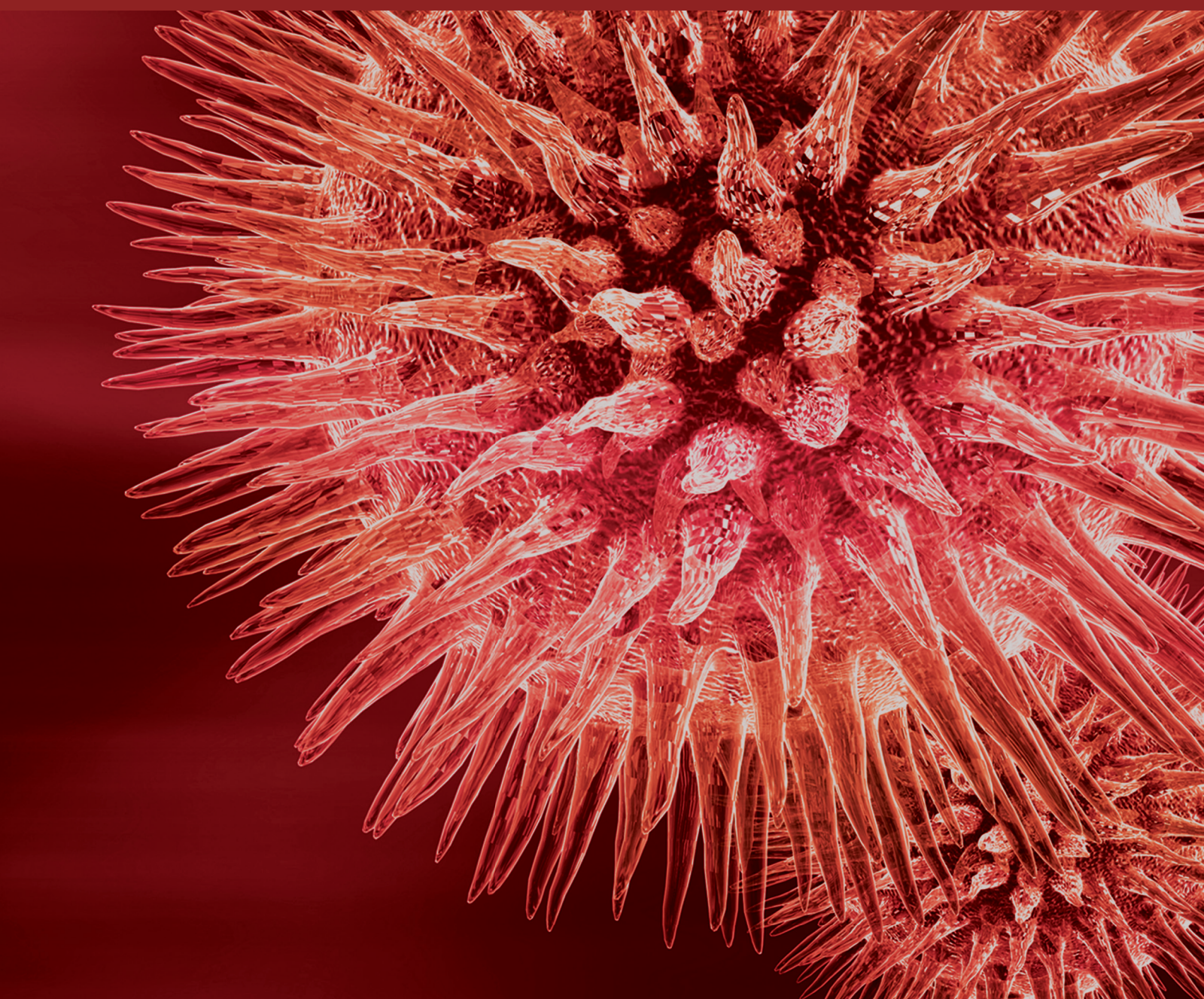
BioMed Research International

Advances in Microbial and Nucleic Acids Biotechnology

Lead Guest Editor: Gamal Enan
Guest Editors: Salah E. Abdel-Ghany, Mohamed E. Osman, and Mahmoud F. Abdel-Haliem



Advances in Microbial and Nucleic Acids Biotechnology

BioMed Research International

Advances in Microbial and Nucleic Acids Biotechnology

Lead Guest Editor: Gamal Enan
Guest Editors: Salah E. Abdel-Ghany, Mohamed E. Osman, and Mahmoud F. Abdel-Haliem

Contents

Hindawi
BioMed Research International
Volume 2018, Article ID 3102374, 2 pages
https://doi.org/10.1155/2018/3102374

Editorial

Advances in Microbial and Nucleic Acids Biotechnology

Gamal Enan,[1] **Mohamed E. Osman**,[2] **Mahmoud E. F. Abdel-Haliem**,[1] **and Salah E. Abdel-Ghany**[1,3]

[1]*Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig 44519, Egypt*
[2]*Department of Botany and Microbiology, Helwan University, Cairo, Egypt*
[3]*Department of Biology, Colorado State University, Fort Collins, CO 80523, USA*

Correspondence should be addressed to Gamal Enan; gamalenan@ymail.com

Received 6 May 2018; Accepted 6 May 2018; Published 24 June 2018

Biotechnology in its broad meaning is the use of organisms or their products for various purposes such as fast diagnosis of infectious diseases, inhibition of the antibiotic resistant bacteria, production of pharmaceuticals, biomaterials, and even energetic materials. Recent challenges in Biotechnology offer state-of-the art studies covering a wide spectrum of biotechnological applications in different organisms for different purposes that serve agriculture, environment, health, and industry. Genomes of both eukaryotes and prokaryotes are recently engineered for advances in food safety and development in all fields.

The call for submission of manuscripts allowed researchers in Biotechnology fields to submit their original and novel findings to this special issue. Nineteen manuscripts were submitted. According to precise peer review processes as well as the policies and standards applied in the journal, only seven of them were accepted and they are in the context of this special issue. The full papers in this issue are categorized in four different subjects based on the scope of the Biotechnology applications.

Three papers in this special issue discussed results in the immunotherapeutic field. The paper titled "Mannose-Binding Lectin: A Potential Therapeutic Candidate against *Candida* Infection" showed the potential therapeutic capability of mannose binding lectin (MBL) against candidiasis as this recombinant MBL induced agglutination of both *Candida albicans* and *C. glabrata*. In their extended studies in the paper titled "Mannose-Binding Lectin Gene Polymorphism and Its Association with Susceptibility to Recurrent Vulvovaginal Candidiasis," the authors studied the MBL gene polymorphism and its association with recurrent vulvovaginal candidiasis (RVVC) and found a close correlation between innate immunity gene mutation and polymorphism in MBL gene and the existence of RVVC. They concluded that MBL genotypic analysis can be used as a surrogate for MBL serum level in order to identify MBL-deficient women for alterative therapeutic options. In the same category, the paper titled "Microcrystalline Cellulose for Delivery of Recombinant Protein-Based Antigen against Erysipelas in Mice" is very interesting as it developed microcrystalline cellulose for delivery of recombinant protein-based antigen against erysipelas in mice. This recombinant surface protein (SPA) from the Gram positive pathogen *Erysipelothrix rhusiopathiae* was fused to cellulose binding domain from *Trichoderma harzianum* (CBD). This CBD-SPA fusion cassette was expressed in *E. coli* successfully.

Molecular cloning and gene expression category include 2 papers. In the paper titled "*Lra*I from *Lactococcus raffinolactis* BGTRK10-1, an Isoschizomer of *Eco*RI, Exhibits Ion Concentration-Dependent Specific Star Activity," the results obtained found that *Lactococcus raffinolactis* BG TRK10-1 produces a novel *Lra*I type II restriction endonuclease which is an isoschizomer of *Eco*R1, and the gene encoding its production was cloned and expressed successfully in the *E. coli* bacterium. In the other paper of this category titled "Cloning and Expression of the Organophosphate Pesticide-Degrading α-β Hydrolase Gene in Plasmid pMK-07 to Confer Cross-Resistance to Antibiotics," the authors were able to clone and express the α-β-hydrolase gene in some strains of bacteria belonging to the genus *Bacillus*; this is a promising result since α-β hydrolase produced by bacteria degrades the organophosphate pesticide pollutants in soil.

Food Biotechnology area of research includes one paper only titled "Genetic Analysis with Random Amplified Polymorphic DNA of the Multiple Enterocin-Producing *Enterococcus lactis* 4CP3 Strain and Its Efficient Role on the Growth of *Listeria monocytogenes* in Raw Beef Meat"; the results obtained showed that the *Enterococcus lactis* 4CP3 strain could be used as meat protective against listerias growth at refrigeration temperatures. The intraspecific genetic analysis of the 4CP3 strain was assessed by random amplified polymorphic DNA polymerase chain reaction analysis.

Tissue culture Biotechnology includes one paper titled "Manipulation of Plant Growth Regulators on Phytochemical Constituents and DNA Protection Potential of the Medicinal Plant *Arnebia benthamii*" which showed a novel protocol for the *in vitro* regeneration of the medicinal plant *Arnebia benthamii* by tissue culture techniques. It was found that the regenerated plants possessed high content of volatile/nonvolatile compounds that showed DNA protection potential against oxidants.

Gamal Enan
Mohamed E. Osman
Mahmoud E. F. Abdel-Haliem
Salah E. Abdel-Ghany

Hindawi
BioMed Research International
Volume 2018, Article ID 7670505, 7 pages
https://doi.org/10.1155/2018/7670505

Research Article

Microcrystalline Cellulose for Delivery of Recombinant Protein-Based Antigen against Erysipelas in Mice

Wooyoung Jeon,[1] Yeu-Chun Kim,[2] Minhee Hong,[1] Sanoj Rejinold,[2] Kyoungmoon Park,[3] Injoong Yoon,[4] Sungsik Yoo,[4] Hongweon Lee,[1,5] and Jungoh Ahn [1,5]

[1]*Biotechnology Process Engineering Center, KRIBB, Cheongju 363-883, Republic of Korea*
[2]*Department of Chemical and Biomolecular Engineering, KAIST, Daejeon 305-701, Republic of Korea*
[3]*Biological and Chemical Engineering, Hongik University, Sejong 339-701, Republic of Korea*
[4]*Choong-Ang Vaccine Laboratory, Daejeon 305-348, Republic of Korea*
[5]*University of Science and Technology (UST), 217 Gajeong-ro, Yuseong-gu, Daejeon 305-350, Republic of Korea*

Correspondence should be addressed to Jungoh Ahn; ahnjo@kribb.re.kr

Received 17 January 2018; Revised 6 April 2018; Accepted 3 May 2018; Published 11 June 2018

Academic Editor: Gamal Enan

The study describes the development of a vaccine using microcrystalline cellulose (Avicel PH-101) as a delivery carrier of recombinant protein-based antigen against erysipelas. Recombinant SpaA, surface protective protein, from a gram-positive pathogen *Erysipelothrix rhusiopathiae* was fused to a cellulose-binding domain (CBD) from *Trichoderma harzianum* endoglucanase II through a S3N10 peptide. The fusion protein (CBD-SpaA) was expressed in *Escherichia coli* and was subsequently bound to Avicel PH-101. The antigenicity of CBD-SpaA bound to the Avicel was evaluated by enzyme-linked immunosorbent (ELISA) and confocal laser scanning microscope (CLSM) assays. For the examination of its immunogenicity, groups of mice were immunized with different constructs (soluble CBD-SpaA, Avicel coated with CBD-SpaA, whole bacterin of *E. rhusiopathiae* (positive control), and PBS (negative control)). In two weeks after immunization, mice were challenged with $1x10^7$ CFU of *E. rhusiopathiae* and Avicel coated with CBD-SpaA induced protective immunity in mice. In conclusion, this study demonstrates the feasibility of microcrystalline cellulose as the delivery system of recombinant protein subunit vaccine against *E. rhusiopathiae* infection in mice.

1. Introduction

Vaccines are the therapeutic formulations given to patients to elicit immune responses entailing antibody production (humoral) or cell-mediated responses that will eventually fight variety of malignancies [1]. There have been many approaches for vaccine delivery via vaccinations, which are considered the most efficient prophylactic method against various infectious diseases. Vaccine delivery systems (i.e., micro- or nanoparticles, liposomes, and virosomes) have been investigated for improving vaccine efficacy [2–5]. In addition, various vaccine types have been developed to overcome the disadvantages of conventional vaccines. Among vaccines, recombinant protein antigens, or their fragments, have been used and considered as novel vaccine candidates. Development of such kinds of vaccines avoids the safety issues with attenuating virus or cell cultures when making conventional viral vaccines [6, 7]. However, some recombinant protein antigens have fatal handicaps (low stability and immunogenicity) caused by lack of key elements that can stimulate immune response [8]. Use of adjuvants, along with recombinant protein vaccines, has been suggested for improved immunogenicity [9]. Different strategies have been extensively explored in order to improve the immunogenicity by protecting antigens through immobilization on inorganic or organic matrices [10].

Cellulose is a primary component of plant cell walls and a linear polymer of glucose residues. It is naturally resistant to biological degradation owing to its insolubility, rigidity, and tendency to pack together to form long crystals. It is chemically inert, pharmaceutically safe, and inexpensive, making it an effective immunosorbent, or carrier material, for protein purification and immobilization [11]. Cellulose-binding domain (CBD) refers to protein modules that bind

firmly to various types of cellulose. The strong attraction between cellulose and CBD has applications in many areas, especially for immunology [12]. In previous studies, cellulose beads (Sigmacell 20® or Orbicell™ cellulose particles) attached to CBD-fused recombinant protein were used for parenteral vaccination for goldfish [13], and the immunogenic properties of CBD in its free form were compared with the CBD-cellulose complex, using cytokine production as an immunological indicator. Small Orbicell beads (1–10 μM) induced antibody levels that were equal to the titers produced by the adjuvanted protein and bacterin formulae compared to the larger Sigmacell particles (10–20 μM). Similarly, Lunin et al. (2009) studied the immobilization of CBD on the cellulose sorbent which enhances the synthesis of specific antibodies and found that cellulose immunosorbent is not immunotolerant and could induce cytokine production involved in the regulation of humoral immune response [8].

Erysipelas is a very serious swine disease causing tremendous economic loss. *E. rhusiopathiae*, causative agent of erysipelas, is a gram-positive, rod shaped, non-spore-forming, non-motile, and bacterial pathogen [14]. Although live-attenuated or recombinant vaccines are currently administered to control this disease, their effectiveness is not consistent. Even the mechanisms of pathogenicity have not yet been elucidated [15–18]. Therefore, it is necessary to have a more advanced and effective system of vaccination against erysipelas.

In this study, we developed Avicel (cellulose microcrystalline) correlated with antigen and investigated its characteristics. In addition, antigen-immobilized Avicel vaccine was subcutaneously injected into mice to analyze the actual immunogenicity of our newly developed antigen-immobilized Avicel recombinant protein vaccine. Overall, the study highlights the development of an efficient recombinant protein vaccine-immunosorbent system to protect swine against *E. rhusiopathiae* infection.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions. The *E. rhusiopathiae* serotype 15 strain was isolated from a diseased piglet in Korea. For it, *E. coli* DH5α [F$^-$, ϕ80d*lac*ZΔM15, *end*A1 *hsd*R17 (rK$^-$, mK$^+$), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96] was used as a host strain for cloning and plasmid maintenance. The *E. coli* BL21 host strain [F$^-$, *hsd*S, *gal*, *omp*T, rB$^-$, mB$^-$] (Novagen, USA), harboring a lambda derivative, *DE3*, was used for gene expression.

2.2. Cloning and Recombinant Plasmid Construction. The gene coding CBD of *Trichoderma harzianum* endoglucanase II, with artificial S_3N_{10} linker, was amplified from pEKPM-EcGAD-Lk-H6 (Ahn, 2004; Hyemin Park, 2012) using specific primers (Forward: 5′-GAAGGAGATATA*CATATG*CAGCAAACTGTTTGGGGG-3′, Reverse: 5′-CTTCTTATC*GGATCC*GTTGTTGTTGTT-GTTGTTGTTGTTGTTGTTGCTGCTGCTAATGCATTG-AGCGTAGTA-3′), in which the underlined characters were introduced for the In-fusion™ Advantage PCR cloning kit (Clontech, USA). The *italic* letters indicate the site of the restriction enzymes. The polymerase chain reaction (PCR) product containing CBD with S_3N_{10} linker was introduced into the pET-28b(+) plasmid (Novagen, USA), previously digested with *Nde*I and *BamH*I. For the isolation of genomic DNA of *E. rhusiopathiae*, a DNeasy Blood and Tissue Kit (Qiagen, Germany) was used according to manufacturer's instructions. The surface protective antigen A (SpaA) gene was amplified from *E. rhusiopathiae* genomic DNA, by PCR using the primers (Forward: 5′-ACTCCGCCAACTAGCTCT*GGATCC*GATTCGACAGAT-ATTTCTG-3′, Reverse: 5′-GTGGTGGTGGTGGTGGTG-*CTCGAG*TTTTAAACTTCCATCGTTC), in which underlined base pairs were introduced for In-fusion ligation. The *italic* letters indicate the sites of *BamH*I and *Xho*I.

2.3. Expression and Purification of CBD-SpaA Protein. The plasmid harboring pKPM-CBD-Lk-SpaA-H6 was transformed into *E. coli* BL21(*DE3*). The transformants were grown in 2YT medium containing 50 μg/ml of kanamycin at 37°C. When an A_{600} of 0.5 was reached, 0.5 mM isopropyl-β-thio-D-galactopyranoside (IPTG) was added and cultures were grown for an additional 3h at 37°C. Cells were harvested by centrifugation and pellets were disrupted by sonication for further analysis. Recombinant fusion protein was purified from the supernatant by Ni-NTA agarose (Qiagen, Germany), using an Econo-Pac Chromatography Column (Bio-Rad, USA) according to the manufacturer instructions. The protein concentration was estimated using the BCA protein assay kit (Pierce, USA).

