction of soluble phenolic compounds was performed as per the method described by Shumoy and Raes [9] with some modifications. One (1) g teff flour was extracted three times with 80% aqueous methanol at 1 : 5 flour/solvent ratio by shaking at 200 rpm for 1 hr followed by 30 min of sonication (Ultrasonicator, Hwa Shin Instrument Co., Seoul, Korea) at room temperature (RT). Then, the extraction mixture was centrifuged at 4,500 cfr (VS-550, Vision Scientific, Daejon, South Korea) for 15 min and all supernatants from the three extractions were combined and transferred to new tubes. Finally, the extracts were filtered through 0.2 μm membrane filters (Roshi Kaisha, Tokyo, Japan) and stored in refrigerator at –20°C until use.

2.10. Determination of Total Phenol. Total phenolic contents (TPC) of samples were determined according to the method described by Chandra et al. [10] with some modifications. A 200 μL methanol extract was mixed with 1 mL of 1 N sodium carbonate, vortexed briefly, and incubated for 2 min at RT. Then, 800 μL of folin–ciocalteu phenol reagent was added, and the mixture was vortexed for 10 sec. After incubation for 30 min at RT in dark condition, absorbance was read at 720 nm using UV-visible spectrophotometer (Biochrom–Libra S22, Cambridge, UK) against a blank (80% methanol). The TPC was calculated based on a calibration curve of gallic acid. Results were expressed as mg of gallic acid equivalent per 100 g of grain flour (mg GAE/100 g dw).

2.11. Determination of Total Flavonoid. Total flavonoid contents (TFC) were determined as per the method described by Zhishen et al. [11] with slight modifications. A 75 μL of 5% sodium nitrite solution was added into 250 μL MeOH extract of each sample and vortexed briefly. After 5 min of incubation at RT, 10% aluminum chloride was added and the mixture was vortexed. Then, the reaction mixture was incubated for 6 min at RT and 500 μL of 1 N NaOH was added. Finally, the total volume was adjusted to 1 mL with distilled water and absorbance was measured at 510 nm against a blank (80% methanol). The TFC was calculated using a calibration curve of quercetin and results were expressed as mg quercetin equivalent (QE) per 100 g of flour sample (mg QE/100 g dw).

2.12. Statistical Analyses. All experiments were performed in triplicate and results were expressed as mean with standard deviation (mean ± SD). Statistical analyses were conducted

Table 1: Acid and bile salt tolerance of LAB isolated from kimchi and fermented teff batter.

Isolates	Mean (SD) survival rates (%)	
	pH 2.5; 3 hrs	0.3% bile salts; 24 hrs
K11	12.92 ± 1.21	74.10 ± 6.93
K12	17.75 ± 4.66	72.90 ± 6.82
K13	19.01 ± 3.78	69.76 ± 6.52
K14	66.73 ± 6.24	39.09 ± 3.66
K15	57.19 ± 5.35	73.96 ± 6.92
K16	16.01 ± 1.50	86.00 ± 8.04
K17	53.48 ± 5.00	81.37 ± 7.61
K18	19.20 ± 1.80	27.59 ± 2.58
K21	53.59 ± 5.01	70.99 ± 6.64
K22	37.41 ± 3.50	66.48 ± 6.22
K25	32.67 ± 3.06	69.49 ± 6.50
K31	78.21 ± 7.31	73.44 ± 6.87
K33	77.85 ± 7.28	47.12 ± 4.41
K34	65.31 ± 6.11	70.60 ± 6.60
K35	47.04 ± 4.40	77.58 ± 7.26
K36	65.82 ± 6.16	70.64 ± 6.61
K37	56.19 ± 5.25	73.38 ± 7.86
K42	34.00 ± 3.18	71.86 ± 6.72
K43	58.13 ± 5.44	54.32 ± 5.08
K44	68.03 ± 6.36	72.49 ± 6.78
K45	43.76 ± 4.09	71.51 ± 6.69
K47	43.33 ± 4.05	59.89 ± 5.60
K48	47.39 ± 9.43	56.71 ± 5.30
K49	49.25 ± 4.61	71.94 ± 6.73
S16	68.02 ± 6.36	72.65 ± 12.79
B11	76.59 ± 7.16	90.98 ± 14.75
B12	80.67 ± 11.54	92.30 ± 6.42
B13	75.22 ± 7.03	88.65 ± 5.77
B14	79.58 ± 8.44	90.17 ± 11.79
B15	81.58 ± 7.63	92.77 ± 5.97
B16	69.62 ± 6.51	92.10 ± 9.14
B17	67.83 ± 6.34	90.42 ± 8.74
B18	76.34 ± 7.14	89.51 ± 10.06
B19	72.95 ± 6.82	93.22 ± 6.45
B110	75.14 ± 7.03	89.94 ± 6.35
B21	82.52 ± 9.72	87.32 ± 8.77
B22	76.41 ± 10.15	87.72 ± 6.56
B23	83.16 ± 7.78	93.15 ± 6.00
B24	74.26 ± 6.94	89.76 ± 11.46
B26	73.98 ± 6.92	94.03 ± 8.71
B27	83.78 ± 7.84	88.16 ± 11.03
B28	75.38 ± 7.05	89.21 ± 8.40
B29	81.42 ± 7.61	87.22 ± 9.77
B210	75.99 ± 8.65	88.95 ± 4.17

Isolate labels starting with 'K' belongs to those isolates obtained from kimchi. Isolate label starting with 'S' belongs to those isolates obtained from fermented food factory wastewater. Isolate labels staring with 'B' belongs to those isolates obtained from fermented teff batter.

with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) for Windows and a one-way analysis of variance (ANOVA). Duncan's multiple range tests were carried out to test any significant difference in the phenolic contents before and after fermentations. Values with $p \leq 0.05$, fixed *a priori*, were considered significantly different.

3. Results and Discussions

About 200 LAB were isolated from five kimchi and four fermented teff batter samples to use them for fermentation of teff flour, thereby exploring the phenolic changes they bring about in fermented teff batter. Then, 167 isolates that showed good growth on MRS agar were screened for primary probiotic properties, mainly acid and bile tolerance. Of these, 44 isolates were able to show considerable acid and bile tolerance. The tolerant isolates were used to investigate their proteolytic and antimicrobial activities. Twenty-two (22) isolates were tested positive for protease activity out of which seven were selected for fermentation of teff and evaluations of phenolics contents after fermentation. Results of the studies are presented below.

3.1. Bacterial Growth and Enumeration. The microbial count after plating on MRS agar (pH 6.5) for each isolate from the kimchi and teff batter samples ranged from 10^5 to 10^9 cfu/g with an average of about 10^6 cfu/g. A total of 200 strains were isolated from 11 samples (from six kimchi, one fermented food factory wastewater, and four fermented teff batter samples). All the kimchi and wastewater isolates showed good growth in MRS broth under aerobic incubation whereas all samples isolated from the teff flour batter could only grow well under microaerobic condition of tightly capped falcon tubes [12].

3.2. Survival of Isolates under GIT Conditions. Of the 200 LAB isolates, the 44 isolates that showed considerable acid and bile salt tolerance were selected for further tests. The survival rates during acid and bile tolerance screening were calculated as follows (where stress represents MRS media with bile salts or pH 2.5):

$$\text{Survival Rate}\,(\%) = \log \text{CFU}_{\text{With Stress}} \times 100/\log \text{CFU}_{\text{Without Stress}}. \quad (1)$$

It can be seen from the results that most of the selected isolates showed significant acid and bile tolerance (Table 1). About 0.3% bile salts concentration has been considered to

Table 2: Identities of LAB isolates as determined by 16s rRNA gene sequencing.

Isolates	Sources	Identification (species)
K11	Kimchi	*Lactobacillus plantarum*
K14	Kimchi	*Lactobacillus plantarum*
K12	Kimchi	*Lactobacillus brevis*
K13	Kimchi	*Lactobacillus brevis*
K15	Kimchi	*Lactobacillus brevis*
K17	Kimchi	*Lactobacillus plantarum*
K18	Kimchi	*Lactobacillus plantarum*
K21	Kimchi	*Lactobacillus brevis*
K22	Kimchi	*Lactobacillus brevis*
K25	Kimchi	*Lactobacillus plantarum*
K31	Kimchi	*Lactobacillus plantarum*
K33	Kimchi	*Lactobacillus plantarum*
K34	Kimchi	*Lactobacillus brevis*
K35	Kimchi	*Lactobacillus brevis*
K36	Kimchi	*Lactobacillus brevis*
K37	Kimchi	*Lactobacillus plantarum*
K42	Kimchi	*Lactobacillus brevis*
K43	Kimchi	*Lactobacillus brevis*
K45	Kimchi	*Lactobacillus plantarum*
B16	Teff batter	*Bacillus velezensis*
B31	Teff batter	*Bacillus amyloliquefaciens*
B19	Teff batter	*Bacillus amyloliquefaciens*

Isolate labels starting with 'K' belongs to those isolates obtained from Kimchi. Isolate labels staring with 'B' belongs to those isolates obtained from fermented teff batter.

Table 3: Proteolytic activity of LAB isolates.

Isolates	Diameter of halo (mm)
K11	16.33 ± 1.53
K14	18.13 ± 1.73
K12	17.67 ± 1.15
K13	13.67 ± 1.06
K15	14.00 ± 2.06
K17	14.84 ± 2.13
K18	15.36 ± 2.08
K21	16.73 ± 1.53
K22	15.41 ± 2.65
K25	17.00 ± 2.07
K31	16.00 ± 0.58
K33	16.50 ± 2.31
K34	14.33 ± 0.58
K35	14.67 ± 1.00
K36	14.00 ± 2.08
K37	16.67 ± 2.65
K42	16.00 ± 1.00
K43	17.30 ± 2.08
K45	16.33 ± 0.67
B16	15.02 ± 1.43
B31	18.50 ± 1.53
B19	13.42 ± 1.15

Values are mean ± SD; n =3. Isolate labels starting with 'K' belongs to those isolates obtained from Kimchi. Isolate labels staring with 'B' belongs to those isolates obtained from fermented teff batter.

be the critical concentration used in the selection of resistant strains for probiotic preparations [13]. It is also well established that the pH of the gastric environment is known to be 2.5 [14]. Previous studies reported similar levels of acid and bile salt tolerance of LAB isolates such as *Lactobacillus plantarum* strains [15].

3.3. Identification of Selected Strains through 16S rRNA Gene Sequencing. All the isolates that showed either acid or bile tolerance were screened for proteolytic activity. Twenty-two (22) of them were positive for protease activity. Then, the 22 isolates were identified at species level using 16S rRNA gene sequencing. Ten (10) isolates were identified as *Lactobacillus brevis*, nine (9) isolates were identified as *Lactobacillus plantarum*, and two (2) were identified as *Bacillus amyloliquefaciens* strains (Table 2). Additionally, one (1) isolate was identified as *Bacillus velezensis* strain.

3.4. Proteolytic Activity of Isolates. The proteolytic activities of the identified strains were determined by skim milk agar assay and were expressed as diameter of halos (clear zones) formed after the degradation of casein by extracellular proteases secreted from or by the isolates. Among the 22 isolated strains tested for proteolytic activity, K14 (Lactobacillus plantarum) and B31 (Bacillus amyloliquefaciens) produced clear zones with bigger diameters (Table 3). Extracellular proteases secreted from LAB hydrolyze the peptide bonds in the casein protein in skim milk and therefore break it down either into smaller peptides or amino acid constituents [16]. Microbial proteases play vital roles in several industrial applications such as leather processing, meat processing and cheese making. In this study, the isolates that showed relatively higher proteolytic activities were used for the fermentation of teff batter in an attempt to enhance its functional properties, particularly phenolic content.

3.5. Antimicrobial Activity of Isolates. The most important probiotic property of LAB is their ability to inhibit the growth of pathogenic bacteria. The pathogenic bacteria used as indicator strains in this study were four *Salmonella enteritidis* strains (HJL344, HJL349, HJL377, and HJL385) and four *Salmonella gallinarium* strains (HJL467, HJL482, HJL510, and HJL517). The results showed that most of the isolates have antimicrobial activities against the *S. gallinarium* indicator strains. The *S. enteritidis* seemed to show some tolerance (Table 4). The inhibitory properties of LAB isolates could be due to the H_2O_2, organic acids, and specific bacteriocins they produce [17, 18].

Table 4: Antimicrobial activity of CFS of LAB isolates against Salmonella sp. indicator strains.

Isolates	Diameter of inhibition Ione (mm) of Indicator strains							
	HJL344	HJL349	HJL377	HJL385	HJL467	HJL482	HJL510	HJL517
K11	+	++	—	—	++	—	++	—
K14	++	+++	—	—	+	—	—	—
K12	—	+++	—	—	—	—	—	—
K13	+	+++	—	—	—	—	—	—
K15	++	+	—	—	—	—	—	—
K17	++	+++	—	—	—	—	—	—
K18	++	+++	—	—	+	—	—	—
K21	++	+++	—	—	—	—	—	—
K22	+++	+++	—	—	—	—	—	—
K25	+++	+++	—	—	—	—	—	—
K31	+++	+++	—	—	—	—	—	—
K33	+	+++	+++	+++	+++	—	+++	+
K34	+	+++	+++	+++	+++	—	+++	+
K35	++	+++	+++	+++	+++	—	+++	+
K36	++	+++	++	+++	+++	—	+++	+
K37	++	+++	+++	+++	+++	—	+++	+
K42	—	—	+++	+++	++	—	—	—
K43	—	—	+++	+++	—	—	+	—
K45	—	+++	+++	+++	—	—	+	—
B16	—	+++	+++	+++	—	—	—	—
B31	—	—	—	—	—	—	—	—
B19	—	—	—	—	—	—	—	—

Symbols for diameter of zone inhibition. +++: >20 mm. ++: 19 to 15 mm. +: 14 to 10 mm.