2.4. Binding of CBD-SpaA onto Avicel. Binding assays were carried out according to methods previously described, with slight modification [19]. Briefly, Avicel (PH-101, Sigma, < 50 μm) (0.1 mg) was mixed with 500 μg of the CBD-SpaA proteins at 4°C for 2 h. The supernatant was collected after centrifugation and used to determine uncombined protein using a BCA protein assay kit. The amount of protein bound to Avicel was determined from the difference between final and starting values in the supernatant. Nonspecific proteins bound to Avicel were removed by washing with 0.5 M NaCl.

2.5. SDS-PAGE and Western Blot Analysis. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed according to the method of Laemmle [20]. For the Western blot analyses, proteins separated by 10% SDS-PAGE gel were transferred to nitrocellulose (NC) membranes. The NC membranes were blocked by phosphate buffered saline (PBS) containing 5% skimmed milk and washed three times with PBST (0.1% Tween 20 in PBS). The membranes were incubated with His-probe monoclonal antibody (Santa Cruz Biotechnology, USA) or mouse-derived antiserum prepared with whole bacteria of *E. rhusiopathiae*, for 1 h at room temperature (RT). The specific antigen reacted with IgG alkaline phosphatase (AP) antibody (Sigma, USA) was visualized using an AP conjugated substrate kit (Bio-Rad, USA).

2.6. Preparation of Polyclonal Mouse Antiserum. To prepare mouse polyclonal antiserum against *E. rhusiopathiae*,

a five-week-old specific-pathogen-free (SPF) mouse was injected subcutaneously with formalinized whole cell vaccine of *E. rhusiopathiae* twice within a 2-week interval. Then, a blood sample was collected two weeks later and sera antibody titer was measured using enzyme-linked immunosorbent assay (ELISA).

2.7. Antigenicity Evaluation. Microtiter assembly strips (Thermo Scientific, Finland) were coated overnight at 4°C with 100 μl per well of immobilized CBD-SpaA on Avicel. Several dilutions (1×10^4, 5×10^4, 1×10^5, and 5×10^5 Avicel particles) of Avicel coated CBD-SpaA were tested in triplet. The plates were washed three times with PBST and blocked with 5% skimmed milk in PBST for 1h at RT. Antiserum derived from mouse against *E. rhusiopathiae* (1:500) was added to the plates and then placed on a rocker platform for 2h at RT. For the detection of immunogenic characteristics, the plates were incubated with a 1:2000 dilution of horseradish peroxidase (HRP) anti-mouse IgG whole antibody (GE Healthcare, UK) for 1 h at RT. Optical density was read at 450 nm using a TECAN Infinity 2000 PRO plate reader (TECAN, Austria).

2.8. Confocal Laser Scanning Microscope (CLSM). Avicel particles (1×10^5) coated with CBD-SpaA were incubated with antiserum against *E. rhusiopathiae* (1:500) for 2h at RT. After washing three times with PBST, the immobilized CBD-SpaA was incubated with Fluorescein- (FITC-) AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Lab, USA) for 30 min at 4°C. The plate was washed again, and Avicel particles coated with CBD-SpaA were fixed with 3.7% paraformaldehyde for 10 min at RT, followed by mounting with VECTASHIELD mount medium (Vector Lab., USA). Immunofluorescence was evaluated using an LSM 510 META Laser Scanning Microscope (Carl Zeiss, Germany).

2.9. Mouse Immunization and Challenge. Forty SPF mice (5 weeks old) were randomly assigned to 4 groups of eight each and injected subcutaneously with 4 μg of soluble CBD-SpaA, Avicel coated with CBD-SpaA, ERT2T-A containing whole bacterin of *E. rhusiopathiae* serovar 15 (positive control), and PBS (negative control) emulsified with an oil-based adjuvant, respectively. Two weeks after injection, all groups were challenged subcutaneously with 1×10^7 CFU of *E. rhusiopathiae* (serovar 15). Mouse mortality was monitored daily for the following ten days.

3. Results

3.1. Expression of the CBD-SpaA Fusion Protein in E. coli. As illustrated in Figure 1, pKPM-CBD-Lk-SpaA-H6 was constructed for the expression of SpaA fused to CBD. In the construct, SpaA was fused to CBD from *T. harzianum* endoglucanase II via an artificial S_3N_{10} peptide known to completely resist *E. coli* endopeptidase at its N-terminus and six histidines at its C-terminus. The fusion protein (CBD-SpaA) was expressed in *E. coli* BL21(*DE3*) harboring pKPM-CBD-Lk-SpaA-H6. The results of the SDS-PAGE and

Figure 1: A schematic representation of the expression vector for CBD-SpaA-H6 fusion protein. SpaA, surface protective antigen A (SpaA) from *E. rhusiopathiae*; CBD, the cellulose-binding domain of *T. harzianum* endoglucanase II; Linker, S_3N_{10} peptide; (His)6, 6x histidine tag sequence; T7lac, T7 promoter sequence; T7t, T7 terminator sequence.

Western blot (Figure 2) indicated that the CBD-SpaA was successfully expressed at the expected molecular weight (77.6 kDa) without being degraded by proteolysis and that the CBD-SpaA was overexpressed at a high level (35%) with respect to the percentage of total cell protein. Moreover, most of the expressed CBD-SpaA were in soluble form, compared to our previous work in which extreme reduction of the solubility of *E. coli*-derived glutamate decarboxylase occurred after fusion with CBD [21].

3.2. Coating of Avicel with CBD-SpaA Protein. The CBD-SpaA proteins from crude cell lysates were purified through a Ni-NTA column with the elution of an imidazole (250 mM). The elution fraction contained CBD-SpaA with a purity of 85.1%. Both the purified CBD-SpaA and crude cell lysates were bound to microcrystalline cellulose Avicel PH-101 and the concentration of CBD-SpaA bound to Avicel was determined by stripping the protein from the beads by boiling. Avicel displayed a binding capacity of 3.11±0.015 $mg_{CBD\text{-}SpaA}/g_{Avicel}$ (purity: 94%) for the purified CBD-SpaA and 1.92±0.001 $mg_{CBD\text{-}SpaA}/g_{Avicel}$ (purity: 81%) for crude cell lysates (Figure 3).

3.3. Antigenicity of CBD-SpaA Bound to Avicel. The antigenicity of CBD-SpaA bound to Avicel was evaluated by indirect ELISA assay. As shown in Figure 4, the increased absorbance values were detected in proportion to the number of Avicel particles coated with CBD-SpaA, whereas the Avicel without CBD-SpaA as a negative control showed a value as low as the PBS. Confocal laser scanning microscope (CLSM) was used for visualization of antigenic properties of the Avicel coated with CBD-SpaA (Figure 5). Purified fusion protein was bound to Avicel and incubated with anti-serum of whole *E. rhusiopathiae*, followed by goat-mouse IgG-FITC. CLSM images demonstrated that green fluorescence dispersed almost equally on the surface of the Avicel coated with CBD-SpaA as shown in Figure 5.

3.4. Protective Immunity in Immunized Mice. To examine whether protection could be induced without side effects by immunization with the Avicel coated with CBD-SpaA, we injected each protein (free CBD-SpaA and Avicel coated with CBD-SpaA, ERT2T-1 containing whole cell bacterin (positive control) from CVAVC in the Republic of Korea, and PBS (negative control)) into mice and challenged the mice with

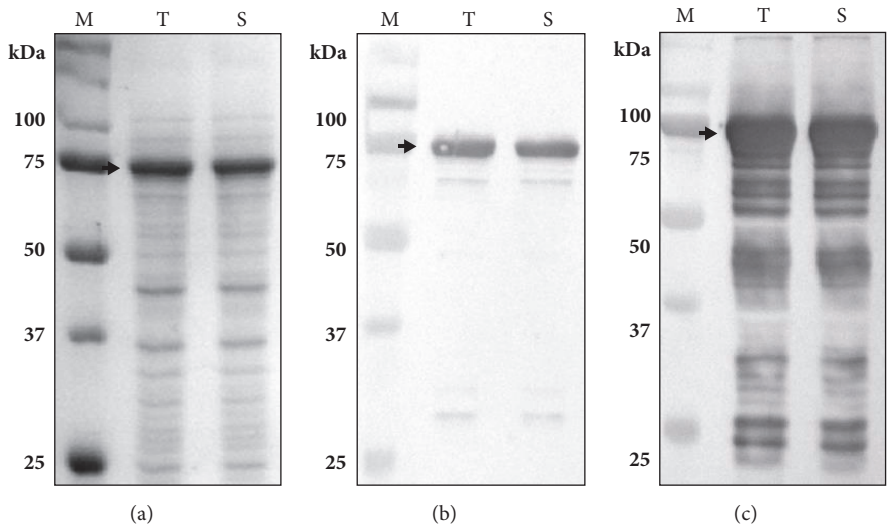


Figure 2: SDS-PAGE and Western blot analyses of the CBD-SpaA-H6 expressed in *E. coli* BL21(DE3)/pKPM-CBD-SpaA-H6: (a) SDS-PAGE; (b) Western blot with anti-histidine antibody; (c) anti-serum derived from mouse inoculated with whole *E. rhusiopathiae*. Lane M: standard molecular weight marker, T: total cell lysates, and S: soluble fraction proteins. The arrows indicate the expressed CBD-SpaA.

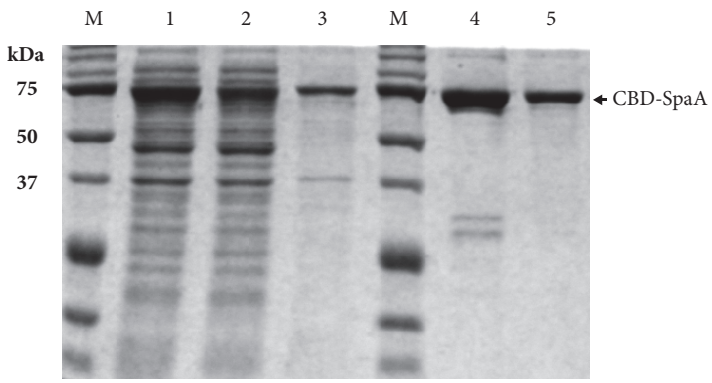


Figure 3: Coating of Avicel with CBD-SpaA protein. Lanes 1-3: crude CBD-SpaA and Lanes 4 and 5: purified CBD-SpaA. Lane M: stand molecular weight marker, Lanes 1 and 4: proteins before binding to Avicel, Lane 2: proteins not bound to Avicel, and Lanes 3 and 5: proteins bound on Avicel.

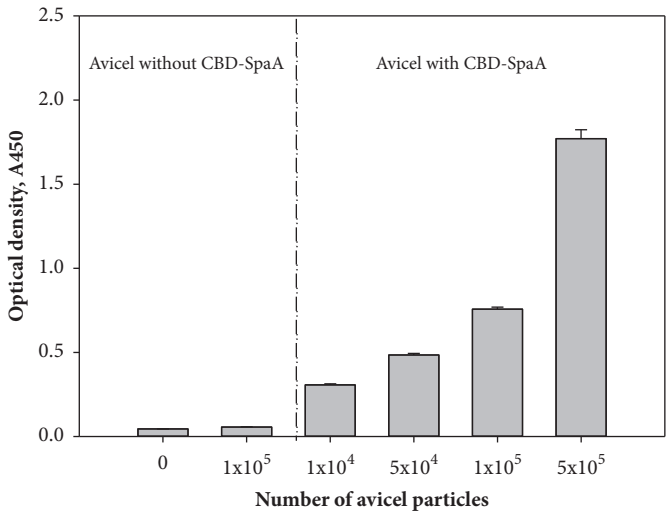
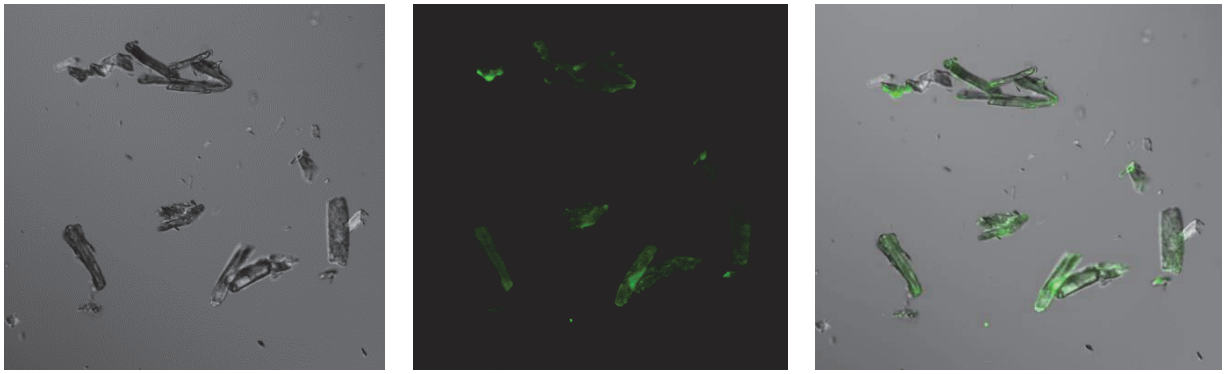


Figure 4: ELISA assay of Avicel coated with CBD-SpaA ($n=3$).



(a) (b) (c)

Figure 5: CLSM images for CBD-SpaA-H6 immobilized on Avicel. Purified CBD-SpaA protein was bound to Avicel and incubated with anti-histidine antibody followed by goat anti-mouse IgG-FITC. The confocal microscope image indicates that CBD-SpaA protein had a high binding affinity to Avicel. (a) Differential interference contrast images; (b) FITC fluorescence; (c) merging of the two split images.

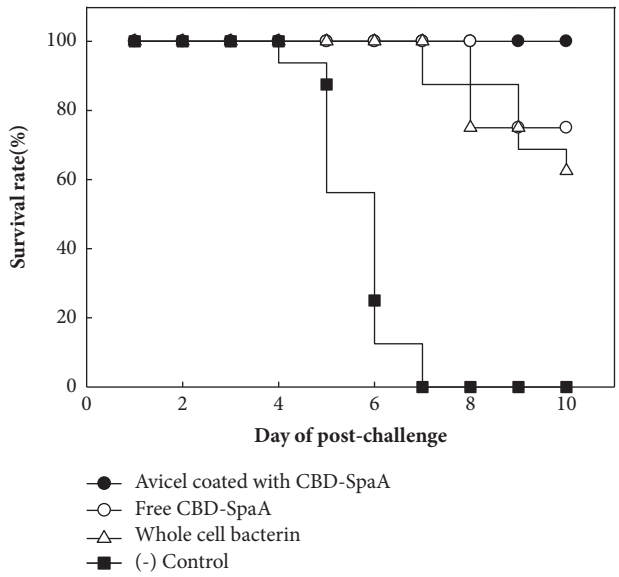


Figure 6: Cumulative mortality of immunized mice after challenge. Mice were vaccinated with free CBD-SpaA, Avicel coated with CBD-SpaA, and ERT2T-A containing whole cell bacterin as a positive control and PBS as a negative control. After 14 days, all the mice were challenged with highly virulent *E. rhusiopathiae* and survival was monitored for ten days.

E. rhusiopathiae. All of the negative control mice died within seven days after challenge. Compared with negative control group, 5 of 8 ($p < 0.0001$, by Fisher's exact test) mice in the ERT2T-1 immunized group, and 6 of 8 ($p < 0.0001$, by Fisher's exact test) mice in the free CBD-SpaA immunized group, survived. In the Avicel coated with CBD-SpaA, the immunized group of mice showed 100% ($p < 0.0001$, by Fisher's exact test) immune-protection against challenge with *E. rhusiopathiae* (Figure 6). However that group did not show significant difference with the free CBD-SpaA immunized group ($p = 0.467$, by Fisher's exact test). These results demonstrated that the microcrystalline cellulose can be used as a delivery carrier of recombinant protein.

4. Discussion

Antigen subunits or synthetic peptides are considered as a promising alternative for viral vaccines. They have been also considered safer vaccine systems than killed/inactivated or live-attenuated whole cells/viruses. Recombinant DNA technology has made the development of subunit vaccines more efficient, because the production and purification procedure can be carefully designed to obtain high yields of a well-defined product [22]. However, some studies have shown that soluble immunogens rarely induce high titers of antibodies without the use of strong adjuvants [23, 24]. To overcome the typical low immunogenicity of protein-based vaccines, and to address the need for effective vaccines and efficient delivery systems, researchers have moved in the direction of molecular biotechnology [25]. Recently, advances in recombinant biotechnology have led to the development of genetically engineered polymers with exact order and accuracy of amino acid residues. Recombinant protein-based polymers such as elastin-like polymers (ELPs), silk-like polymers (SLPs), and silk-elastin-like protein polymers (SELPs) have been reported to bring controlled release, longer circulating therapeutics, and tissue-specific treatment options [26]. Nanoscale structures such as gold nanoparticles and virus-like particles have also recently peaked interests for drug delivery as they offer manifest benefits [27, 28].

In our study, Avicel was selected as immunosorbent for purification and delivery of recombinant protein subunit vaccine, because it is highly inert, inexpensive, and safe biomaterial. This should protect the protein subunit vaccine from degradation in harsh conditions (extreme pH or temperature). As shown in Figure 4, immune response increased with increasing Avicel particle concentration, confirming its suitability as a better immunosorbent for vaccine systems. The higher immunity achieved with increased Avicel concentration could be due to selective coating of SpaA by Avicel.

Our findings clearly demonstrate that Avicel is specific for the CBD-SpaA with good binding capacity for vaccine delivery. The binding capacity was visualized using CLSM imaging as shown in Figure 5. It has been well known that

the Spa proteins are potent protective antigens against *E. rhusiopathiae* infection [29]. Recent reports show that SpaA is the major Spa-type of serotypes 1a, 1b, and 2 [30] which are most commonly implicated in swine erysipelas. The protective domain of SpaA lies between amino acids 29-414 [30]. This particular amino acid sequence itself can induce highly protective antibodies against *E. rhusiopathiae* infection [15]. Being a well delivery agent and immune sorbent, Avicel further enhanced its potency as we found in our experiments.

5. Conclusions

In this study, we developed a vaccine using microcrystalline cellulose, Avicel PH-101 to deliver recombinant protein-based antigen against erysipelas. The recombinant protein, CBD-SpaA, was expressed in *E. coli* and bound to Avicel PH-101. Our data perceptibly showed that a 100% survival rate could be achieved using the Avicel coated with antigen in *E. rhusiopathiae*-challenged mice. Thus, the in vitro immunogenicity test has been validated by the in vivo challenge experiment.

In our newly developed vaccine system, protein purification is unnecessary. This cuts down production costs and enables a cost-effective, recombinant protein vaccine system. Specifically, our delivery system using Avicel coated with CBD-SpaA could provide an appealing vaccination strategy against erysipelas.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Wooyoung Jeon and Yeu-Chun Kim contributed equally to this work.

Acknowledgments

This research was supported by a grant from KRIBB Research Initiative Program. This work was supported by the Advanced Biomass R&D Center (ABC) of the Global Frontier Project funded by the Ministry of Science and ICT (ABC-2015M3A6A2074238).

References

[1] C. Saroja, P. Lakshmi, and S. Bhaskaran, "Recent trends in vaccine delivery systems: A review," *International Journal of Pharmaceutical Investigation*, vol. 1, no. 2, pp. 64–74, 2011.

[2] C.-J. Chiou, L.-P. Tseng, M.-C. Deng et al., "Mucoadhesive liposomes for intranasal immunization with an avian influenza virus vaccine in chickens," *Biomaterials*, vol. 30, no. 29, pp. 5862–5868, 2009.

[3] C. Moser, M. Amacker, A. R. Kammer, S. Rasi, N. Westerfeld, and R. Zurbriggen, "Influenza virosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations," *Expert Review of Vaccines*, vol. 6, no. 5, pp. 711–721, 2007.

[4] S. T. Reddy, A. J. van der Vlies, E. Simeoni et al., "Exploiting lymphatic transport and complement activation in nanoparticle vaccines," *Nature Biotechnology*, vol. 25, no. 10, pp. 1159–1164, 2007.

[5] C. Wang, Q. Ge, D. Ting et al., "Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines," *Nature Materials*, vol. 3, pp. 190–196, 2004.

[6] M. Hansson, P.-A. Nygren, and S. Stahl, "Design and production of recombinant subunit vaccines," *Biotechnology and Applied Biochemistry*, vol. 32, no. 2, pp. 95–107, 2000.

[7] S. Liljeqvist and S. Stahl, "Production of recombinant subunit vaccines: Protein immunogens, live delivery systems and nucleic acid vaccines," *Journal of Biotechnology*, vol. 73, no. 1, pp. 1–33, 1999.

[8] V. G. Lunin, N. E. Sharapova, T. V. Tikhonova et al., "Research into the immunogenic properties of the recombinant cellulose-binding domain of Anaerocellum thermophilum in vivo," *Molekuliarnaia Genetika, Mikrobiologiia I Virusologiia*, pp. 21–27, 2009.

[9] A. S. McKee, M. W. Munks, and P. Marrack, "How do adjuvants work? Important considerations for new generation adjuvants," *Immunity*, vol. 27, no. 5, pp. 687–690, 2007.

[10] G. Georgiou, C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, and R. Curtiss, "Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines," *Nature Biotechnology*, vol. 15, no. 1, pp. 29–34, 1997.

[11] D. Klemm, B. Heublein, H. P. Fink, and A. Bohn, "Cellulose: fascinating biopolymer and sustainable raw material," *Angewandte Chemie*, vol. 44, no. 22, pp. 3358–3393, 2005.

[12] M. Linder and T. T. Teeri, "The roles and function of cellulose-binding domains," *Journal of Biotechnology*, vol. 57, no. 1–3, pp. 15–28, 1997.

[13] S. Maurice, M. Dekel, O. Shoseyov, and A. Gertler, "Cellulose beads bound to cellulose binding domain-fused recombinant proteins; an adjuvant system for parenteral vaccination of fish," *Vaccine*, vol. 21, no. 23, pp. 3200–3207, 2003.

[14] Y. Shimoji, "Pathogenicity of Erysipelothrix rhusiopathiae: Virulence factors and protective immunity," *Microbes and Infection*, vol. 2, no. 8, pp. 965–972, 2000.

[15] Y. Imada, N. Goji, H. Ishikawa, M. Kishima, and T. Sekizaki, "Truncated surface protective antigen (SpaA) of Erysipelothrix rhusiopathiae serotype 1a elicits protection against challenge with serotypes 1a and 2b in pigs," *Infection and Immunity*, vol. 67, no. 9, pp. 4376–4382, 1999.

[16] H. I. Cheun, K. Kawamoto, M. Hiramatsu et al., "Protective immunity of SpaA-antigen producing Lactococcus lactis against Erysipelothrix rhusiopathiae infection," *Journal of Applied Microbiology*, vol. 96, no. 6, pp. 1347–1353, 2004.

[17] Y. Shimoji, Y. Mori, and V. A. Fischetti, "Immunological characterization of a protective antigen of Erysipelothrix rhusiopathiae: Identification of the region responsible for protective immunity," *Infection and Immunity*, vol. 67, no. 4, pp. 1646–1651, 1999.

[18] G. J. Eamens, J. C. Chin, B. Turner, and I. Barchia, "Evaluation of Erysipelothrix rhusiopathiae vaccines in pigs by intradermal challenge and immune responses," *Veterinary Microbiology*, vol. 116, no. 1-3, pp. 138–148, 2006.

[19] J. A. Santiago-Hernández, J. M. Vásquez-Bahena, M. A. Calixto-Romo et al., "Direct immobilization of a recombinant invertase to Avicel by E. coli overexpression of a fusion protein containing the extracellular invertase from Zymomonas mobilis and the carbohydrate-binding domain CBDCex from Cellulomonas fimi," *Enzyme and Microbial Technology*, vol. 40, no. 1, pp. 172–176, 2006.

[20] F. Hong, N. Q. Meinander, and L. J. Jönsson, "Fermentation strategies for improved heterologous expression of laccase in Pichia pastoris," *Biotechnology and Bioengineering*, vol. 79, no. 4, pp. 438–449, 2002.

[21] J. O. Ahn, E. S. Choi, H. W. Lee et al., "Enhanced secretion of *Bacillus stearothermophilus* L1 lipase in *Saccharomyces cerevisiae* by translational fusion to cellulose-binding domain," *Applied Microbiology and Biotechnology*, vol. 64, no. 6, pp. 833–839, 2004.

[22] C. Andersson, *Production and Delivery of Recombinant Subunit Vaccines*, Department of Biotechnology. Royal Institute of Technology (KTH), 2000.

[23] O. Pérez, A. Batista-Duharte, E. González et al., "Human prophylactic vaccine adjuvants and their determinant role in new vaccine formulations," *Brazilian Journal of Medical and Biological Research*, vol. 45, no. 8, pp. 681–692, 2012.

[24] S. Wang, H. Liu, X. Zhang, and F. Qian, "Intranasal and oral vaccination with protein-based antigens: Advantages, challenges and formulation strategies," *Protein & Cell*, vol. 6, no. 7, pp. 480–503, 2015.

[25] I. P. Nascimento and L. C. C. Leite, "Recombinant vaccines and the development of new vaccine strategies," *Brazilian Journal of Medical and Biological Research*, vol. 45, no. 12, pp. 1102–1111, 2012.

[26] J. L. Frandsen and H. Ghandehari, "Recombinant protein-based polymers for advanced drug delivery," *Chemical Society Reviews*, vol. 41, no. 7, pp. 2696–2706, 2012.

[27] F. Y. Kong, J. W. Zhang, R. F. Li, Z. X. Wang, W. J. Wang, and W. Wang, "Unique roles of gold nanoparticles in drug delivery, targeting and imaging applications," *Molecules*, vol. 22, no. 9, p. 1445, 2017.

[28] B. D. Hill, A. Zak, E. Khera, and F. Wen, "Engineering virus-like particles for antigen and drug delivery," *Current Protein & Peptide Science*, vol. 19, no. 1, pp. 112–127, 2018.

[29] J. E. Galan and J. F. Timoney, "Cloning and expression in Escherichia coli of a protective antigen of Erysipelothrix rhusiopathiae," *Infection and Immunity*, vol. 58, no. 9, pp. 3116–3121, 1990.

[30] H. To and S. Nagai, "Genetic and antigenic diversity of the surface protective antigen proteins of Erysipelothrix rhusiopathiae," *Clinical and Vaccine Immunology*, vol. 14, no. 7, pp. 813–820, 2007.

Hindawi
BioMed Research International
Volume 2018, Article ID 5827986, 10 pages
https://doi.org/10.1155/2018/5827986

Research Article

Genetic Analysis with Random Amplified Polymorphic DNA of the Multiple Enterocin-Producing *Enterococcus lactis* 4CP3 Strain and Its Efficient Role in the Growth of *Listeria monocytogenes* in Raw Beef Meat

Olfa Ben Braïek,[1,2] **Slim Smaoui**,[3] **Karim Ennouri**,[3] **Khaled Hani**,[4] **and Taoufik Ghrairi**[1,2]

[1]*Laboratory of Microorganisms and Active Biomolecules (LMBA), Faculty of Sciences of Tunis, University of Tunis El-Manar, Tunisia*
[2]*Research Laboratory of Environmental Science and Technology (RLEST), ISSTE, Technopole de Borj Cedria, Tunisia*
[3]*Laboratory of Microorganisms and Biomolecules of the Centre of Biotechnology of Sfax, Tunisia*
[4]*UR012-ES03, Department of Biochemistry, Faculty of Medicine Ibn El Jazzar of Sousse, Tunisia*

Correspondence should be addressed to Olfa Ben Braïek; olfa_bbraiek@yahoo.fr

Received 2 February 2018; Revised 9 March 2018; Accepted 4 April 2018; Published 10 June 2018

Academic Editor: Gamal Enan

In this manuscript, a multiple enterocin-producing *Enterococcus lactis* strain named 4CP3 was used to control the proliferation of *Listeria monocytogenes* in refrigerated raw beef meat model. Also, the intraspecific genetic differentiation of 4CP3 strain was assessed by Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) analysis. *E. lactis* 4CP3 strain was found to produce the enterocins A, B, and P. It displayed activity against *L. monocytogenes* EGDe 107776 by agar-well diffusion method. The application of *E. lactis* 4CP3 culture at 10^7 CFU/g in raw beef meat was evaluated using both ANOVA and ANCOVA linear models in order to examine its effect on the growth of the pathogen *L. monocytogenes* during refrigerated storage. Hence, a very interesting result in decreasing ($P<0.05$) and suppressing the growth of *L. monocytogenes* in refrigerated raw beef meat was shown during 28 days of storage. In conclusion, *E. lactis* 4CP3 strain might be useful for prevention of the proliferation and survival of *L. monocytogenes* in raw meat during refrigerated storage.

1. Introduction

Contamination and growth of *Listeria monocytogenes* in raw beef meats during refrigerated storage have been intensively reported [1–4]. Effectively, *L. monocytogenes* is known to be a major concern for the meat processing industry causing listeriosis in humans [4, 5]. This fact constitutes a significant public health issue [6]. Indeed, this virulent foodborne pathogen is psychrophile which is able to grow at temperatures as low as 0°C, adapted to several food systems and the contaminated foods do not present unusual odour, texture, or taste which evade control in human foodstuffs and increase its danger in products [7]. In this context, many researches were performed in order to develop natural agents other than antibiotics and chemically synthesised additives to ensure the safety and maintain the security of foods as public health issues [8]. Among these natural agents, lactic acid bacteria (LAB) have received great attention in terms of food safety and are mainly used in foods for different technological effects because of their potent Generally Recognised as Safe (GRAS) status [9]. In fact, LAB are implicated in the biopreservation and prolongation of the shelf-life of diverse food products owing to their production of antimicrobial substances [10, 11].

Bacteriocins are among the most studied antimicrobial substances produced by LAB [12]. These antimicrobial peptides (bacteriocins) may be added as biopreservatives to improve the microbial stability and safety of chill-stored fresh meat [13, 14]. Among the studied bacteriocins in meat and meat products we can cite the nisin. Produced by *Lactococcus*

lactis, nisin was used successfully as food preservative in more than 50 countries [4]. This purified bacteriocin, nisin, showed bactericidal effect against *Listeria monocytogenes* in fresh meat and its application at 500 IU/ml engenders a significant reduction in the *L. monocytogenes* in meat [4]. On the other hand, direct use of bacteriocin-producing cells is one of the most practical strategies that seem to be more feasible from an economic point of view and lesser legal restrictions compared to the direct addition of purified bacteriocins. This can benefit the food industry in terms of microbiological quality and safety as well as cost since it reduces food losses caused by microbiological spoilage. Enterococci, isolated from diverse food sources, are among the most evaluated LAB as protective cultures in different foods due to their produced bacteriocins that are able to inhibit several key foodborne pathogens such as *L. monocytogenes* [15, 16]. Effectively, there are many strains of *Enterococcus* spp. that have been applied to the control of *L. monocytogenes* in different food systems [17–19]. Nowadays, advanced technologies have been developed for starters and protective cultures to enhance their efficacy and applicability in food products such as bioactive packaging and encapsulation [20]. Even though enterococci have been found naturally in different types of foods, their use in food products is controversial because they are considered as opportunistic pathogens implicated in several nosocomial infections and constitute a source of multiple antibiotic resistances [16].

The objectives of this work were to characterise genetically the multiple enterocin-producing *Enterococcus lactis* 4CP3 strain using RAPD-PCR analysis and evaluate its effect on the growth of *L. monocytogenes* in refrigerated raw beef meat.

2. Material and Methods

2.1. Strains and Growth Conditions. *E. lactis* 4CP3 strain was isolated from a raw shrimp (*Palaemon serratus*). The kinetic of bacteriocin production by 4CP3 strain was evaluated in MRS (de Man, Rogosa and Sharpe, Biokar Diagnostics, Beauvais, France) broth under aerobic conditions at 30°C [21]. This isolate was a multiple enterocin-producing strain able to produce the enterocins A, B, and P [21]. Also, it has been shown to display bactericidal mode of action against the pathogenic Gram-positive strain of *L. monocytogenes* EGDe 107776. It was grown overnight in MRS broth at 30°C.

E. faecium VC185 strain was isolated from Italian cheese [22]. This isolate is a non-bacteriocin-producing strain and is used in this study as the control strain in the meat challenge experimentation. It was also grown in MRS broth.

L. monocytogenes EGDe 107776 strain was used as the indicator strain for antimicrobial activity tests and the target microorganism in the microbiological challenge test. It was grown in BHI (Brain Heart Infusion, Biokar Diagnostics, Beauvais, France) broth and cultured on ALOA (Agar *Listeria* Ottaviani and Agosti, BIO-RAD, Marnes-la-Coquette, France) medium for enumeration [23].

E. faecium MMT21 strain was isolated from Tunisian rigouta cheese [24]. This isolate is used as the target strain in the direct detection of antimicrobial activity by overlaying with MRS soft agar in order to examine the capacity of *E. lactis* 4CP3 to produce bacteriocins in beef samples during the challenge test.

2.2. Random Amplified Polymorphic DNA-PCR (RAPD-PCR) Analysis. Genomic DNA used for RAPD-PCR amplification was extracted from overnight culture of *E. lactis* 4CP3 in M17 broth at 30°C according to Cremonesi et al., 2006 [25]. RAPD-PCR amplification was realised using the universal primers M13 and D8635 as described by Andrighetto et al., 2001 [26]. Amplification products were separated by electrophoresis on agarose gel (1.5%) in 1 × TAE buffer at 100 mV for 99 min. The gels were stained in ethidium bromide and photographed on a UV transilluminator. Photopositives were scanned into a computer and were analysed using the BioNumeric 5.0 software package (Applied Maths NV, Sint-Martens-Latem, Belgium). Grouping of the RAPD-PCR patterns was performed using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) cluster analysis. The reproducibility value of the RAPD-PCR assay, calculated from two repetitions of independent amplification of type strains, was higher than 90%. The RAPD-PCR profiles obtained with both primers (M13 and D8635) were analysed together to obtain a single dendrogram.

2.3. Antimicrobial Activity against L. monocytogenes. Overnight culture of *E. lactis* 4CP3 strain incubated at 30°C in MRS broth was centrifuged at 10,000 × *g* for 10 min to obtain a cell free supernatant which was neutralised at pH 6 with NaOH (1 M) in order to eliminate the inhibitory effect of organic acids, and sterilised by filtration (0.22 μm, Millipore, Bedford, MA) [21]. The antimicrobial activity of the cell free supernatant of *E. lactis* 4CP3 against *L. monocytogenes* EGDe 107776 was assayed by the agar-well diffusion method according to Ben Braïek et al., 2017 [27]. The BHI agar plate was incubated at 37°C for 24 h and the diameter of the inhibition zone was measured in millimetres (mm).

2.4. Influence of E. lactis 4CP3 Strain addition on the Growth of L. monocytogenes EGDe 107776 in Raw Beef Meat

2.4.1. Preparation and Inoculation of Raw Beef Samples. Raw beef meat was bought from a local supermarket in the region of Sousse (Tunisia) and transported to the laboratory under refrigerated conditions to be processed immediately. The prepared beef meat was aseptically cut into five equal portions of 200 g each (BF1-BF5). In order to reduce to the lowest possible levels the number of intrinsic microorganisms attached to the surface of beef meat portions, each piece was immersed in boiling sterile water for 5 min [28]. The cooked surface of the meat samples was eliminated with sterile knives under aseptic conditions [28]. These meat portions were further cut into small pieces of about 2 × 2 × 0.5 cm. Prior to beef meat contamination with *L. monocytogenes* and inoculation with LAB strains, beef portions were examined for any contamination by mesophilic and psychrotrophic flora. Total mesophilic bacteria were determined on plate count agar (PCA; Difco Laboratories, Detroit, MI, USA), incubated at 30°C for 48 h. Psychrotrophic counts were

determined as described above for mesophilic bacteria except that the incubation was at 4°C for 7 days [29].