3.6. Changes in Phenolic Contents of Teff during Fermentation with LAB. Out of the 22 isolates that tested positive for protease activity, seven (7) of them were selected to be used as starter cultures for white and brown teff fermentations. The effect of fermentation on phenolic content of teff batter was monitored at 24 and 48 hrs fermentation times. The results of changes in TPC and TFC after fermentation with each strain are presented in Figure 1 (refer also to Table 5).

All isolates were able to increase TPC in both grain types (Figures 1(a) and 1(b)). However, the effects of the fermentation on phenolic contents showed noticeable differences between the white and brown teff samples as well as due to fermentation time. Generally, increasing the fermentation time from 24 to 48 hrs significantly increased the phenolic content. Exceptions were isolates of K43 and B31 that gave higher TPC in the brown teff samples in 24 hrs fermentation. These result could have been caused by the growth patterns of the isolates or the stability of their proteases after secretion into the media. This can be confirmed by testing at different time intervals but we cannot conduct that experiment at this time. On the other hand, K21 (in white teff samples) and K12 isolates (in brown teff samples) did not show significant difference due to fermentation time. The highest (7-fold) increase in TPC of white teff was observed in K43 isolate in 48 hrs fermentation ($1{,}331.40 \pm 56.32$ mg GAE/100 g) followed by B31 (6.5-fold increase), K14 (6-fold increase), K33 (5-fold increase), and K25 (about 4-fold increase) compared with the nonfermented sample. In the case of brown teff, 48 hrs fermentation with isolate K14 gave the highest increase (4.5-fold) in TPC followed by isolate K21 and isolates K25 and K33 that yielded 3.6-fold and 2.5-fold increase in TPC, respectively.

Increments in TPC contents were reported for some cereals such as rye, barley, and oat after fermentation with LAB [19, 20]. However, there is no previous empirical data on the effects of LAB fermentation on teff phenolics. LAB contain various sets of enzymes that can transform phenolic compounds. Extracellular enzymes of LAB are known to hydrolyze complex polyphenols and their glycosylated compounds into free forms of phenolic acids during sourdough fermentations [21, 22]. More than 20-fold increase in phenolic contents were recorded in fermentation of barely and oat with probiotic LAB such *L. casei*, *L. brevis*, and *L. plantarum* strains. In a study that examined the effects of fermentation on red sorghum dough reported an increase in phenolic contents through release of bound phenolic compounds, partial hydrolysis of glycerol esters of phenolic compounds, and partial conversion of flavonoid hexosides into the corresponding flavonoids [23]. The difference in the patterns of phenolic changes between white and brown teff may be accounted to the genetic variations between the two grain types. There are different sets of indigenous enzymes that may contribute to hydrolysis of polyphenols in the two grain types. Moreover, molecules that may induce or suppress LAB enzymes

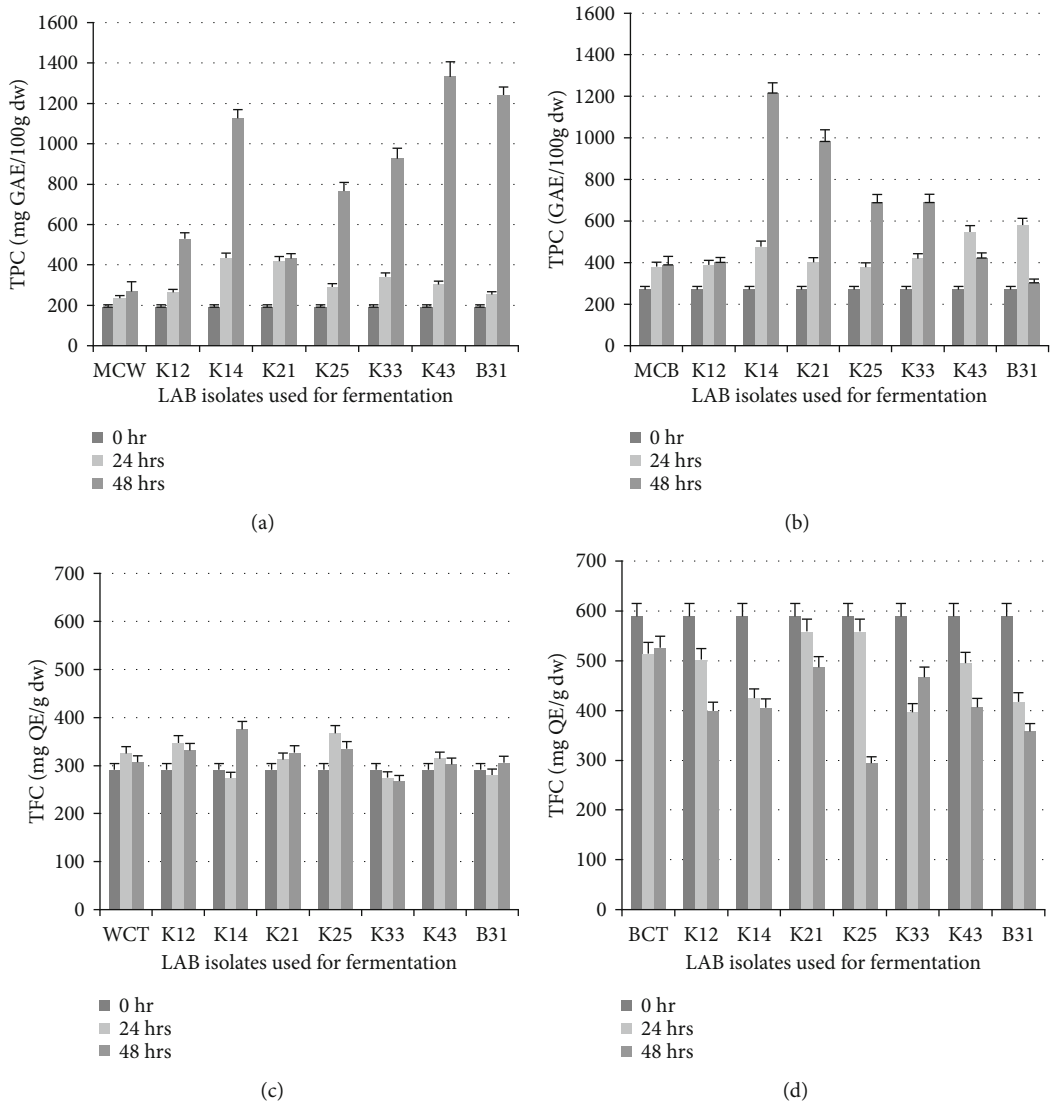

Figure 1: Changes in phenolic and flavonoid contents of teff during fermentation with lactic acid bacteria. (a) TPC in white teff; (b) TPC in brown teff; (c) TFC in white teff; (d) TFC in brown teff. MCW: matched control white teff; MCB: matched contol brown teff; WCT: white teff contol; BCT: brown teff control.

may also contributed to the different patterns in phenolic changes.