E. lactis 4CP3 strain was grown in MRS broth at 30°C for 24 h to reach the maximum of its bacteriocin production (1400 AU/ml) [21]. *L. monocytogenes* EGDe 107776 was subcultured in BHI broth firstly at 37°C for 18 h to reach the early stationary phase with cells at the same physiological state, then at 10°C (temperature of the meat storage) for 3 days as adaptation step to the storage conditions. The *in situ* influence of the application of *E. lactis* 4CP3 strain on the survival of *L. monocytogenes* EGDe 107776 in raw beef meat was assessed according to the slightly modified method of Dortu et al., 2008 [6]. Briefly, the portions BF2 and BF3 were firstly surface inoculated at 10^7 CFU/g of meat with *E. lactis* 4CP3 and *E. faecium* VC185 strains, respectively. After absorption of the LAB inocula at room temperature, the BF1, BF2, and BF3 meat portions were surface contaminated with 10^5 CFU of *L. monocytogenes* EGDe 107776/g of meat. A sterile spreader was used to distribute homogeneously the inocula. The portion BF1 served as control (artificially contaminated only with *L. monocytogenes* EGDe 107776). The portion BF4 and BF5 were not contaminated with *L. monocytogenes* EGDe 107776 but were inoculated only with 4CP3 and VC185 strains, respectively, at 10^7 CFU/g of meat.

2.4.2. Storage and Enumerations. The raw beef meat portions were placed separately in sterile plastic bags and stored for 28 days at 10°C. The choice of this storage temperature relies firstly on the growth conditions of enterococcal strains used in this study that are not able to grow at temperatures lower than 10°C [30]. Secondly, meat storage at 10°C aimed to mimic the worst-case scenario for cold storage according to Kennedy et al., 2005 [31].

L. monocytogenes EGDe 107776 plate counts were determined on ALOA agar plates according to NF EN ISO 11290-2: 2005 [23]. The LAB counts were determined on MRS agar plates after incubation at 30°C for 24 h. Microbial enumerations were expressed as $\log_{10}$ CFU/g of beef meat. Plates containing 25-250 colonies were selected and counted, and the average number of CFU/g was calculated. These cell counts were performed every 6 h during 48 h, every day up to day 7 and every 7 days until day 28.

To detect enterocin production by *E. lactis* 4CP3 in raw beef meat, homogenates from the portions BF2 and BF4 stored at days 0, 7, 14, 21, and 28 were plated on MRS agar. After aerobic incubation at 30°C for 24 h, the plates were further overlaid with the indicator strain *E. faecium* MMT21 in soft agar and incubated overnight at 30°C. Bacteriocin production was indicated by clear inhibition zones around the colonies.

2.5. Statistical Analysis. Measurements were carried out in triplicates and repeated three times. A one-way analysis of variance (ANOVA) was applied for each parameter by using SPSS 19 statistical package (SPSS Ltd., Woking, UK). Means and standard errors were calculated and a probability level of $P<0.05$ was used in testing the statistical significance of all experimental data. Tukey's post hoc test was used to determine significance of mean values for multiple comparison at $P<0.05$. On the other hand, we used linear mixed models assuming the error to compare the CFU values among treatments with different days. Mixed models were fitted using SPSS 19 and followed by post hoc contrasts through the origin. The interpretation of the statistical output of a mixed model requires an understanding of how to explain the relationships among the fixed and random effects in terms of the hierarchy levels.

Table 1: Inhibitory spectrum of *E. lactis* 4CP3, CR4, CL, 5CP2, C15, and C23 strains against *L. monocytogenes* EGDe 107776.

Test strain	Diameter of inhibition zones (mm)
E. lactis 4CP3	12.00 ± 1.00^{d}
E. lactis CR4	10.00 ± 1.00^{c}
E. lactis CL	5.00 ± 0.00^{b}
E. lactis 5CP2	5.00 ± 0.00^{b}
E. lactis C15	0.00 ± 0.00^{a}
E. lactis C23	0.00 ± 0.00^{a}
PC	18.00 ± 2.00^{e}
NC	0.00 ± 0.00^{a}

Results are reported as means ± standard error of three replicates.[a–e]: averages with different letters in the same column, for each diameter of inhibition zones, are significantly different ($P<0.05$). PC: positive control (Novobiocin 1 mg/ml) and NC: negative control (noninoculated MRS broth medium).

3. Results and Discussion

3.1. RAPD-PCR Analysis. *E. lactis* 4CP3 strain was previously identified by different genetic methods: 16S rRNA gene sequencing, *rpoA* and *pheS* gene sequencing, and 16S-23S rRNA intergenic spacer analysis (RSA) [21]. Indeed, RSA analysis demonstrated that *E. lactis* 4CP3 strain displayed the same 16S-23S profile as the type strain *E. lactis* DSM 23655^{T} (BT159), while in this study, they presented different RAPD-PCR patterns as shown in Figure 1. Accordingly, two clusters (I and II) could be detected at a similarity level of 45% arbitrarily chosen for defining species. Interestingly, *E. lactis* 4CP3 and the type strain *E. lactis* BT159 were found to belong to different clusters even though they belong to the same species (Figure 1). This genetic differentiation between *E. lactis* 4CP3 and BT159 strains as illustrated by their clustering in the dendrogram could be related to their different isolation sources. Effectively, our *E. lactis* 4CP3 strain was isolated from a fresh shrimp sample of *Palaemon serratus* [21], while, *E. lactis* BT159 strain was isolated from an Italian cheese sample [32]. Therefore, RAPD-PCR analysis constitutes a rapid molecular method that could detect genetic diversity at a strain level with accuracy.

3.2. Antilisterial Activity. *In vitro* antibacterial assay of *E. lactis* 4CP3 strain showed high antilisterial activity ($P<0.05$) against *L. monocytogenes* EGDe 107776 with a clear growth inhibition zone diameter of 12 mm on BHI agar (Table 1). This result corroborates previous finding described for *E. faecium* strains [17]. This antagonistic activity towards *L. monocytogenes* was due to the production of the enterocins

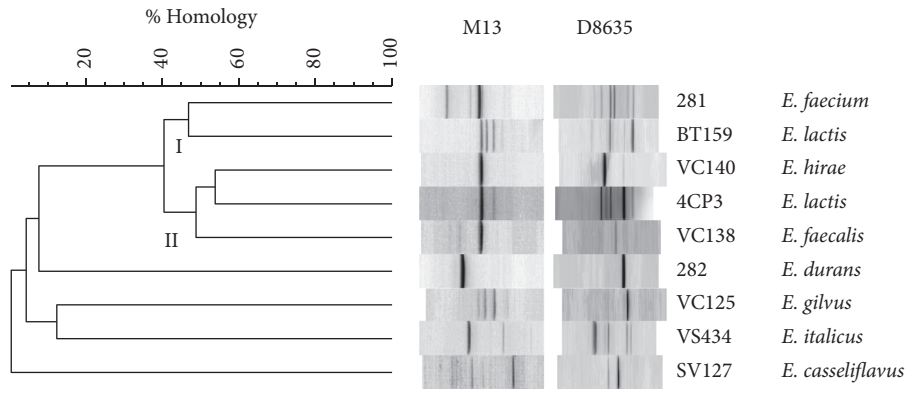


Figure 1: Unweighted pair group method using arithmetic averages (UPGMA) based dendrogram derived from the combined RAPD-PCR profiles generated with primers M13 and D8635 of *E. lactis* 4CP3 strain, type strains, and other enterococcal strains. The type strains used in this analysis were *E. lactis* DSM 23655^T (BT159), *E. faecium* DSM 20477^T (281), and *E. durans* DSM 20633^T (282) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany). The other enterococcal strains were *E. gilvus* (VC125), *E. italicus* (VS434), *E. hirae* (VC140), *E. faecalis* (VC138), and *E. casseliflavus* (SV127) from the bacterial collection of ISPA-CNR (Milan, Italy).

(A, B, and P) as previously demonstrated by Ben Braïek et al., 2018 [21]. In fact, the enterocins A, B, and P are among the most characterised bacteriocins and are known to be active against *Listeria* spp. as reported by Vandera et al. 2017 [16] and Rehaiem et al. 2014 [33].

3.3. Influence of E. lactis 4CP3 Strain addition on the Growth of L. monocytogenes EGDe 107776 in Raw Beef Meat Using ANOVA. Meat is considered to be one of the most frequently contaminated foods with *L. monocytogenes* [3]. According to Rapid Alert System for Food and Feed in 2016 [34], 20% of *L. monocytogenes* notifications were due to the contamination of meats other than poultry. In this context, a challenge test to control the growth of *L. monocytogenes* in raw beef meat inoculated with an enterocin-producing *E. lactis* strain was carried out. Furthermore, it is important to mention that high levels of intrinsic nonpathogenic microorganisms may have an inhibitory effect on pathogens present in meat by outcompeting them [28]. For this reason, our meat samples were subjected to boiling treatment with sterile water as described above in order to reduce the number of factors that could be implicated in the listerial growth in beef food models and to ovoid interferences of colonies on plating agar.

It should be noted that the analysis of mesophilic and psychrotrophic bacteria from meats treated separately with *E. lactis* 4CP3 and *E. faecium* VC185 strains showed an inhibition of these bacteria (mesophilic and psychrotrophic) since only the LAB, *E. lactis* and *E. faecium*, were identified (data not shown). In fact, the microbial load of aerobic mesophilic plate count and psychrotrophic count was zero, demonstrating the effective process of the boiling sterile water immersion intervention to eliminate these bacteria (aerobic mesophilic and psychrotrophic) from meat portions.

As demonstrated by Figure 2, there were no significant differences ($P>0.05$) in the growth of *E. lactis* 4CP3 strain and *E. faecium* VC185 strain in raw beef meat showing comparable growth rates increasing by 3.43 logs and 3.35 logs, respectively, in 28 days of storage. The population of *L. monocytogenes* EGDe 107776 in portion BF1 (positive control: artificially contaminated with 10^5 CFU/g of meat) underwent an increase from 10^5 CFU/g to 2.87×10^9 CFU/g after 28 days (Figure 3).

Statistical evaluation of the data relating to the growth behaviour of *L. monocytogenes* EGDe 107776 in raw beef meat inoculated with *E. lactis* 4CP3 strain showed significant reduction ($P<0.05$) of listerial population by 6.77 log units compared with the untreated control after 7 days of storage (Figure 3). Then, the growth of *L. monocytogenes* was completely inhibited from day 14 to the end of the experiment.

The application of the non-bacteriocin-producing *E. faecium* VC185 strain led to a very low reduction of *L. monocytogenes* populations. These counts were only 0.46 log units and 0.55 log units lower than the control counts after 7 and 28 days of storage, respectively. Moreover, no significant growth ($P>0.05$) of *L. monocytogenes* EGDe 107776 was observed in the portions BF4 and BF5 which were only inoculated with LAB strains at 10^7 CFU/g and not contaminated with the listerial pathogen.

3.4. In Situ Detection of Enterocin Production in Raw Beef Meat. Overlay assays with MRS agar plates were realised in order to detect *in situ* production of enterocins by *E. lactis* 4CP3 strain in beef meat samples during the refrigerated storage period. After incubation, enterocin production was indicated by observation of obvious inhibition zones around the colonies grown on MRS agar medium. Generally, it was shown that the application of the multiple enterocin-producing *E. lactis* 4CP3 strain in raw beef meat led to a greater ($P<0.05$) inhibition of *L. monocytogenes* EGDe 107776 than that of the non-bacteriocin-producing *E. faecium* VC185 strain (Figure 3). Also, it was demonstrated in this study that this enterococcal culture strongly ($P<0.05$) inhibited the growth of *L. monocytogenes* in beef meat after the first 7 days of the challenge test and then suppressed dramatically the pathogen. This potent inhibitory behaviour of *E. lactis* 4CP3 towards *L. monocytogenes* could be explained

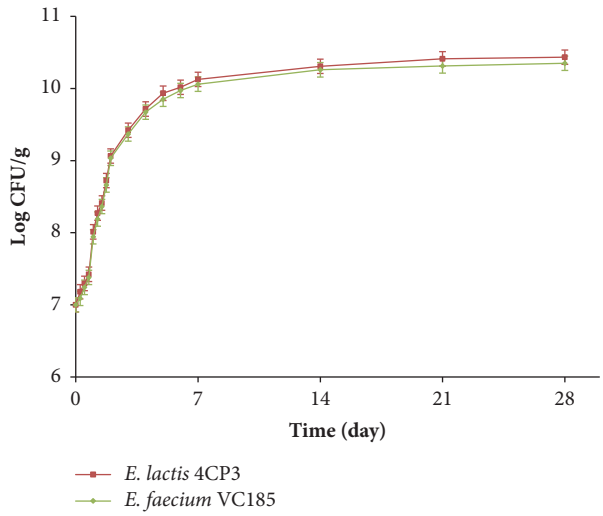


Figure 2: Growth of LAB strains in raw beef meat. Red square: *E. lactis* 4CP3 (enterocin-producing LAB strain) and green diamond: *E. faecium* VC185 (non-bacteriocin-producing LAB strain).

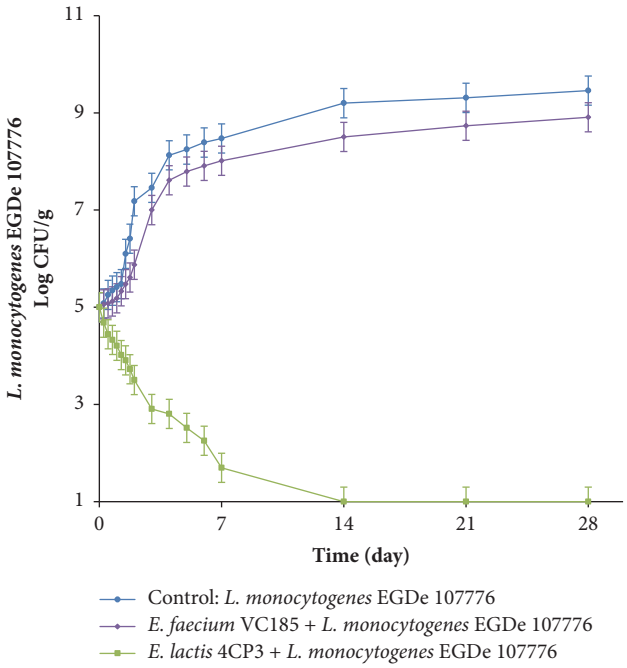


Figure 3: Influence of inhibitory LAB cultures on the growth of *L. monocytogenes* EGDe 107776 in raw beef meat during storage at 10°C. Blue circle: control (*L. monocytogenes* EGDe 107776 without enterocin-producing LAB strain), violet diamond: *E. faecium* VC185 (non-bacteriocin-producing LAB strain), and green square: *E. lactis* 4CP3 strain (enterocins A, B and P-producing strain).

by the enterocin production as confirmed above. In fact, enterocins A and P have strong antilisterial activity against *L. monocytogenes*; however, enterocin B displays synergistic activity with enterocin A [16, 33]. Thus, our present results corroborate these previous findings indicating that enterocins A and B may synergistically inhibit *L. monocytogenes* growth. Likewise, a synergistic interaction between the three produced enterocins (A, B, and P) by *E. lactis* 4CP3 could be proposed reflecting thus its effectiveness in raw beef meat preservation. Similar results reporting the biocontrol of *L. monocytogenes* in different meat products with bacteriocinogenic LAB were previously described by Dortu et al., 2008 [6], Pragalaki et al., 2013 [35], and Giello et al., 2018 [36]. Therefore, it is clear that application of bacteriocin-producing LAB in meats and meat products have been attracting considerable interest as alternative natural food preservatives to extend shelf-life and safety of meats these recent years [37, 38]. Effectively, direct application of bacteriocin-producing LAB is among the most advanced and practical approaches from economic and regulatory status point of views. Indeed, this bacterial use does not need many processing steps such as purification and has fewer legal restrictions and limits compared to the direct application of purified bacteriocins [39].

3.5. Influence of E. lactis 4CP3 Strain addition on the Growth of L. monocytogenes EGDe 107776 in Raw Beef Meat Using General Linear Model (ANCOVA). Analysis of covariance (ANCOVA) is a general linear model which blends ANOVA and regression. ANCOVA evaluates whether the means of dependent variables (11 sampling days: 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 days of storage at 10°C) which are equal across levels of categorical independent variables (five trials: Trial 1: BF1, Trial 2: BF2, Trial 3: BF3, Trial 4: BF4, and Trial 5: BF5) and inversely. In order to simplify the obtained results, for each meat product, firstly (i) we analysed parameters between 0 and 7 days and secondly (ii) all parameters were evaluated between 7 and 28 days.

3.5.1. ANCOVA Parameter Analyses between 0 and 7 Days. As in ANCOVA, writing out the full regression model and then simplifying tells us that the intercept for day zero was 4.000 (4.194436–0.194436) and this was lower than $\log_{10}$ CFU at the seventh day group (t= -0.053). Similarly, we knew that

Table 2

(a) Raw beef meat estimates of trials fixed effects between 0 and 7 days.