Changes in TFC also showed marked differences between grain types. Only few isolates showed slight increases of TFC in the white teff while all the brown samples showed sever reductions after fermentation with every isolate (Figures 1(c) and 1(d)). The most significant reduction in TFC was observed in isolate K25 where it decreased the TFC by almost half after 48 hrs of fermentation compared with the control (Figure 1(d)). This indicates that as the fermentation progressed, hydrolysis of the flavonoid compounds by enzymes released by the starter culture increased. Flavonoid compounds were reported to be converted to smaller molecules such as phenolic acids and other metabolites by bacterial enzymes [24]. The difference between the white and brown teff samples is obvious considering their genetic variations and the higher initial flavonoid content in the brown one. Flavonoids are major group of dietary phenolic compounds [25]. Some studies consider hydrolysis of flavonoids by probiotic bacteria as a good outcome. Prior metabolism of flavonoids is very useful as they are poorly absorbed in the gut. Gut microbiota are known to metabolize flavonoids so that their metabolites reach circulation system and exert their anti-inflammatory effects [26].

4. Concluding Remarks

This study isolated LAB from Korean kimchi and spontaneously fermented Ethiopian teff flour batter and screened them for probiotic characteristics. A major fraction of the isolates showed considerable acid and bile tolerance. Many isolates from the acid and bile tolerant groups also showed significant antimicrobial activities, especially against the *S. enteritidis* indicator strains. Isolates with good protease

Table 5: ANOVA results of data on changes in TPC and TFC of teff during fermentation with LAB.

(a) Total phenolic contents

Teff variety	LAB isolate	Mean (SD) TPC (mg GAE/100 g dw) of fermented teff batter		
		0 hr	24 hrs	48 hrs
White	MCW	191.94 (10.75)^{a}	235.20 (13.17)^{a}	268.14 (48.62)^{a}
	K12	191.94 (10.75)^{a}	264.18 (14.79)^{ab}	529.62 (29.66)^{c}
	K14	191.94 (10.75)^{a}	434.28 (24.32)^{e}	1,126.02 (43.06)^{f}
	K21	191.94 (10.75)^{a}	418.32 (23.43)^{e}	431.76 (24.18)^{b}
	K25	191.94 (10.75)^{a}	291.06 (16.30)^{bc}	765.66 (42.88)^{d}
	K33	191.94 (10.75)^{a}	341.46 (19.12)^{d}	926.10 (51.86)^{e}
	K43	191.94 (10.75)^{a}	302.82 (16.96)^{c}	1,331.40 (74.56)^{h}
	B31	191.94 (10.75)^{a}	253.26 (14.18)^{a}	1,241.52 (39.53)^{g}
Brown	MCB	270.06 (15.12)^{a}	380.52 (21.31)^{a}	389.98 (39.84)^{b}
	K12	270.06 (15.12)^{a}	388.92 (21.78)^{a}	401.94 (22.51)^{b}
	K14	270.06 (15.12)^{a}	476.70 (26.7)^{b}	1,217.16 (48.16)^{e}
	K21	270.06 (15.12)^{a}	401.10 (22.47)^{a}	984.06 (55.11)^{d}
	K25	270.06 (15.12)^{a}	377.58 (21.14)^{a}	689.22 (38.60)^{c}
	K33	270.06 (15.12)^{a}	419.16 (23.47)^{a}	690.06 (38.64)^{c}
	K43	270.06 (15.12)^{a}	547.26 (30.65)^{c}	422.94 (23.68)^{b}
	B31	270.06 (15.12)^{a}	580.44 (32.50)^{c}	303.66 (17.00)^{a}

Means in the same column with different letters are statistically significantly different at $p \leq 0.05$.

(b) Total flavonoid contents

Teff variety	LAB isolate	Mean (SD) TFC (mg GAE/100 g dw) of fermented teff batter		
		0 hr	24 hrs	48 hrs
White	MCW	291.26 (12.82)^{a}	325.04 (14.30)^{bc}	306.78 (13.50)^{bc}
	K12	291.26 (12.82)^{a}	346.96 (15.27)^{cd}	331.43 (14.58)^{cd}
	K14	291.26 (12.82)^{a}	273.91 (12.05)^{a}	375.26 (16.51)^{e}
	K21	291.26 (12.82)^{a}	312.26 (13.74)^{b}	326.87 (14.38)^{bcd}
	K25	291.26 (12.82)^{a}	367.04 (16.15)^{d}	335.09 (14.74)^{d}
	K33	291.26 (12.82)^{a}	274.83 (12.09)^{a}	267.52 (11.77)^{a}
	K43	291.26 (12.82)^{a}	314.09 (13.82)^{b}	302.22 (13.30)^{b}
	B31	291.26 (12.82)^{a}	280.30 (12.33)^{a}	305.87 (13.46)^{bc}
Brown	MCB	588.91 (25.91)^{a}	514.04 (22.62)^{b}	525.91 (23.14)^{e}
	K12	588.91 (25.91)^{a}	502.17 (22.10)^{b}	399.00 (17.56)^{c}
	K14	588.91 (25.91)^{a}	424.57 (18.68)^{a}	405.39 (17.84)^{c}
	K21	588.91 (25.91)^{a}	558.78 (24.59)^{c}	486.65 (21.41)^{d}
	K25	588.91 (25.91)^{a}	558.78 (24.59)^{c}	294.00 (12.94)^{a}
	K33	588.91 (25.91)^{a}	396.26 (17.44)^{a}	466.57 (20.53)^{d}
	K43	588.91 (25.91)^{a}	494.87 (21.77)^{b}	406.30 (17.88)^{c}
	B31	588.91 (25.91)^{a}	417.26 (18.36)^{a}	357.91 (15.75)^{b}

Means in the same column with different letters are statistically significantly different at $p \leq 0.05$.

activities were selected for teff fermentation to examine how fermentation affects phenolic contents. Fermentation using the isolates caused significant increase in TPC in teff. But the reverse was true for TFC where fermentation with majority of the isolates lowered the TFC content. In conclusion, fermentation of teff flour batter with a suitable starter LAB can enhance its functional property by increasing the contents of phenolic acids and enhancing the bioavailability of flavonoids.

Data Availability

The data used to support this study can be obtained from the first author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Screening of Antioxidant and Antimicrobial Activity of *Micromeria fruticosa serpyllifolia* Volatile Oils: A Comparative Study of Plants Collected from Different Regions of West Bank, Palestine

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Background. The investigation of volatile oils used in traditional medicine is vital to enhance the quality of healthcare. This study is aimed at screening the antioxidant and antimicrobial properties of *Micromeria fruticosa serpyllifolia* volatile oils from three different regions in Palestine (north, middle, and south). *Methods*. Volatile oils of three samples of *M. fruticosa serpyllifolia* were extracted using the microwave-ultrasonic apparatus. The antioxidant activity of the volatile oils was assessed by inhibition of DPPH free radical. The antimicrobial activity was examined using the broth microdilution method. Assessment of antifungal activity was achieved using the agar dilution method. *Results*. Screening the biological activity of plant extracts revealed that the sample from Ramallah (middle region) possessed the most potent antioxidant activity with an IC_{50} value of 0.45 μg/mL. The three samples exhibited broad antimicrobial activity and showed potential antifungal activity. The sample from the southern region showed the highest potency against *Shigella sonnei* with the lowest reported MIC; the sample from the northern region demonstrated the least potency against clinical isolate of *Staphylococcus aureus* and "methicillin"-resistant *Staphylococcus aureus*. *Conclusions*. The study showed that *Micromeria fruticosa serpyllifolia* volatile oil samples from different regions in Palestine possess different potential antioxidant and antimicrobial activities that were in line with traditional uses of the plant extracts.

1. Background

Volatile oils (VOs) have antioxidant, anti-inflammatory, anticancer, anthelmintic, antimalarial, antiviral, antibacterial, cholesterol inhibition, and insecticide activities [1, 2]. The chemical structures of VOs determine their therapeutic activities [3]. Recently, there is great attention towards natural antioxidants from plants. Antioxidants can act as a radical scavenger, promote health, and produce anticancer activity [4].

There is a growing concern about antimicrobial resistance issue [5]. The complications of multidrug resistance enforced scientists to search for new antimicrobial agents from various sources such as medicinal plants [6]. In recent time, there has been considerable interest in VOs and extracts of medicinal plants for the development of alternatives to prevent or to delay the growth of pathogens [7]. Many scientific investigations reported that the chemical composition, total yield, and the aroma of VOs may be different due to growing conditions (climate, type of soil and

composition, and altitude), plant age, geoclimatic location, and environmental conditions of collection time and site [8].

Micromeria fruticosa subspecies *serpyllifolia* (Lamiaceae), also known as White *Micromeria*, is an aromatic herb [9], dominant in the eastern Mediterranean regions including Palestine, which has a pleasant minty fragrance that in hot summer provides a sensation of coolness [10, 11]. In Palestinian society known as Duqat ʻAdas, ʻIshbit esh-shai, Qurnya and as Thyme-leave savory in English, the aerial parts of the plant (flower, leaves, and stalk) are used in folk medicine [12]. It has been widely used in traditional medicine for the treatment of hypertension, heart disorders, diarrhea, abdominal pains, colds, headache, wounds, and infections such as skin and eye infections and as an anti-inflammatory agent [9, 11, 13–17]. *M. fruticosa serpyllifolia* VOs also exhibit antibacterial, antifungal, antioxidant, insecticide, analgesic, anticonvulsant, and CNS depressant activity [11, 15].

The authors of the current study conducted several tests on wild-growing *M. fruticosa serpyllifolia* in three regions in the West Bank area in Palestine. Part of these tests has been published previously [18]. Specifically, structural elucidation and identification of the chemical composition using the GC-MS analysis section represent shared results with this study, therefore elaborating and discussing some aspects linked to the findings of the published manuscript. As a continued step, the aims of this study were to screen antioxidant and antimicrobial activities of *M. fruticosa serpyllifolia* collected from three different regions of West Bank, Palestine.

2. Methods

2.1. Chemicals and Reagents. Calcium chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Trolox were purchased from Sigma-Aldrich, Germany. Dimethyl sulfoxide (DMSO) was purchased from CARLO ERBA, France; Nutrient Agar, Mannitol, MacConKey Agar, and Mueller Hinton Broth were purchased from HiMedia Laboratories, Mumbai, India; Sabouraud Dextrose Agar was purchased from Oxoid, UK; 3-[N-morpholino] propanesulfonic acid (MOPS) buffer and RPMI-1640 medium (with L-glutamine, without sodium bicarbonate) (developed at Roswell Park Memorial Institute) were purchased from Sigma-Aldrich, UK. Tween 80 (0.05%) was purchased from ACROS Organics, Belgium. Sodium hydroxide and ethyl alcohol 99.9% were purchased from Sun Pharm. Drug Stars, Nablus, Palestine; cefuroxime 250 mg (as axetil) tablet and doxycycline 100 mg (hyclate) tablet (Jerusalem Pharmaceutical Company, Albiereh, Ramallah, Palestine), levofloxacin 500 mg tablet (Birzeit Pharmaceutical Company, Birzeit, Ramallah, Palestine), azithromycin 250 mg capsules (Pharmacare Company, Birzeit, Ramallah, Palestine), tinidazole 500 mg tablet (Jerusalem Pharmaceutical Company, Albiereh, Ramallah, Palestine), and terbinafine hydrochloride 250 mg tablet (Birzeit Pharmaceutical Company, Birzeit, Ramallah, Palestine), all these pharmaceuticals were donations from Military Medical Services Ramallah Palestine.

2.2. Instrumentation. Grinder (Moulinex model, Uno, China) was used to fracture the dried herbs. A balance (Radway ag, AS 220/c/2, Poland) was used to weigh the plant material; ultrasonic-microwave cooperative extractor/reactor (CW-2000, China) was employed for the extraction of volatile oil. A balance (Boeco, 4500 g, Germany), a UV-visible spectrophotometer (Jenway 7315, UK) for the assessment of the antioxidant activity of VOs, a water bath (Memmert, Germany), micropipettes (Finnpipette, Finland), a heater (Lab-Tech, Korea), and a balance (Sartorius AY 303, Canada) were used.

2.3. Plant Material Collection and Extraction Procedures. The aerial parts of *M. fruticosa serpyllifolia* were collected in April of 2017 from three cities in the West Bank (WB) in Palestine: Nablus, Ramallah, and Hebron representing north, middle, and south of the WB in Palestine, respectively. The samples were botanically identified and coded by Dr. Nidal Jaradat, the pharmacognosist at the Department of Pharmacy in Pharmacognosy and Herbal Products Laboratory, An-Najah National University, and the voucher specimen code was Pharm-PCT-1575. The extraction of VOs was performed following the procedure in accordance to [19, 20].