Parameter	Estimate	Std. Error	Df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	4.194436	2.595846	24	1.616	0.119 (ns)	-1.163127	9.551998
Day 0	-0.194436	3.671080	24	-0.053	0.958 (ns)	-7.771173	7.382302
Day 1	-0.699838	3.671080	24	-0.191	0.850 (ns)	-8.276576	6.876899
Day 2	-0.041189	3.671080	24	-0.011	0.991 (ns)	-7.617927	7.535548
Day 3	-0.064947	3.671080	24	-0.018	0.986 (ns)	-7.641685	7.511790
Day 4	0.392455	3.671080	24	0.107	0.916 (ns)	-7.184282	7.969193
Day 5	0.285756	3.671080	24	0.078	0.939 (ns)	-7.290981	7.862494
Day 6	0.235276	3.671080	24	0.064	0.949 (ns)	-7.341462	7.812013
Day 7	0[a]	0	.	.	.	.	.
Trial	1.160110	6.194913	94193.706	0.187	0.851 (ns)	-10.981849	13.302068
Day 0 × Trial	-0.560110	8.760930	94193.706	-0.064	0.949 (ns)	-17.731432	16.611213
Day 1 × Trial	-0.273876	8.760930	94193.706	-0.031	0.975 (ns)	-17.445198	16.897447
Day 2 × Trial	-0.233863	8.760930	94193.706	-0.027	0.979 (ns)	-17.405185	16.937460
Day 3 × Trial	-0.126228	8.760930	94193.706	-0.014	0.989 (ns)	-17.297550	17.045095
Day 4 × Trial	-0.160129	8.760930	94193.706	-0.018	0.985 (ns)	-17.331451	17.011194
Day 5 × Trial	-0.097362	8.760930	94193.706	-0.011	0.991 (ns)	-17.268685	17.073960
Day 6 × Trial	-0.067141	8.760930	94193.706	-0.008	0.994 (ns)	-17.238464	17.104181
Day 7 × Trial	0[a]	0	.	.	.	.	.

[a]: this parameter is set to zero because it is redundant. Std. Error: standard error, df: the degrees of freedom, t: Student's t-statistic, and Sig.: the p-value (associated with the correlation). ns: $P>0.05$.

(b) Raw beef meat estimates of days fixed effects.

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	7.467589	0.246726	26.643	30.267	0.000 (∗∗∗)	6.961032	7.974147
Trial 1	-2.190545	0.334916	23.708	-6.541	0.000 (∗∗∗)	-2.882229	-1.498862
Trial 2	-3.072563	0.334916	23.708	-9.174	0.000 (∗∗∗)	-3.764247	-2.380879
Trial 3	-2.595970	0.334916	23.708	-7.751	0.000 (∗∗∗)	-3.287653	-1.904286
Trial 4	0.028207	0.334916	23.708	0.084	0.934 (ns)	-0.663477	0.719891
Trial 5	0[a]	0	.	.	.	.	.
Day	0.489768	0.070900	21.369	6.908	0.000 (∗∗∗)	0.342479	0.637057
Trial 1 × Day	0.103902	0.080060	23.708	1.298	0.207 (ns)	-0.061443	0.269246
Trial 2 × Day	-0.836490	0.080060	23.708	-10.448	0.000 (∗∗∗)	-1.001834	-0.671146
Trial 3 × Day	0.080347	0.080060	23.708	1.004	0.326 (ns)	-0.084998	0.245691
Trial 4 × Day	0.005842	0.080060	23.708	0.073	0.942 (ns)	-0.159502	0.171186
Trial 5 × Day	0[a]	0	.	.	.	.	.

[a]: this parameter is set to zero because it is redundant. Std. Error: standard error, df: the degrees of freedom, t: Student's t-statistic, and Sig.: the p-value (associated with the correlation). Trial 1: BF1 (control sample), Trial 2: BF2, Trial 3: BF3, Trial 4: BF4, and Trial 5: BF5. ns: $P>0.05$, ∗∗∗: $P<0.001$.

the days 0, 1, 2, 3, 4, 5, and 6 had lower intercepts than the 7th day. The trial coefficient of 1.160110 represented the average for each subsequent trial for the baseline on day 7. The interaction estimates tell the difference in slope for other day groups compared to the seventh day groups (Table 2(a)). We are particularly interested in the conclusion that we are 95% confident that the control sample had an effect on the CFU that was between 16.611213 points more and -17.731432 points less than treatment for beef meat (Table 2(a)).

Equally, ANCOVA indicated that there were no statistically significant differences ($P>0.05$) among the treatments between 0 and 7 days (Table 2(a)).

Table 3: Estimates of covariance parameters in raw beef meat samples between 0 and 7 days.

Parameter		Estimate	Wald Z	Sig.
Residual		0.134603	3.443	0.001
Day [subject = id]	Variance	0.006163	1.067	0.286
Residual		6.125832	3.464	0.001
Trial [subject = id]	Variance	37.764364[a]	.	.

[a]: this covariance parameter is redundant. The test statistic and confidence interval cannot be computed. Sig.: the p-value (associated with the correlation).

As shown in Table 2(b), writing out the full regression model then simplifying tells us that the intercept for trial 1 was 5.277044 (7.467589–2.190545). Similarly, we knew the trials 2, 3, 4, and 5. The day coefficient of 0.489768 represented the average for each subsequent trial for the baseline on the trial 5 (Table 2(b)). The interaction estimates tell the difference in slope for other trial groups compared to the fifth groups (Table 2(b)).

The treatments BF1 (control sample), BF3 (*E. faecium* VC185 strain at 10^7 CFU/g of meat + 10^5 CFU of *L. monocytogenes* EGDe 107776/g of meat), BF4 (only *E. lactis* 4CP3 strain at 10^7 CFU/g of meat), and BF5 (only *E. faecium* VC185 strain at 10^7 CFU/g of meat) had no significant differences ($P>0.05$) between them. However, at the $P<0.001$ confidence level, the treatment of *E. lactis* 4CP3 strain at 10^7 CFU/g of meat + 10^5 CFU of *L. monocytogenes* EGDe 107776/g of meat (BF2) was statistically different and was more sensitive to dose than the other trials (Table 2(b)).

It is very important to realise that the parameter estimates given in the fixed effects were estimates of mean parameters. The covariance parameters are presented in Table 3. Equally, the intercepts' variances were estimated as 0.134603 and 6.125832 (Table 3). The null hypothesis for this parameter was a variance of zero, which would indicate that a random effect was not needed. The statistical test is called Wald Z statistic. On the other hand, the hypothesis (Wald Z = 0.000, $P = 1.00$) was accepted and the null hypothesis (Wald Z = 1.067, $P = 0.286$) was rejected. In fact, we conclude that we do need a random intercept (Table 3). This suggests that there are important unmeasured explanatory variables for each subject that raise or lower their performance in a way that appears random because we do not know the values of the missing explanatory variables.

3.5.2. ANCOVA Parameter Analyses between 7 and 28 Days. For a period ranged between 7 and 28 days of storage, the ANCOVA intercept for day seven was 4.194435 (4.664446–0.470011) and this was lower than $\log_{10}$ CFU at the twenty-eighth day group (t= -0.081) (Table 4(a)). Similarly, the days 7, 14, and 21 had lower intercepts than the day 28. The trial coefficient of 1.121930 represented the average for each subsequent trial for the baseline on the day 28 (Table 4(a)). Furthermore, the treatment control sample (Trial 1) had an effect on the CFU (Table 4(a)).

As shown in Table 4(a), there were no significant differences ($P>0.05$) among the trials and days 7, 14, 21, and 28.

Indeed, the lower and upper bound of the confidence interval for the mean difference ranged from -25.174660 points to 25.251019 points (Table 4(a)). The full regression model then simplifying the intercept for the control sample was 8.344604 (10.015610–1.671006) (Table 4(b)). Similar results were shown for 2, 3, 4, and 5 trial groups. The day coefficient was 0.013178.

The effects of treatments, time, and their interaction on the inhibition of *L. monocytogenes* are shown in Table 4(b). no significant interaction ($P>0.05$) between treatments BF3 and BF4, and the time of storage in meat. However, interestingly, at the $P<0.01$ confidence level, BF2 and time of storage were found to have a highly significant effect regarding inhibition of *L. monocytogenes* EGDe 107776 in meat (Table 4(b)).

Moreover, the intercepts' variances were estimated as 0.029149 and 15.235632. Besides, the hypothesis (Wald Z = 0.000, $P = 1.00$) was accepted for beef meat samples (Table 5).

3.6. Practical Aspects. Enterococcal strains with a view to be used as protective or starter/adjunct cultures in biopreservation of foods, must usually be selected on the basis of the safety aspects which frequently are the absence of virulence and antibiotic resistance traits. Effectively, *E. lactis* 4CP3 strain was previously verified as nonhaemolytic, gelatinase negative, sensitive to vancomycin and other clinically relevant antibiotics and lacked known antibiotic resistance genes and several significant virulence factors [21]. Therefore, the presence of *E. lactis* 4CP3 in meat does not appear to represent a health risk.

4. Conclusion

To the best of our knowledge, this is the first report on the application of a multiple enterocin-producing *E. lactis* strain to control *L. monocytogenes* in artificially contaminated raw beef meat during refrigerated storage. Based on the obtained results, *E. lactis* 4CP3 strain might be useful as natural biopreservative against *L. monocytogenes* in meat products.

Conflicts of Interest

The authors have declared that there are no conflicts of interest.

Acknowledgments

This work was supported by a grant from the Ministry of High Education, Tunisia. The authors thank Dr. Stefano Morandi (Institute of Sciences of Food Production, Italian National Research Council (CNR ISPA), Milan, Italy) for providing them with the non-bacteriocin-producing *E. faecium* VC185 strain and the RAPD-PCR analysis.

Table 4

(a) Raw beef meat estimates of trials fixed effects between 7 and 28 days.

Parameter	Estimate	Std. Error	Df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	4.664446	4.093800	12	1.139	0.277 (ns)	-4.255177	13.584069
Day 7	-0.470011	5.789507	12	-0.081	0.937 (ns)	-13.084262	12.144241
Day 14	-0.239269	5.789507	12	-0.041	0.968 (ns)	-12.853521	12.374983
Day 21	-0.138680	5.789507	12	-0.024	0.981 (ns)	-12.752932	12.475572
Day 28	0[a]	0	.	.	.	.	.
Trial	1.121930	2.383010	0.000	0.471	1.000 (ns)	-16.706240	18.950100
Day 7 × Trial	0.038180	3.370085	0.000	0.011	1.000 (ns)	-25.174660	25.251019
Day 14 × Trial	0.021102	3.370085	0.000	0.006	1.000 (ns)	-25.191737	25.233942
Day 21 × Trial	0.020506	3.370085	0.000	0.006	1.000 (ns)	-25.192333	25.233345
Day 28 × Trial	0[a]	0	.	.	.	.	.

[a]: this parameter is set to zero because it is redundant. Std. Error: standard error, df: the degrees of freedom, t: Student's t-statistic, and Sig.: the p-value (associated with the correlation).
ns: $P>0.05$.

(b) Raw beef meat estimates of days fixed effects.

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	10.015610	0.209103	10	47.898	0.000 (∗∗∗)	9.549700	10.481519
Trial 1	-1.671006	0.295716	10	-5.651	0.000 (∗∗∗)	-2.329902	-1.012110
Trial 2	-8.316640	0.295716	10	-28.124	0.000 (∗∗∗)	-8.975536	-7.657744
Trial 3	-2.204440	0.295716	10	-7.455	0.000 (∗∗∗)	-2.863336	-1.545544
Trial 4	0.047959	0.295716	10	0.162	0.874 (ns)	-0.610937	0.706855
Trial 5	0[a]	0	.	.	.	.	.
Day	0.013178	0.010908	10	1.208	0.255 (ns)	-0.011126	0.037482
Trial 1 × Day	0.030534	0.015426	10	1.979	0.046 (∗)	-0.003837	0.064905
Trial 2 × Day	-0.043134	0.015426	10	-2.796	0.009 (∗∗)	-0.077504	-0.008763
Trial 3 × Day	0.028453	0.015426	10	1.845	0.095 (ns)	-0.005917	0.062824
Trial 4 × Day	0.001487	0.015426	10	0.096	0.925 (ns)	-0.032884	0.035857
Trial 5 × Day	0[a]	0	.	.	.	.	.

[a]: this parameter is set to zero because it is redundant. Std. Error: standard error, df: the degrees of freedom, t: Student's t-statistic, and Sig.: the p-value (associated with the correlation). Trial 1: BF1 (control sample), Trial 2: BF2, Trial 3: BF3, Trial 4: BF4, and Trial 5: BF5. ns: $P>0.05$, ∗: $P<0.05$, ∗∗: $P<0.01$, and ∗∗∗: $P<0.001$.

Table 5: Estimates of covariance parameters in raw beef meat samples between 7 and 28 days.

Parameter		Estimate	Wald Z	Sig.
Residual		0.029149	2.236	0.025
Day [subject = id]	Variance	0.000000[a]	.	.
Residual		15.235632	2.449	0.014
Trial [subject = id]	Variance	4.155172	0.000	1.000

[a]: this covariance parameter is redundant. The test statistic and confidence interval cannot be computed. Sig.: the p-value (associated with the correlation).

References

[1] M. S. Islam, A. A. Husna, M. A. Islam, and M. M. Khatun, "Prevalence of *Listeria monocytogenes* in beef, chevon and chicken in Bangladesh," *American Journal of Food Science and Health*, vol. 2, no. 4, pp. 39–44, 2016.

[2] J. A. Lennox, P. O. Etta, G. E. John, and E. E. Henshaw, "Prevalence of *Listeria monocytogenes* in fresh and raw fish, chicken and beef," *Journal of Advances in Microbiology*, vol. 3, no. 4, pp. 1–7, 2017.

[3] A. A.-R. Ismaiel, A. E.-S. Ali, and G. Enan, "Incidence of *Listeria* in Egyptian meat and dairy samples," *Food Science and Biotechnology*, vol. 23, no. 1, pp. 179–185, 2014.

[4] S. H. Abdellatif, S. Abdel-Shafi, A. E. Ali, and A. A. Ismaiel, "Inhibition of two *Listeria* strains *in vitro* and *in situ* by nisin, onion and garlic juices separately and in combinations," *Wulfenia Journal*, vol. 25, no. 1, pp. 194–206, 2018.

[5] B. Martín, A. Perich, D. Gómez et al., "Diversity and distribution of *Listeria monocytogenes* in meat processing plants," *Food Microbiology*, vol. 44, pp. 119–127, 2014.

[6] C. Dortu, M. Huch, W. H. Holzapfel, C. M. A. P. Franz, and P. Thonart, "Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat," *Letters in Applied Microbiology*, vol. 47, no. 6, pp. 581–586, 2008.

[7] EFSA, "The european union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011," *EFSA Journal*, vol. 11, no. 4, p. 3129, 2013.

[8] P. M. Davidson, H. B. Cekmer, E. A. Monu, and C. Techathuvanan, "The use of natural antimicrobials in food: an overview," *Handbook of Natural Antimicrobials for Food Safety and Quality*, pp. 1–27, 2015.

[9] Y. Widyastuti and A. Febrisiantosa, "The role of lactic acid bacteria in milk fermentation," *Journal of Food and Nutrition Sciences*, vol. 05, no. 04, pp. 435–442, 2014.

[10] M.-F. Pilet and F. Leroi, "Applications of protective cultures, bacteriocins and bacteriophages in fresh seafood and seafood products," in *Protective Cultures, Antimicrobial Metabolites and Bacteriophages for Food And Beverage Biopreservation*, pp. 324–347, Woodhead Publishing, Cambridge, UK, 2011.

[11] B. Melero, R. Vinuesa, A. M. Diez, I. Jaime, and J. Rovira, "Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere," *Poultry Science*, vol. 92, no. 4, pp. 1108–1116, 2013.

[12] M. E. F. Abdel-Haliem, E. Tartour, and G. Enan, "Characterization, production and partial purification of a bacteriocin produced by *Lactobacillus plantarum* LPS10 isolated from pickled lives," *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, vol. 7, no. 5, pp. 2362–2371, 2016.

[13] S. Smaoui, L. Elleuch, R. Ben Salah et al., "Efficient role of BacTN635 on the safety properties, sensory attributes, and texture profile of raw minced meat beef and chicken breast," *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, vol. 31, no. 2, pp. 218–225, 2014.

[14] S. Smaoui, A. B. Hsouna, A. Lahmar et al., "Bio-preservative effect of the essential oil of the endemic *Mentha piperita* used alone and in combination with BacTN635 in stored minced beef meat," *Meat Science*, vol. 117, pp. 196–204, 2016.

[15] A. Paari, P. Kanmani, R. Satishkumar, N. Yuvaraj, V. Pattukumar, and V. Arul, "Potential function of a novel protective culture *Enterococcus faecium*-MC13 isolated from the gut of *Mughil cephalus*: safety assessment and its custom as biopreservative," *Food Biotechnology*, vol. 26, no. 2, pp. 180–197, 2012.

[16] E. Vandera, A. Lianou, A. Kakouri, J. Feng, A.-I. Koukkou, and J. Samelis, "Enhanced control of *Listeria monocytogenes* by *Enterococcus faecium* KE82, a multiple enterocin-producing strain, in different milk environments," *Journal of Food Protection*, vol. 80, no. 1, pp. 74–85, 2017.

[17] M. Aspri, D. Field, P. D. Cotter, P. Ross, C. Hill, and P. Papademas, "Application of bacteriocin-producing *Enterococcus faecium*isolated from donkey milk, in the bio-control of *Listeria monocytogenes*in fresh whey cheese," *International Dairy Journal*, vol. 73, pp. 1–9, 2017.

[18] A. Chakchouk-Mtibaa, L. Elleuch, S. Smaoui et al., "An antilisterial bacteriocin BacFL31 produced by *Enterococcus faecium* FL31 with a novel structure containing hydroxyproline residues," *Anaerobe*, vol. 27, pp. 1–6, 2014.

[19] M. C. Coelho, C. C. G. Silva, S. C. Ribeiro, M. L. N. E. Dapkevicius, and H. J. D. Rosa, "Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria," *International Journal of Food Microbiology*, vol. 191, pp. 53–59, 2014.

[20] J. Weiss, Q. Zhong, F. Harte, and P. M. Davidson, "Micro- and nanoparticles for controlling microorganisms in foods," in *Liposomes, Lipid Bilayers and Model Membranes: From Basic Research to Technology*, G. Pabst, N. Kucerka, M. P. Nieh, and J. Katsaras, Eds., CRC Press, Boca Raton, FL, USA, 2014.

[21] O. Ben Braïek, P. Cremonesi, S. Morandi, S. Smaoui, K. Hani, and T. Ghrairi, "Safety characterisation and inhibition of fungi and bacteria by a novel multiple enterocin-producing *Enterococcus lactis* 4CP3 strain," *Microbial Pathogenesis*, vol. 118, pp. 32–38, 2018.

[22] S. Morandi, T. Silvetti, J. M. Miranda Lopez, and M. Brasca, "Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk valtellina casera cheese," *Journal of Food Safety*, vol. 35, no. 2, pp. 193–205, 2015.

[23] French Standardization Association (AFNOR), "Microbiology of food and animal feeding stuffs: Horizontal method for the detection and enumeration of *Listeria monocytogenes*-Part 2: Enumeration method," NF EN ISO 11290, Plaine Saint-Denis, France, 2005.

[24] T. Ghrairi, J. Frere, J. M. Berjeaud, and M. Manai, "Purification and characterization of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese," *Food Control*, vol. 19, no. 2, pp. 162–169, 2008.

[25] P. Cremonesi, B. Castiglioni, G. Malferrari et al., "Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk," *Journal of Dairy Science*, vol. 89, no. 1, pp. 163–169, 2006.

[26] C. Andrighetto, L. Zampese, and A. Lombardi, "RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy)," *Letters in Applied Microbiology*, vol. 33, no. 1, pp. 26–30, 2001.

[27] O. Ben Braïek, H. Ghomrassi, P. Cremonesi et al., "Isolation and characterisation of an enterocin P-producing *Enterococcus lactis* strain from a fresh shrimp (*Penaeus vannamei*)," *Antonie van Leeuwenhoek Journal of Microbiology*, vol. 110, no. 6, pp. 771–786, 2017.

[28] N. El Abed, B. Kaabi, M. I. Smaali et al., "Chemical composition, antioxidant and antimicrobial activities of thymus capitata essential oil with its preservative effect against *Listeria monocytogenes* inoculated in minced beef meat," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 152487, 11 pages, 2014.

[29] International Organization for Standardization (ISO), "Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of psychrotrophic microorganisms," Tech. Rep. 17410, 2001.

[30] U. V. John and J. Carvalho, "*Enterococcus*: Review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology," *Frontiers in Biology*, vol. 6, no. 5, pp. 357–366, 2011.

[31] J. Kennedy, V. Jackson, I. S. Blair, D. A. McDowell, C. Cowan, and D. J. Bolton, "Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators," *Journal of Food Protection*, vol. 68, no. 7, pp. 1421–1430, 2005.

[32] S. Morandi, T. Silvetti, and M. Brasca, "Biotechnological and safety characterization of *Enterococcus lactis*, a recently described species of dairy origin," *Antonie van Leeuwenhoek-Journal of Microbiology*, vol. 103, no. 1, pp. 239–249, 2013.

[33] A. Rehaiem, Z. B. Belgacem, M. R. Edalatian et al., "Assessment of potential probiotic properties and multiple bacteriocin encoding-genes of the technological performing strain *Enterococcus faecium* MMRA," *Food Control*, vol. 37, no. 1, pp. 343–350, 2014.

[34] RASFF, *The Rapid Alert System for Food and Feed: Annual Report*, Publications Office of the European Union, Luxembourg, Luxembourg, 2017.

[35] T. Pragalaki, J. G. Bloukas, and P. Kotzekidou, "Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in liquid broth medium and during processing of fermented sausage using autochthonous starter cultures," *Meat Science*, vol. 95, no. 3, pp. 458–464, 2013.

[36] M. Giello, A. La Storia, F. De Filippis, D. Ercolini, and F. Villani, "Impact of *Lactobacillus curvatus* 54M16 on microbiota composition and growth of *Listeria monocytogenes* in fermented sausages," *Food Microbiology*, vol. 72, pp. 1–15, 2018.

[37] F. P. Rivas, M. P. Castro, M. Vallejo, E. Marguet, and C. A. Campos, "Sakacin Q produced by *Lactobacillus curvatus* ACU-1: functionality characterization and antilisterial activity on cooked meat surface," *Meat Science*, vol. 97, no. 4, pp. 475–479, 2014.

[38] M. de Souza Barbosa, S. D. Todorov, I. Ivanova, J.-M. Chobert, T. Haertlé, and B. D. G. de Melo Franco, "Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate," *Food Microbiology*, vol. 46, pp. 254–262, 2015.

[39] W. Woraprayote, Y. Malila, S. Sorapukdee, A. Swetwiwathana, S. Benjakul, and W. Visessanguan, "Bacteriocins from lactic acid bacteria and their applications in meat and meat products," *Meat Science*, vol. 120, pp. 118–132, 2016.

Hindawi
BioMed Research International
Volume 2018, Article ID 1535209, 13 pages
https://doi.org/10.1155/2018/1535209

Research Article

Cloning and Expression of the Organophosphate Pesticide-Degrading α-β Hydrolase Gene in Plasmid pMK-07 to Confer Cross-Resistance to Antibiotics

Kirubakaran Rangasamy,[1] Murugan Athiappan,[1] Natarajan Devarajan,[2] Javid A. Parray,[3] Nowsheen Shameem,[4] K. N. Aruljothi,[5] Abeer Hashem,[6,7] Abdulaziz A. Alqarawi,[8] and Elsayed Fathi Abd_Allah[8]

[1]*Department of Microbiology, Periyar University, Salem, Tamil Nadu, India*
[2]*Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India*
[3]*Department of Environmental Science, Government SAM Degree College Budgam, Jammu & Kashmir 191111, India*
[4]*Department of Environmental Science, Cluster University Srinagar, Jammu & Kashmir 190001, India*
[5]*Department of Genetic Engineering, SRM University, Chennai, Tamil Nadu, India*
[6]*Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia*
[7]*Mycology and Plant Disease Survey Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt*
[8]*Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia*

Correspondence should be addressed to Murugan Athiappan; amuruganpu@gmail.com

Received 23 January 2018; Accepted 8 April 2018; Published 16 May 2018

Academic Editor: Gamal Enan

Pesticide residual persistence in agriculture soil selectively increases the pesticide-degrading population and transfers the pesticide-degrading gene to other populations, leading to cross-resistance to a wide range of antibiotics. The enzymes that degrade pesticides can also catabolize the antibiotics by inducing changes in the gene or protein structure through induced mutations. The present work focuses on the pesticide-degrading bacteria isolated from an agricultural field that develop cross-resistance to antibiotics. This cross-resistance is developed through catabolic gene clusters present in an extrachromosomal plasmid. A larger plasmid (236.7 Kbp) isolated from *Bacillus* sp. was sequenced by next-generation sequencing, and important features such as α-β hydrolase, DNA topoisomerase, DNA polymerase III subunit beta, reverse transcriptase, plasmid replication rep X, recombination U, transposase, and S-formylglutathione hydrolase were found in this plasmid. Among these, the α-β hydrolase enzyme is known for the degradation of organophosphate pesticides. The cloning and expression of the α-β hydrolase gene imply nonspecific cleavage of antibiotics through a cross-resistance phenomenon in the host. The docking of α-β hydrolase with a spectrum of antibiotics showed a high G-score against chloramphenicol (−3.793), streptomycin (−2.865), cefotaxime (−5.885), ampicillin (−4.316), and tetracycline (−3.972). This study concludes that continuous exposure to pesticide residues may lead to the emergence of multidrug-resistant strains among the wild microbial flora.

1. Introduction

Plasmid-borne drug resistance and its persistence among soil bacteria cause great public health hazards. Although other genetic elements such as nonconjugative, mobilizable plasmids contribute to multidrug resistance [1–3], bacterial plasmid DNA also confers drug resistance [4, 5]. Multidrug resistance is very common among soil bacteria that are exposed to pesticides [6, 7]. Moreover, the indiscriminate use of pesticides favours the microbial population that can metabolize those pesticides. Higher exposure to pesticides enables bacteria to produce suitable enzymes for the degradation of pollutants. Consequently, pesticide-metabolizing populations tend to overgrow [8–10]. Pesticide-degrading bacteria metabolize the pesticides through hydrolytic cleavage for their carbon and energy sources [11, 12]. Degradation

of pesticides may also occur through oxidation, reduction, hydrolysis, peroxidise, or oxygenase mechanisms [13, 14].

Enzymes produced by pesticide-degrading bacteria can catabolize drugs and other xenobiotics. Breaking C=C bonds in the drug by nonspecific cleavage leads to cross-resistance to antibiotics [15, 16]. This could be an explanation regarding how susceptible soil bacteria become multidrug resistant. A previous study on formaldehyde resistance with *Enterobacteriaceae* has shown high formaldehyde dehydrogenase activity leading to multidrug resistance through nonspecific activity [17, 18]. A study conducted on formaldehyde dehydrogenase enzyme-mediated multidrug resistance provided the notion that cross-resistance is possible via nonspecific degradation. Another study proved that enzymes produced by soil bacteria in pesticide-contaminated soil can degrade other xenobiotics [19–21]. Assessing the catabolic properties of α-β hydrolase, an enzyme-degrading pesticide, would answer whether these enzymes play a role in multidrug resistance. It is very common among soil bacteria to adapt themselves to changing environmental conditions. Evolutionarily, bacteria in a stressed environment tend to produce putative enzymes via induced mutations that can degrade a wide range of xenobiotics [22].

Therefore, the present study aimed to understand the bacterial isolates with an extrachromosomal plasmid carrying pesticide-resistant genes that also confer the cross-resistance of multidrug resistance. In silico analyses were performed to understand the mechanism of cross-resistance via the α-β hydrolase enzyme.

2. Materials and Methods

2.1. Sample Collection. Soil samples were collected from up to 5 cm of the upper layer from different pesticides applied to an agriculture field located in the Salem district (11.7794°N, 78.2034°E), Tamil Nadu, India. The collected samples were transported to the laboratory and stored at 4°C until further analysis.

2.2. Plasmid Isolation and DNA Sequencing. Owing to the increased use of the plasmid, the sample was kept for plasmid enrichment by incubating with 1% pesticide (monocrotophos) supplemented medium for two days. The bacterial strains were isolated from the enriched sample, and it was found that *Bacillus* sp. MK-07 (KU510395.1) was predominant. Plasmid DNA (pMK-07) was isolated by the alkaline lysis method from the isolate [23]. The plasmid was treated with plasmid-safe, adenosine 5-triphosphate- (ATP-) dependent DNase to remove any genomic DNA contamination. The plasmid DNA was processed for library preparation using an Illumina Nextera XT DNA library preparation kit. SnapGene version 4.0.2 software was used to create a plasmid DNA map [24]. The library was sequenced on MiSeq using 2 × 300 bp to generate approximately 1 GB of data. The Draft assemblies of short Illumina sequence reads (2 × 300 MiSeq library) were analysed with a 4200-tape station system, Eurofins Genomics, Bangalore, India (Agilent Technologies, USA) [25].

2.3. Assembling the Plasmid DNA Sequence. Raw data were processed using Trimmomatic v 0.35 to remove adapter sequences, ambiguous reads (reads with unknown nucleotides "N" larger than 5%), and low-quality sequences (reads with more than 10% quality thresholds (QV) < 20 phred score). Clear sequences with a size of 1,073,566 (2 × 300 bp) high-quality reads were retained for further analysis and were used for de novo assembly [2, 26].

2.3.1. Gene Prediction and Functional Annotation. Sequences were predicted using prodigal with default parameters. In total, 225 genes were predicted with an average gene size of 816 bp, while the maximum and minimum sizes of the genes were 15,033 bp and 105 bp, respectively [27, 28]. Gene ontology annotations of the predicted [29] genes were determined by the Blast2GO program (https://www.Blast2GO.com). Gene ontology assignments were used to classify the functions of the predicted genes. Functional annotations of the genes were performed using BLASTx, part of the NCBI-Blast-2.3.0 standalone tool. BLASTx was used to find the homologous sequence of genes against NR (nonredundant protein database) within *Bacillus cereus* (MK-07).

2.4. Phylogenetic Distinct Clades and Cloning of α-β Hydrolase Gene. The scaffold sample of plasmid (pMK-07) was aligned against plasmids of all the *Bacillus* species using BlastN. A Newick file was downloaded from the Blast Tree View and plotted further using an interactive tree of life (http://itol.embl.de/upload.cgi). Different parameters were adjusted according to the visualization requirements and were exported [29]. The α-β hydrolase gene was amplified using a gene-specific primer that was designed by the net primer (Premier Biosoft): α-β hydrolase MK-FP: ATGGCTAAAGAAATGTTTGTGC and MK-RP: CGCACTAACTACTACTTCTGGT. The polymerase chain reaction mixtures (50 μl) contained 10 μM of each primer, PCR Invitrogen Master Mix (PCR buffer, 5 U of Taq polymerase, 10 μM of BSA and 2 μl of DNA). The thermocycling conditions included a denaturation step at 94°C for 3 min, 34 amplification cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 1 min, and a final extension step for 8 min using an Eppendorf thermocycler (Eppendorf AG 22331). Electrophoresis was continued for 30 min at 100 V (Tarson electrophoresis unit). The size of the fragment was determined by comparing it with a 1 kb marker (NEB). The gene product was inserted into the pXcm vector using a ligation (Fermentas) enzyme.

The α-β hydrolase gene was released from the pXcm vector using Bam H1 and Hind III. Expression of α-β hydrolase in *E. coli* DH5α was achieved by subcloning it into pET-20. Transformation of recombinant DNA into *E.coli/DH5α*: pET-20b was carried out by standard methods [30]. Preliminary screening was performed based on the blue-white colonies on x-gal medium, followed by PCR amplification of the α-β hydrolase gene.

The recombinant bacterial strains were cultured overnight. The cells were harvested by centrifugation, and α-β hydrolase was recovered by sonication (10–15 min). Crude enzyme was electrophoresed by slope gel electrophoresis along with marker protein (SERVA) and then analysed. Next,

100 μl of crude enzyme was mixed with 1 ml of 30 μg/ml chloramphenicol, followed by incubation at 37°C for 48 hours. After the incubation, the metabolites were purified with twofold ethyl acetate and were evaporated under vacuum conditions. The extracted residues were dissolved in methanol to a volume of 2 ml and were stored at 4°C until GC-FID analysis. The extract was analysed in an Agilent gas chromatograph (Model 7820A Series USA) equipped with a flame ionization detector [31].

2.5. Docking with Ligands. The Crystal Structure of α-β hydrolase (PDB ID: 1I6W) was retrieved from the protein data bank, and the ligands were downloaded from PubChem (http://www.ncbi.nlm.nih.gov/pccompound) with a PubChem ID (Table 1). The ligands were retrieved from the PubChem (http://www.ncbi.nlm.nih.gov/pccompound) database based on a literature survey. These compounds were subjected to ligand preparation by the Ligprep wizard application of the Maestro 9.2. Corrections such as the addition of hydrogen, 2D to 3D conversion, corrected bond lengths and bond angles, low energy structure, stereochemistries, and ring conformation, followed by minimization and optimization in the optimized potential for the liquid simulation force field [32–34] were performed. One conformation for each ligand was generated with other parameters used as the default in Maestro 9.2. Protein-ligand binding sites were predicted by the core-attachment based method (COACH) (http://zhanglab.ccmb.med.umich.edu/COACH/) using the meta-server approach. Complementary ligand binding site predictions were achieved using two comparative methods, TM-SITE and S-SITE, which recognize ligand binding templates from the BioLiP protein function database by binding-specific substructure and sequence profile comparisons. Docking was performed using the Glide software package (http://www.schrodinger.com/), which searches for favourable interactions between one or more typically small ligand molecules and a larger receptor molecule, usually a protein. The retrieved structures were subjected to the removal of water up to 5-Å distances, assigning lone pair electron atoms using a protein preparation wizard. The receptor grid was set up and generated to specify the binding pocket where the ligand binds using the receptor grid generation panel. Molecular docking of the prepared protein and ligand was carried out using Glide.

3. Results and Discussion

Continuous usage and accumulation of pesticide in the agricultural field lead to the development of cross-resistance to antibiotics among soil bacteria. Plasmid DNA (pMK-07) from *Bacillus* sp. was sequenced and analysed using in silico tools, revealing that the plasmid DNA sequences and their relatedness lead to cross-resistance to pesticide and antibiotics. A triclosan-resistant bacterial population showing resistance to antimicrobial agents [35, 36] due to self-transmissible genes that can jump between plasmids and chromosomes [37, 38] and the accumulation of multidrug resistance genes in the soil bacterial community through horizontal gene transfer were common among pesticide degraders [39, 40]. Thus, these studies proved the phenomenon of cross-resistance in bacteria.

Table 1: List of ligands used for the docking analysis with α-β hydrolase.

Compounds	Molecular Weight	Pubchem ID
Chloramphenicol	323.132	5959
Streptomycin	581.574	19649
Cefotaxime	455.47	6540461
Ampicillin	349.41	6249
Tetracycline	444.435	54675776
Monocrotophos	223.16	5371562

Note. http://www.ncbi.nlm.nih.gov/pccompound.

3.1. Plasmid DNA Sequence. The sequencing of plasmid pMK-07 of *Bacillus* species isolated from pesticide-exposed agriculture soil revealed that the plasmid shares genes from 6 different strains of *Bacillus cereus* (MSX-A12, NC7401, AH187, MSX-D12, IS845/00, and H3081.97), *B. weihenstephanensis,* and *S. pneumoniae.* Phylogenetic and dendrogram analyses of pMK-07 revealed that the plasmid shares 100% sequence similarity with *Bacillus* species (Figure 1), and the sequence was deposited in GenBank (KY940428.1).