2.4. Antioxidant Activity. The scavenging activity of *M. fruticosa serpyllifolia* VOs of the three samples from three regions in WB in Palestine was assessed using the methods described in [20, 21]. Stock solutions at a concentration of 1 mg/mL in methanol and Trolox were prepared from *M. fruticosa serpyllifolia* VOs that were collected from three Palestinian regions. Each one of these stock solutions was diluted in methanol to prepare 12 of the working solutions with the following concentrations: 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, and 100 μg/mL. A freshly prepared DPPH solution (0.002% w/v) was mixed with both methanol and with each of the abovementioned working solutions at 1 : 1 : 1 ratio. In addition, a negative control solution was prepared by mixing the mentioned DPPH solution with methanol in a 1 : 1 ratio. All of these solutions were incubated at room temperature in a dark cabinet for 30 min. By the end of the incubation period, the optical density of these solutions was determined using spectrophotometric absorbances at a wavelength of 517 nm using methanol as the blank solution.

Antioxidant activity was monitored by measuring the absorbance at 517 nm wavelength. The antioxidant activities of *M. fruticosa serpyllifolia* VOs and Trolox were assessed by their ability to donate a hydrogen atom or electron and were identified from converting the deep violet color of a methanol solution of DPPH to colorless or pale yellow; for that, the inhibition percentage of DPPH activity was used to determine the antioxidant activity of *M. fruticosa serpyllifolia* VOs and Trolox using the following equation (Inhibition% of antioxidant activity [6]):

$$\text{In}\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100, \quad (1)$$

where A_{blank} represented the absorption of the control reaction (all reagent without the sample) and A_{sample} represented the absorbance of the sample.

2.5. Antimicrobial Screening. The antibacterial activities of *M. fruticosa serpyllifolia* VOs were investigated against the growth of nine reference bacterial strains obtained from the American Type Culture Collection (ATCC): *Escherichia coli* (ATCC 25922), *Enterococcus faecium* (ATCC 700221, USA), *Klebsiella pneumoniae* (ATCC 13883, UK), *Pseudomonas aeruginosa* (ATCC 27853, USA), *Shigella sonnei* (ATCC 25931, USA), and *Staphylococcus aureus* (ATCC 25923, USA). In addition, diagnostically proven clinical isolates *Proteus mirabilis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA) were tested. Four antibiotics were used to test the sensitivity of bacterial strains: azithromycin, levofloxacin, cefuroxime, and doxycycline; the antibiotics were dissolved in a suitable solvent according to solubility test to obtain stock solution [22–24]. The antifungal activity of VOs was examined against the growth of two fungal strains acquired, from the American Type Culture Collection (ATCC), *Candida albicans* (ATCC 90028, USA) and *Epidermophyton floccosum* (ATCC 52066, UK). The antifungal agents (terbinafine and tinidazole) were used for susceptibility tests to *Candida albicans*, used with *M. fruticosa serpyllifolia* VOs [25]. The antibacterial activity of three VO samples was determined using broth microdilution method described in Jaradat et al. [20].

Each one of the isolated *M. fruticosa serpyllifolia* VOs was dissolved in DMSO (100%) at a concentration of 50 mg/mL. The prepared *M. fruticosa serpyllifolia* VO solutions were filter sterilized and then were serially microdiluted (2-fold) eleven times in sterile nutrient broth. In 96-well plates, the dilution processes were carried out under aseptic conditions. In the microwells that were assigned to evaluate the antibacterial activities of the extracted *M. fruticosa serpyllifolia* VOs, the concentration of these oils ranged from 0.024 to 25 mg/mL. On the other hand, the concentrations of these essential oils in the microwells assigned to evaluate their antifungal activities ranged from $8.467 * 10^{-3}$ to 16.666 mg/mL. In these plates, microwell number 11 contained essential oil-free nutrient broth, which was used as a positive control for microbial growth. In addition, the microwell number 12 contained essential oil-free nutrient broth that was left uninoculated with any of the test microbes. This well was used as a negative control for microbial growth. Microwell numbers 1 to 11 were inoculated aseptically with the test microbes. At the time of inoculation, the final concentrations of microbial cells were about 2.5×10^5 and $0.333 - 1.666 \times 10^3$ colony-forming unit (CFU)/mL for the tested bacterial pathogens and *Candida albicans*, respectively. Each of the included microbes in this study was examined in duplicate for being inhibited by the *M. fruticosa serpyllifolia* essential oils. At 35°C, all the inoculated plates were incubated and the incubation period lasted for about 16-20 hours for the plates inoculated with the test bacterial strains and for about 48 hours for the plates inoculated with *Candida albicans*. The lowest concentration of *M. fruticosa serpyllifolia* essential oils, at which there was no visible microbial growth in that microwell, was observed.

2.6. Statistical Analysis. IC_{50} values of antioxidant activity and I% of DPPH free radical were determined in triplicate for *M. fruticosa serpyllifolia* VOs obtained from three different regions in Palestine. The results were expressed as mean ± standard deviation (SD), and the obtained data were compared using one-way ANOVA with post hoc Tukey-Kramer HSD multiple comparison calculation; p values of 0.05 or less were considered statistically significant [26].

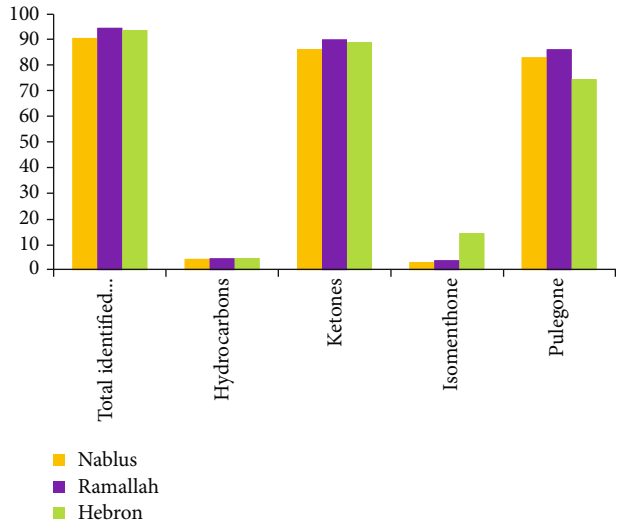

FIGURE 1: Chemical analysis of three samples of *M. fruticosa serpyllifolia* VOs.

3. Results

3.1. Yields and Chemical Composition. Volatile oils of the three samples of *M. fruticosa serpyllifolia* were extracted using microwave-ultrasonic apparatus; still, the results of this section can be obtained from the previously published work by the same research group [18]. However, these results are shown in Figure 1 modified from [18]. Briefly, Figure 1 represents the chemical composition obtained using GC-MS analysis in which the most abundant components in all three samples were pulegone and isomenthone. The total identified components in the three samples were almost consistent in which 90.48, 94.44, and 93.55% of the constituents were identified in Nablus, Ramallah, and Hebron districts, respectively.