In total, 225 genes were annotated from the plasmid, among which 221 genes found hits in the nucleotide database and four genes did not have a matching sequence in the database. Based on gene ontology annotation, the genes from the plasmid were categorized into three domains: biological process, cellular component, and molecular function. Similar categorization was performed for a plasmid (pNUC and p11601MD) that includes cellular and molecular component genes of a clinical multidrug resistance in *S. typhimurium* and *Campylobacter jejuni* strain 11601MD [2, 3] (Table 2).

3.2. Biological and Molecular Function of Genes. The genes responsible for the biological process of the bacteria, including the genes for spore formation, germination, sporulation-specific N-acetylmuramoyl-L-alanine amidase, small, acid-soluble spore protein C_5, and germination protein-*Ger* (x) C family protein, were found. These genes enable the bacteria to withstand the adverse conditions. The genes that are essential for DNA recombination were also present in the plasmid: site-specific recombinase, resolvase family, Tn1546 resolvase recombination protein U, and integrase *(Bacillus cereus)* [4]. Thus, the plasmid underwent random recombination with different strains of *Bacillus* sp. The presence of genes such as thetraG/traD family, Flp pilus assembly protein, and *cpa*B determines the horizontal gene transfer through conjugation [41, 42]. The presence of the ars R regulatory element makes the bacteria sense the presence of metal ions in the surroundings [43] and develop tolerance against the metal ions. The gene *rep X* present in the plasmid is known for plasmid DNA replication. The presence of the IS3 and IS605 transposase families allows the DNA-mediated recombination and insertion of random sequences in the bacterial genome and extrachromosomal plasmid DNA. The presence of RNA-mediated DNA polymerase (reverse transcriptase)

Table 2: Various genes present in the pMK-07 plasmid.

ORF	Sequence description	Gene length (bp)
AP007210.1_9	transposase	699
AP007210.1_11	group II intron reverse transcriptase maturase	1233
AP007210.1_14	SAM-dependent methyltransferase	1092
AP007210.1_18	chitin-binding protein	1275
AP007210.1_20	F0F1 ATP synthase subunit alpha	420
AP007210.1_21	acid-soluble spore C5	216
AP007210.1_22	chemotaxis protein	819
AP007210.1_23	Bacillolysin precursor	1671
AP007210.1_28	peptidase S8	762
AP007210.1_29	precorrin-3B C(17)-methyltransferase	468
AP007210.1_32	conserved hypothetical protein	144
AP007210.1_33	integrase	951
AP007210.1_37	nucleotidyltransferase	378
AP007210.1_38	cytotoxin	264
AP007210.1_40	DNA-binding protein	414
AP007210.1_41	nucleotidyltransferase	387
AP007210.1_43	thiamine biosynthesis	849
AP007210.1_50	DNA polymerase III subunit beta	1113
AP007210.1_56	membrane protein	285
AP007210.1_59	MULTISPECIES: membrane	264
AP007210.1_63	S1 RNA binding domain	984
AP007210.1_64	inosine-uridine preferring nucleoside hydrolase family	951
AP007210.1_65	reverse transcriptase	1647
AP007210.1_66	conjugation family	3501
AP007210.1_67	conserved hypothetical plasmid	387
AP007210.1_68	conserved hypothetical plasmid	1287
AP007210.1_69	Plasmid replication repX	1308
AP007210.1_70	conserved hypothetical protein	351
AP007210.1_72	IS605 family	1113
AP007210.1_78	IS605 family transposase	1335
AP007210.1_79	surface layer	1278
AP007210.1_81	integrase core domain	786
AP007210.1_82	DNA-binding	522
AP007210.1_84	Flp pilus assembly	861
AP007210.1_85	SAF domain family	852
AP007210.1_86	type II secretion system	1425
AP007210.1_87	membrane	933
AP007210.1_88	conserved hypothetical protein	867
AP007210.1_90	conserved domain	204
AP007210.1_91	conserved domain	207
AP007210.1_92	IS605 family transposase	1119
AP007210.1_95	membrane protein	624
AP007210.1_98	sortase	702
AP007210.1_99	LPXTG-motif cell wall anchor domain	528
AP007210.1_100	ATP-binding protein	189
AP007210.1_103	conserved hypothetical protein	252
AP007210.1_104	cell division	165
AP007210.1_106	recombination U	126
AP007210.1_113	transposase for insertion sequence element D	1275
AP007210.1_115	transposon resolvase	561
AP007210.1_116	S-layer homology domain ribonuclease	3396
AP007210.1_117	barnase inhibitor	276

Table 2: Continued.

ORF	Sequence description	Gene length (bp)
AP007210.1_119	putative membrane protein	198
AP007210.1_124	MULTISPECIES: ATPase	924
AP007210.1_127	prgI family	351
AP007210.1_130	reverse transcriptase	1833
AP007210.1_132	Reticulocyte binding	3981
AP007210.1_133	M23 M37 family	2208
AP007210.1_139	CAAX amino protease	705
AP007210.1_141	penicillin-binding partial	414
AP007210.1_142	membrane protein	1146
AP007210.1_143	thiol reductase thioredoxin	483
AP007210.1_147	family transcriptional regulator	279
AP007210.1_148	transcriptional regulator	327
AP007210.1_149	integrase recombinase	1056
AP007210.1_150	Transposase (plasmid)	165
P007210.1_151	conserved domain	513
AP007210.1_152	transcriptional regulator	255
AP007210.1_153	conserved domain	156
AP007210.1_155	type VII secretion	1233
AP007210.1_156	SMI1 KNR4 family	450
AP007210.1_157	lumazine binding domain	381
AP007210.1_160	transcriptional family	300
AP007210.1_163	transposase, partial	630
AP007210.1_164	transposon resolvase	576
AP007210.1_165	family transcriptional regulator	846
AP007210.1_166	transposon resolvase	495
AP007210.1_168	N-acetylmuramoyl-L-alanine amidase	699
AP007210.1_169	phosphoglycerate mutase	570
AP007210.1_170	IS21 family	1254
AP007210.1_171	ATPase AAA	759
AP007210.1_172	Two-component response regulator	864
AP007210.1_173	glyoxalase family	354
AP007210.1_174	tn3 transposase DDE domain	3054
AP007210.1_175	site-specific recombinase	627
AP007210.1_177	XRE family transcriptional regulator	231
AP007210.1_178	alpha beta hydrolase	732
AP007210.1_180	cardiolipin synthetase	1494
AP007210.1_181	membrane yetF	549
AP007210.1_183	lipo	486
AP007210.1_184	stage V sporulation AC	477
AP007210.1_185	stage V sporulation AD	1017
AP007210.1_186	stage V sporulation AE	351
AP007210.1_187	NADH dehydrogenase	207
AP007210.1_189	ATP-dependent Clp protease proteolytic subunit	582
AP007210.1_190	resolvase	552
AP007210.1_191	spore germination C	1134
AP007210.1_192	spore germination	657
AP007210.1_193	spore germination family	1536
AP007210.1_194	phospholipase D competence helix-hairpin-helix domain	222
AP007210.1_195	transposase	1500

Table 2: Continued.

ORF	Sequence description	Gene length (bp)
AP007210.1_196	transposase, partial	1695
AP007210.1_197	transposase for transposon	741
AP007210.1_198	transcriptional regulator	357
AP007210.1_199	S-(hydroxymethyl)glutathione dehydrogenase-like	1116
AP007210.1_200	S-glutathione hydrolase	834
AP007210.1_201	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	273
AP007210.1_202	Glutamate--cysteine ligase	2268
AP007210.1_204	spore germination XA	696
AP007210.1_205	germination %2C Ger(x)C family	1155
AP007210.1_206	Spore germination	1161
AP007210.1_207	cell surface	15033
AP007210.1_209	Isochorismatase	531
AP007210.1_211	Two-component response regulator	681
AP007210.1_212	two-component sensor histidine kinase	1848
AP007210.1_214	conserved domain	1011
AP007210.1_215	inosine-uridine preferring nucleoside hydrolase family	333
AP007210.1_217	cell surface	3711
AP007210.1_218	cell surface	6834
AP007210.1_219	RNA-binding Hfq	186
AP007210.1_220	family transcriptional regulator	294
AP007210.1_221	2-oxoglutarate dehydrogenase	429
AP007210.1_225	DNA topoisomerase I	2664

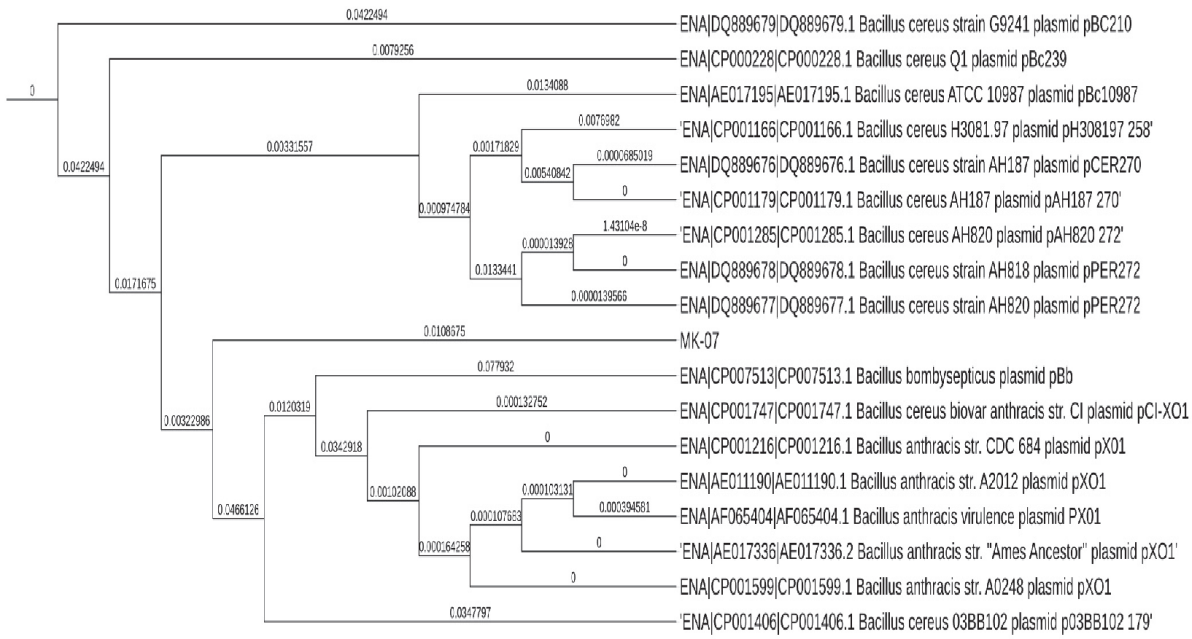


Figure 1: Homology cladogram of the plasmid DNA (sequence similarity of pMK-07 with *Bacillus cereus*).

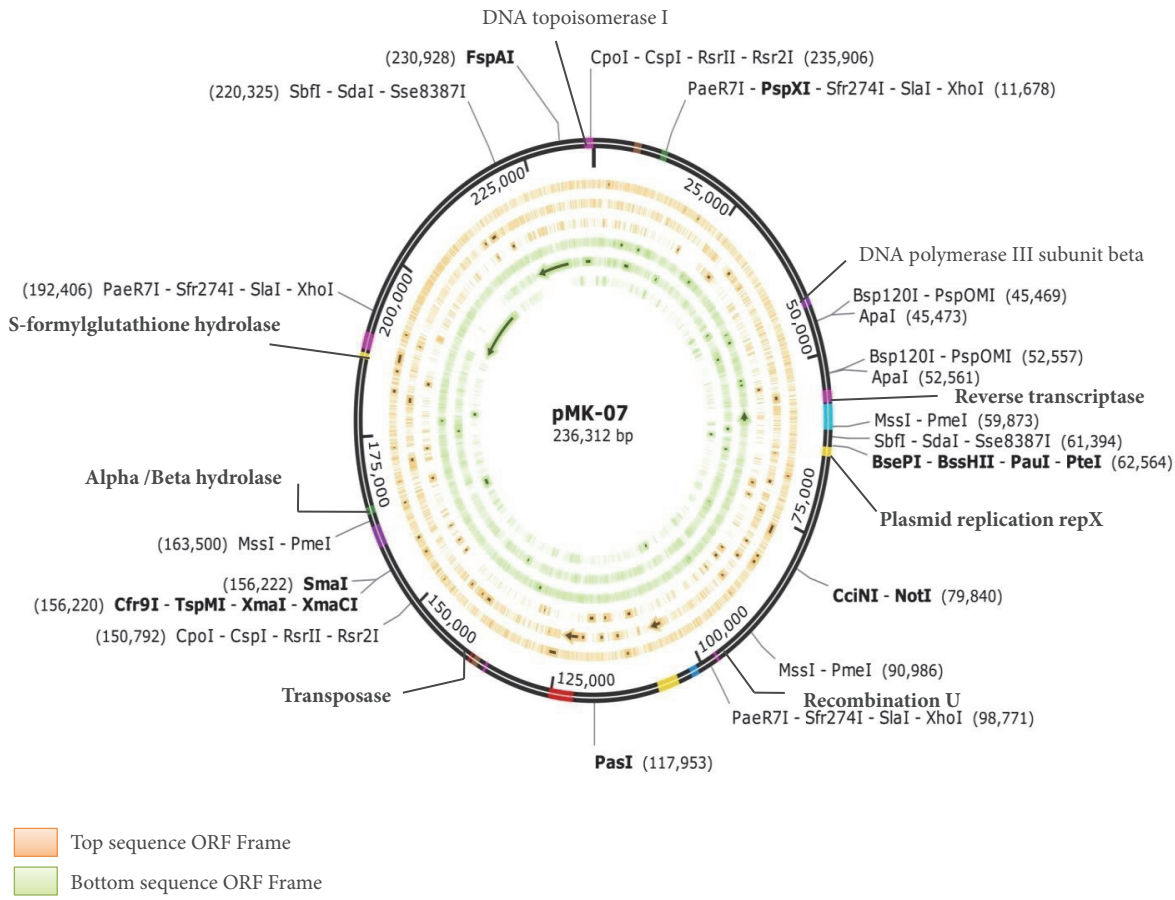


Figure 2: Gene map of plasmid DNA (pMK07) (distribution of α-β hydrolase, DNA topoisomerase, DNA polymerase III subunit beta, reverse transcriptase, plasmid replication rep X, recombination U, transposase, and S-formylglutathione hydrolase).

genes indicated a history of involvement of viral transduction in the route of de novo plasmid generation.

DNA sequence analysis of the plasmid (pMK-07) DNA revealed the genes harboured in the novel de novo plasmid pMK-07 (Figure 2). Previous studies have shown that α-β hydrolase can hydrolyse a wide range of pesticides [44], and glutathione S-transferases (GSTs) were found to hydrolyse DDT [45, 46], organochlorine, and organophosphorus insecticides [47, 48]. Hydrolases and hypothetical protein existence in the plasmid suggest that it was a degradative plasmid, especially pesticides [49].

3.3. Cellular Components. It was observed that approximately 10% of genes are present in the plasmid codes for membrane components. The bacteria possess an LPXTG anchoring domain and sortase enzyme genes, whose coexistence affirms that the bacteria carrying this pMK-07 plasmid might also be pathogenic [50].

3.4. Evolution of Newer Characters. The increased uses of pesticides in the agricultural field serve as the selection pressure for the evolution of soil microbial flora. The bacteria in the soil tend to develop tolerance by acquiring new genes or plasmids from other bacterial sources by either vertical or horizontal gene transfer. Surprisingly, plasmids carry all the essential genes required for survival under adverse or stressed conditions, a finding that has been confirmed in *C. jejuni* and *E. coli* [51]. A similar observation was noted among plant pathogenic Gram-negative bacteria carrying genes essential for their infection in plants [52]. The results from our study agree with those in previous studies.

3.5. Cloning of the α-β Hydrolase Gene. The α-β hydrolase gene (700 bp) was cloned into pXcm and was confirmed for their presence by running it on a 1.0% agarose gel. The expression of the gene for the α-β hydrolase enzyme was also verified by SDS-PAGE, with the protein size corresponding to 45 kDa (Figure 3). The α-β hydrolase gene from the pXcm vector was then excised and subcloned into pET-20b. After transformation, the bacterial cells were screened on LB agar medium supplemented with ampicillin, IPTG, and X-gal. Plates showing white colonies (transformants; pET-α-β hydrolase plasmid) were picked and processed for further use.

3.6. Nonspecific Degradation of Chloramphenicol. It was predicted that α-β hydrolase could degrade chloramphenicol by nonspecific cleavage and break the C=C bond in the ring

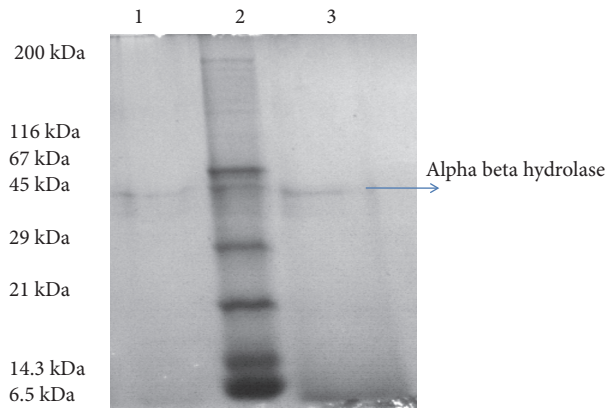


Figure 3: Characterization of α-β hydrolase SDS-PAGE gels (lane-1: XL1 blue MRF':pET α-β hydrolase gene expression; lane-2: SERVA unstained SDS-PAGE protein marker; lane-3: *Bacillus* sp. expression enzyme).