3.2. Antioxidant Activity. DPPH assay was used as in vitro approach to determine the free radical-scavenging activity and to screen for the possible antioxidant activity of the *M. fruticosa serpyllifolia* VOs from different regions in Palestine. IC_{50} values were used to assess the ability of the examined samples to inhibit DPPH. The assay revealed that the VO samples exhibited higher antioxidant potency compared to Trolox but they showed lower efficacy (maximum inhibition) (Table 1 and Figure 2). Statistical analysis using one-way ANOVA was performed to compare the antioxidant potency (IC_{50}) and efficacy among samples. There were significant differences in antioxidant potency and efficacy of VOs compared to Trolox ($p < 0.05$ or <0.01). There were significant differences in antioxidant efficacy of VOs compared to each other ($p < 0.05$ or <0.01), but there were no significant

Table 1: IC_{50} of DPPH radical scavenging activity of *M. fruticosa serpyllifolia* VOs from different regions of Palestine and Trolox.

	Trolox	Nablus	Ramallah	Hebron
IC_{50} (μg/mL)	0.64 ± 0.12	0.47 ± 0.02[a]	0.45 ± 0.01[a]	0.47 ± 0.01[d]
Max. I% DPPH radical scavenging activity	96.80 ± 2.83	49.25 ± 0.33[d]	50.19 ± 0.65[bd]	45.01 ± 0.86[cde]

[a]$p < 0.05$ compared to Trolox, [b]$p < 0.05$ compared to Nablus, [c]$p < 0.01$ compared to Ramallah, [d]$p < 0.01$ compared to Trolox, and [e]$p < 0.01$ compared to Nablus; *mean ± SD, $n = 3$.

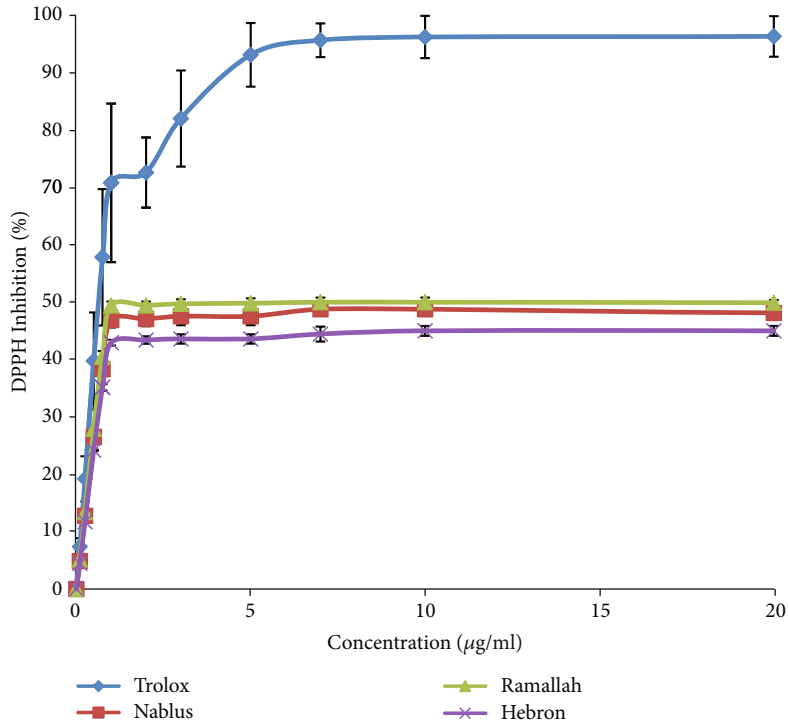

Figure 2: DPPH radical scavenging activity of the three samples of *M. fruticosa serpyllifolia* VOs and Trolox.

differences in antioxidant potency of VOs compared to each other ($p > 0.05$).

3.3. Antimicrobial Activity. The minimum inhibitory concentrations (MICs) of *M. fruticosa serpyllifolia* VOs from different regions of Palestine were reported in Table 2. The majority of Gram (+) and Gram (-) bacterial strains were sensitive to *M. fruticosa serpyllifolia* VOs at MIC of 3.13 mg/mL. There were no statistically significant differences in activity against nine bacterial strains between *M. fruticosa serpyllifolia* VOs from the three regions in Palestine. There were significant differences of Hebron VO sample compared to Nablus and Ramallah VO samples against the American Type Culture Collection *Shigella sonnei* (ATCC 25931), $p < 0.01$, and the VO sample from Hebron had the highest potency at a MIC value of 1.56 mg/mL. There were significant differences in Hebron and Ramallah VO samples compared to Nablus VO samples against two clinical isolate of Gram (+) bacterial strains, *Staphylococcus aureus* and MRSA, $p < 0.01$, the VO sample from Nablus provided the lowest potency at a MIC value of 6.250 mg/mL. To evaluate the sensitivity of bacterial strains, four antibacterial drugs were used: azithromycin 250 mg, levofloxacin 500 mg, doxycycline 100 mg, and cefuroxime 250 mg. The MIC values of the drugs were in the range $1.28 * 10^{-6}$ mg/mL – $22.5 * 10^{-3}$ mg/mL; Table 3 lists the MICs for drugs. In addition, the antifungal activity against the fungal strains was tested for sensitivity to *M. fruticosa serpyllifolia* VOs; the yeast was the most sensitive followed by the fungus; the American Type Culture Collection *C. albicans* (ATCC 90028) yeast was found to be the most sensitive to *M. fruticosa serpyllifolia* VO samples at a MIC value of 0.206 mg/mL followed by the fungus *Epidermophyton floccosum* (ATCC 52066) at a VO MIC value of 0.78 mg/mL (Table 2). To evaluate the sensitivity of fungal strains, two antifungal dugs were used: terbinafine 250 mg and tinidazole 500 mg, and the MIC value of antifungal drugs was 18.52 μg/mL.

4. Discussion

Natural antioxidants have been widely investigated to find protective compounds against damages and diseases developed from free radicals and oxidative stress. *Micromeria* species were identified as a rich source of antioxidant agents [27]. Different results were reported by Güllüce and coauthors in Turkey in which the antioxidant activity of the

Table 2: Antimicrobial activity (MIC in mg/mL) of *M. fruticosa serpyllifolia* VOs from different regions of Palestine based on the broth microdilution method and agar dilution method.

	MIC Nablus	MIC Ramallah	MIC Hebron	DMSO 100%
Yeast				
C. albicans (ATCC 90028)	0.206	0.206	0.206	3.70%
Fungus				
Epidermophyton floccosum (ATCC 52066)	0.781	0.781	0.781	6.25%
Bacterial strains				
Staphylococcus aureus (ATCC 25923)	3.125	3.125	3.125	12.50%
Staphylococcus aureus (CI)	6.250	3.125[a]	3.125[a]	12.50%
MRSA (CI)	6.250	3.125[a]	3.125[a]	12.50%
Enterococcus faecium (ATCC 700221)	3.125	3.125	3.125	6.25%
Escherichia coli (ATCC 25922)	3.125	3.125	3.125	12.50%
Pseudomonas aeruginosa (ATCC 27853)	3.125	3.125	3.125	12.50%
Shigella sonnei (ATCC 25931)	3.125	3.125	1.5625[ab]	12.50%
Proteus mirabilis (CI)	3.125	3.125	3.125	12.50%
Klebsiella pneumoniae (ATCC 13883)	3.125	3.125	3.125	12.50%

[a]$p < 0.01$ compared to Nablus, [b]$p < 0.01$ compared to Ramallah.

Table 3: Minimum inhibitory concentration (μg/mL) of some antimicrobial drugs.

	Azithromycin	Levofloxacin	Doxycycline	Cefuroxime
Bacterial strains				
Staphylococcus aureus (ATCC 25923)	0.352	$5.125 * 10^{-3}$	0.012	2.356
Staphylococcus aureus (CI)	0.352	$6.4 * 10^{-3}$	0.097	4.713
MRSA (CI)	0.176	$6.4 * 10^{-3}$	0.097	4.713
Proteus mirabilis (CI)	5.625	$1.28 * 10^{-3}$	0.387	4.713
Pseudomonas aeruginosa (ATCC 27853)	0.703	$1.28 * 10^{-3}$	0.387	2.356
Escherichia coli (ATCC 25922)	0.703	$1.28 * 10^{-3}$	0.012	2.356
Klebsiella pneumoniae (ATCC 13883)	1.406	0.012	0.387	4.713
Shigella sonnei (ATCC 25931)	0.703	—	0.387	2.356
Enterococcus faecium (ATCC 700221)	22.5	1.64	0.0.097	4.713
	Terbinafine	Tinidazole		
Candida albicans (ATCC 90028)	18.5185	—		

VOs of *M. fruticosa serpyllifolia* was observed with an IC_{50} value of 98.2 μg/mL [28]. The study showed that VOs abundant with oxygenated monoterpene such as pulegone have antioxidant activity [29, 30]. However, this result may support the antioxidant potency of Ramallah sample VOs, which contained the highest amount of total oxygenated compounds and pulegone (89.88 and 86.04%, respectively) among the three samples of VOs.

Interestingly, multidrug resistance of bacterial species causes health difficulties. Extracts of volatile oils have been investigated as new potential antimicrobial agents, biopreservative products, and promising antiseptic enhancers for topical uses [31]. *Micromeria* species VOs were considered to have strong broad-spectrum antimicrobial activity [32]. The results listed in Table 2 showed that the VOs of the three samples exhibited considerable antifungal potency but little antibacterial potency. The results of the antimicrobial activity of the VOs of three samples revealed that this activity was specific against *Shigella sonnei*, *Staphylococcus aureus* (CI), and MRSA and nonspecific against the rest of the microbial organisms. Screening the potential antimicrobial activity of *M. fruticosa serpyllifolia* VOs and methanolic extract, growing in Turkey conducted by Güllüce et al. [28], concluded that the VO provided stronger antimicrobial properties than methanolic extract (methanolic extract did not show any antimicrobial activity); the MIC values of 0.5 mg/mL volatile oil stock solution for bacterial species which were susceptible to the oil ranged from 31.25 to 125 μg/mL and for fungi which were susceptible to VO ranged from 31.25 to 62.50 μg/mL. A study conducted by Omari et al. [33] evaluating the antifungal activity of *M. barbata* growing in Lebanon, using different fungal strains and yeasts, including *Epidermophyton floccosum* and *Candida albicans*, concluded that

the *M. barbata* VOs showed a high fungistatic activity. Investigating the antimicrobial activity of *Micromeria cilicica* VOs growing in Turkey resulted in the finding that the *Micromeria cilicica* VOs and pulegone crude compound (the main component) showed a significant antifungal and antibacterial activity; the activities increased relying on the amount of pulegone and VOs and *Candida albicans* was the most sensitive to pulegone [34]. *Micromeria congesta* VOs were considered as a significant antibacterial due to abundant components such as pulegone and isomenthone [35]. Studying the chemical ingredients and antibacterial and antifungal activity of the volatile oils of four plants including *Mentha spicata* growing in Iran by Kazemi et al. [36], and crude menthone (the dominant component) for antimicrobial activity, reported that VOs showed very strong antimicrobial properties against *Staphylococcus aureus*, all of *Shigella species*, *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas aeruginosa*, *Proteus sp.*, *Candida albicans*, and other strains and concluded that menthone (isomenthone) exhibited strong antibacterial properties with MIC 1.5-3.5 μg/mL. These findings could be linked with our results obtained from Hebron in which the VOs of *M. fruticosa serpyllifolia* owned the highest quantity of isomenthone (14.41%) which is thought to be effective against *Shigella sonnei*. On the contrary, isomenthone represented the lowest amount of constituents (3.16%) in Nablus and therefore exhibited the lowest potency against *Staphylococcus aureus* (CI) and MRSA.

5. Conclusions

M. fruticosa serpyllifolia VOs from different regions in Palestine represented by three cities showed variable antioxidant and antimicrobial activities depending on the phytochemical constituents of the volatile oils. The sample from middle Palestine (Ramallah) showed the most potent antioxidant properties. The plant extract exhibited strong antifungal activities and minimal antibacterial activities. The sample of the south region showed higher potency against *Shigella sonnei* while the sample of the northern region showed lower potency against *Staphylococcus aureus* (CI) and MRSA. These findings enable *M. fruticosa serpyllifolia* VOs to be good agents in curing or preventing oxidative stress and healing wounds and skin dermatitis and a good food preservative agent.

Further *in vivo* studies are needed to evaluate the potential pharmacological activities, to isolate the basic components responsible for potential pharmacological activities, and to evaluate the safety and toxicity of plant extract.

Abbreviations

CNS: Central nervous system
DMSO: Dimethyl sulfoxide
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
GC-MS: Gas chromatography mass spectrometry
MIC: Minimum inhibitory concentration
MRSA: Methicillin-resistant *Staphylococcus aureus*
VOs: Volatile oils.

Data Availability

All raw data are available upon request from the corresponding author.

Conflicts of Interest

The authors declare that they have no competing interest.

Authors' Contributions

NS performed the experimental part and drafted the manuscript; NYS designed, supervised, coordinated the study, and drafted the manuscript; NJ designed and conceptualized the study; ME and RA conducted the antimicrobial tests; SK, MA, and LA conducted the GC analysis; AR and MA drafted the manuscript. All authors read and approved the final manuscript.

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Supplementary Materials

Supplementary data 1: GC-MS chromatograms of volatile oil samples of *M. fruticosa serpyllifolia* collected from different regions. Supplementary data 2: GC-MS analysis and component identification and quantification of volatile oil samples. Supplementary data 3: detailed procedures for antibacterial agent preparation. Supplementary data 4: detailed protocol for antifungal agent preparation. Supplementary data 5: antimicrobial effect of *M. fruticosa serpyllifolia* volatile oils collected from different regions using the agar dilution method. *(Supplementary Materials)*

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