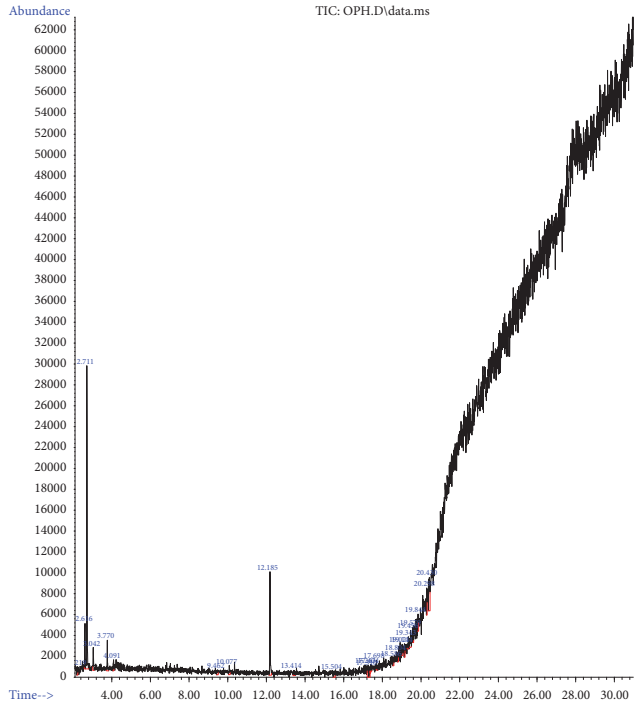


Figure 4: Nonspecific degradation of chloramphenicol by α-β hydrolase analysed by GC-MS (nonspecific degradation of antibiotics with α-β hydrolase analysed by GC-MS).

structure. Similar phenomena were observed in our study, when the cell lysate was mixed with 30 μg/ml of chloramphenicol, the lysate degraded the antibiotic, an observation that was proven through GC-MS analysis (Figure 4). GC-MS analysis revealed the breakdown compounds present in the pET α-β hydrolase-treated sample (methane, oxybis dichloro, phenol, indole-2-one) (Figure 5). Based on previous works on the characterization of the catabolic ability of α-β hydrolase, the side chain of the nucleophilic amino acid residue of the enzyme attacks the electropositive carbon atom of the substrate [53–55]. The findings of the present study suggest that this bacterial strain, *Bacillus* sp. MK-07, which survived all the sublethal concentration of pesticides, potentially has the cross-resistance property to degrade the antibiotic chloramphenicol. The cross-resistance mechanisms may be due to ribosomal gene alteration to evolve cross-resistance [7].

3.7. Protein-Ligand Binding Site Prediction. The α-β hydrolase ligand binding sites were predicted by COACH. The number of templates as the Cluster size was 69, the confidence score (c-score) was 0.96, and the binding residues were VAL9,

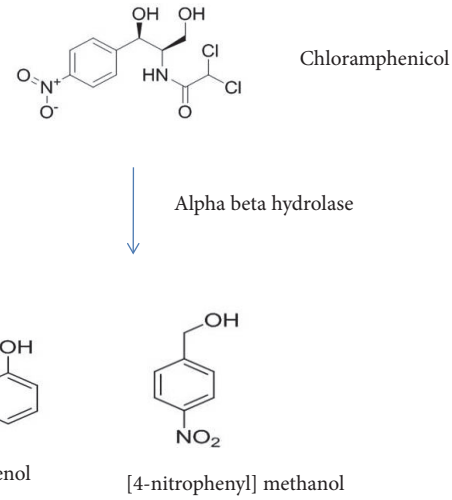


Figure 5: Pathway depicting chloramphenicol metabolism by α-β hydrolase (nonspecific degradation of chloramphenicol produces 4-nitrophenyl methanol and phenol as intermediates).

Table 3: Docking score of α-β hydrolase with various ligands.

Ligands/Pesticide	Gscore Kcal/mol	Number of Hydrogen Bonds	Amino Acid Interacting with Ligand
Chloramphenicol	−3.793	4	Gly11, Ile12, Ser 77
Streptomycin	−2.865	3	Gly13, Ile12
Cefotaxime	−5.885	2	Asn18, Ser 77
Ampicillin	−4.316	2	Asn18, Ser 77
Tetracycline	−3.972	1	Ile12
Monocrotophos	−4.464	2	Ile12, Ser 77

HIS 10, VAL 74, ALA 75, HIS 76, ASP 103, ASP 133, and VAL 154 (Table 3).

3.7.1. Protein-Ligand Interaction. α-β hydrolase (PDB Id: 1I6W) was docked with antibiotics (chloramphenicol, streptomycin, cefotaxime, ampicillin, and tetracycline) and the pesticide monocrotophos using Glide Maestro 9.2. Identification of the best-fit antibiotic was performed based on the G-score and number of hydrogen bonds involved. A similar study showed that a sublethal concentration of herbicides would result in the development of multidrug resistance among soil bacteria [56]. Because of the toxicity of the pesticide, the bacteria develop resistance, which allows them to adapt to such components [57].

The strong interaction of α-β hydrolase with chloramphenicol showed a Glide score of −3.793 Kcal/mol. Chloramphenicol interacts with ILE 12, GLY 11, and SER 77 with distances of 2.214 Å and 2.176 Å, 2.255 Å, and 2.430 Å, respectively, at the active site of the enzyme (Figure 6. I.a). In the surface view of the α-β hydrolase-chloramphenicol complex, chloramphenicol is highlighted with green (Figure 6. I.b). In the 2D interaction of the α-β hydrolase-chloramphenicol complex, the purple dotted line represents the hydrogen bond with the side chain (Figure 6. I.c and Table 3).

The surface view of α-β hydrolase with streptomycin showed a Glide score of −2.865 Kcal/mol and interaction of streptomycin with ILE 12, GLY 13, and HIS 76 at the active site (Figure 6. II.a). In the surface view of the α-β hydrolase-streptomycin complex, streptomycin is highlighted in green (Figure 6. II.b). In the 2D interaction of the α-β hydrolase-streptomycin complex, the purple dotted line represents the hydrogen bond with the side chain (Figure 6. II.c and Table 3).

The interaction formed between α-β hydrolase and cefotaxime showed a Glide score of −5.885 Kcal/mol. This interaction of cefotaxime at the active site of the enzyme with ASN 18 and with SER 77 revealed distances of 2.077 Å and 2.022 Å, respectively (Figure 6. III.a). In the surface view of the interaction of the α-β hydrolase-cefotaxime complex, cefotaxime is highlighted in green (Figure 6. III.b). In the 2D interaction of the α-β hydrolase-cefotaxime complex, the purple dotted line represents the hydrogen bond with the side chain (Figure 6. III.c and Table 3).

The interaction formed between α-β hydrolase and ampicillin showed a Glide score of −4.316 Kcal/mol. This interaction involves two hydroxyl bonds between the hydrogen atom of ampicillin with ASN 18 and an oxygen atom of ampicillin with SER 77 with a distance of 1.987 Å and 2.181 Å (Figure 6. IV.a). In the surface view of the α-β hydrolase-ampicillin complex, ampicillin is highlighted in green (Figure 6. IV.b). In the 2D interaction of the α-β hydrolase-ampicillin complex, the purple dotted line represents the hydrogen bond with the side chain (Figure 6. IV.c and Table 3).

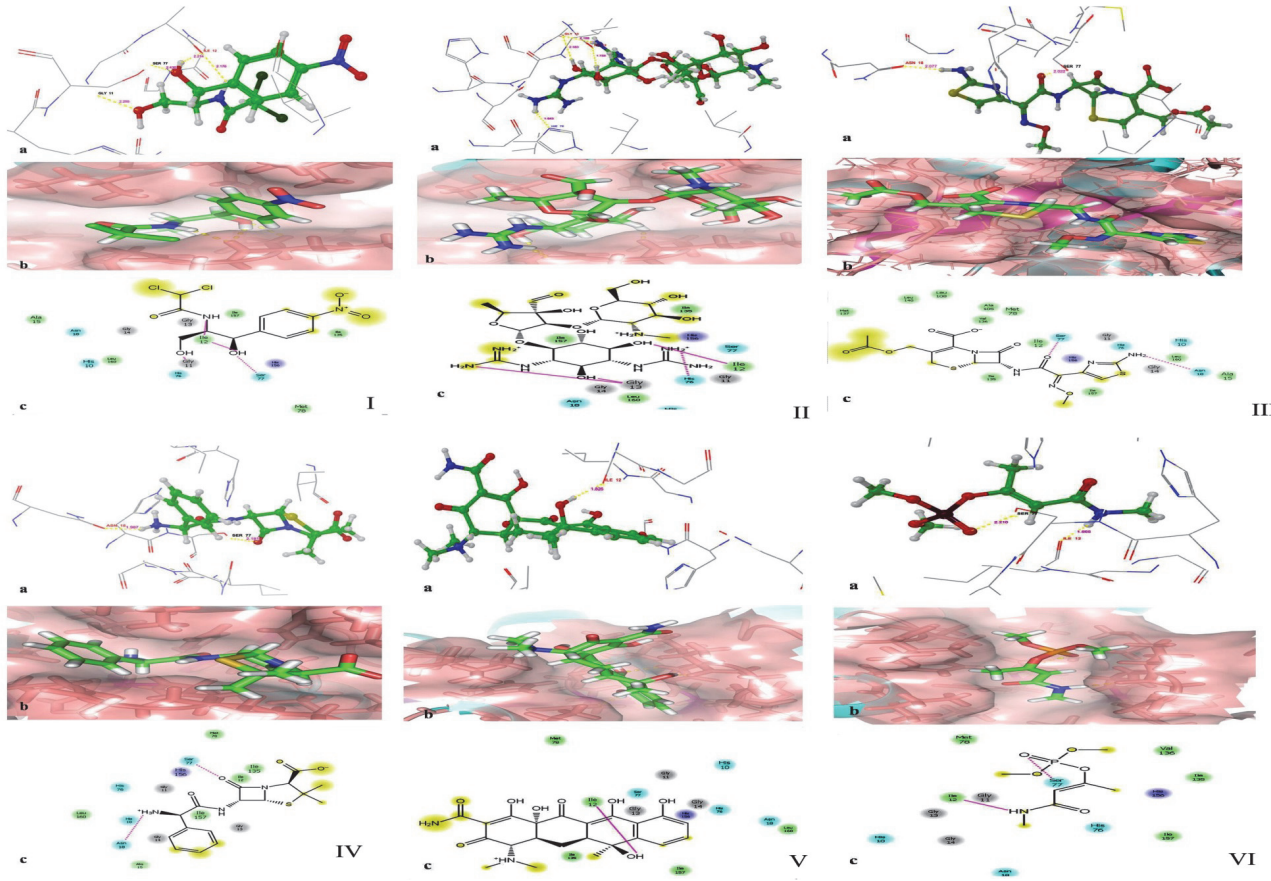


Figure 6: Docking of α-β hydrolase with antibiotics. Interaction of GLY 11 and SER 77 with the OH group. Similarly, ILE 12 binds to NH and OH; II: interaction of ILE 12 with the OH group. HIE 76 binds to NH, and GLY 13 binds to both the NH and OH groups; III: binding interaction of ASN 18 with the OH group and SER 77 with the NH group; IV: interaction of ASN 18 with the NH_3 group. SER 77 binds to the OH group; V: docking interaction of ILE 12 binding to the OH group; VI: docking interaction of ILE 12 binding to the NH group. SER 77 interacts with the OH group.

The interaction formed between α-β hydrolase and tetracycline showed a Glide score of −3.972 Kcal/mol. This interaction involved ILE 12 hydroxyl bonds between tetracycline and ILE 12 with a distance of 1.826 Å (Figure 6. V.a). In the surface view of the α-β hydrolase-tetracycline complex, tetracycline is highlighted in green (Figure 6. V.b). In the 2D interaction of the α-β hydrolase-tetracycline complex, the purple dotted line represents the hydrogen bond with the side chain (Figure 6. V.c and Table 3).

The interaction of α-β hydrolase with monocrotophos showed a Glide score of −4.464 Kcal/mol. The monocrotophos interacts with ILE 12 and with SER 77 with a distance of 1.865 Å and 2.210 Å at the active site of the enzyme (Figure 6. VI.a). In the surface view of the α-β hydrolase-monocrotophos complex, monocrotophos is highlighted in green (Figure 6. VI.b). In the 2D interaction of the α-β hydrolase-monocrotophos complex, the pink dotted line represents the hydrogen bond with the side chain (Figure 6 VI.c and Table 3). Among these antibiotics, based on the docking scores, it can be concluded that all five antibiotics can be degraded through nonspecific cleavage by α-β hydrolase.

A similar observation in the present study has proven that the hydrolase enzymes could bind with chloramphenicol and hydrolyse it into a nontoxic substance [58, 59]. Excessive pesticide usage resulted in the accumulation of pesticide residues in crops, soils, and the biosphere, creating ecological stress [60, 61].

4. Conclusion

The present work focuses on the pesticide-degrading bacteria isolated from an agricultural field that develop cross-resistance to antibiotics. This cross-resistance is developed through catabolic gene clusters present in an extra chromosomal plasmid. It can be concluded from the current study that existence of pesticide-resistant plasmids among soil bacteria can also confer cross-resistance to antibiotics through natural selection exerted by pesticide accumulation in the agriculture field. The enzymes that degrade pesticides can also catabolize the antibiotics by inducing changes in the gene or protein structure through induced mutations. Hence, an alternate way to control pests may

pave the way for limiting the emergence of multidrug resistance.

Conflicts of Interest

All the authors declare no conflicts of interest.

Acknowledgments

The authors acknowledge the Periyar University, Salem, Tamil Nadu, India, for a University Research Fellowship and DST-FIST Grant no. SR/FST/LSI-640/2015(c). The authors would also like to thank SERB-DST GoI vide Grant no. YSS/2014/000590 for providing financial support. The authors would also like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this Research Group (RGP-271).

References

[1] Y. Wang, "Spatial distribution of high copy number plasmids in bacteria," *Plasmid*, vol. 91, pp. 2–8, 2017.

[2] M. Oliva, R. Monno, P. D'Addabbo et al., "A novel group of IncQ1 plasmids conferring multidrug resistance," *Plasmid*, vol. 89, pp. 22–26, 2017.

[3] M. D. Crespo, E. Altermann, J. Olson, W. G. Miller, K. Chandrashekhar, and S. Kathariou, "Novel plasmid conferring kanamycin and tetracycline resistance in the turkey-derived Campylobacter jejuni strain 11601MD," *Plasmid*, vol. 86, pp. 32–37, 2016.

[4] K. Smalla, S. Jechalke, and E. M. Top, "Plasmid detection, characterization and ecology," *Microbiology Spectrum*, vol. 3, no. 1, pp. 1–21, 2015.

[5] R. Anjum and N. Krakat, "A review: improper antibiotic utilization evokes the dissemination of resistances in biotic environments - a high risk of health hazards," *Pharmaceutica Analytica Acta*, vol. 6, no. 12, 2015.

[6] S. Shafiani and A. Malik, "Tolerance of pesticides and antibiotic resistance in bacteria isolated from wastewater-irrigated soil," *World Journal of Microbiology and Biotechnology*, vol. 19, no. 9, pp. 897–901, 2003.

[7] J. Bergman, "Does the acquisition of antibiotic and pesticide resistance provide evidence for evolution?" *Journal of Creation*, vol. 1, no. 17, pp. 26–32, 2003.

[8] R. Kirubakaran, A. Murugan, P. Chinnathambi, and J. A. Parray, "Influence of residual pesticide on plant growth promoting bacteria isolated from agriculture field," *Journal of Basic Applied Plant Sciences*, vol. 1, no. 2, p. 110, 2017.

[9] J. Davison, "Genetic exchange between bacteria in the environment," *Plasmid*, vol. 42, no. 2, pp. 73–91, 1999.

[10] C. Smillie, M. P. Garcillan-Barcia, M. V. Francia, E. P. Rocha, and F. de la Cruz, "Mobility of Plasmids," *Microbiology and Molecular Biology Reviews*, vol. 74, no. 3, pp. 434–452, 2010.

[11] S. J. Sørensen, M. Bailey, L. H. Hansen, N. Kroer, and S. Wuertz, "Studying plasmid horizontal transfer in situ: A critical review," *Nature Reviews Microbiology*, vol. 3, no. 9, pp. 700–710, 2005.

[12] L. Yao, X. Jia, J. Zhao et al., "Degradation of the herbicide dicamba by two sphingomonads via different O-demethylation mechanisms," *International Biodeterioration & Biodegradation*, vol. 104, pp. 324–332, 2015.

[13] S. Xie, R. Wan, Z. Wang, and Q. Wang, "Atrazine biodegradation by Arthrobacter strain DAT1: Effect of glucose supplementation and change of the soil microbial community," *Environmental Science and Pollution Research*, vol. 20, no. 6, pp. 4078–4084, 2013.

[14] B. Ramakrishnan, M. Megharaj, K. Venkateswarlu, N. Sethunathan, and R. Naidu, "Mixtures of environmental pollutants: Effects on microorganisms and their activities in soils," *Reviews of Environmental Contamination and Toxicology*, vol. 211, pp. 63–120, 2011.

[15] R. Capita, F. Riesco-Peláez, A. Alonso-Hernando, and C. Alonso-Calleja, "Exposure of Escherichia coli ATCC 12806 to Sublethal Concentrations of Food-Grade Biocides Influences Its Ability To Form Biofilm, Resistance to Antimicrobials, and Ultrastructure," *Applied and Environmental Microbiology*, vol. 4, no. 80, pp. 1268–1280, 2014.

[16] R. J. W. Lambert, "Comparative analysis of antibiotic and antimicrobial biocide susceptibility data in clinical isolates of methicillin-sensitive Staphylococcus aureus, methicillin-resistant," *Journal of Applied Microbiology*, vol. 97, no. 4, pp. 699–711, 2004.

[17] H. T. Tu, F. Silvestre, N. T. Phuong, and P. Kestemont, "Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses," *Environmental Toxicology and Chemistry*, vol. 29, no. 4, pp. 929–938, 2010.

[18] M. Heinzel, "Phenomena of biocide resistance in microorganisms," *International Biodeterioration & Biodegradation*, vol. 41, no. 3-4, pp. 225–234, 1998.

[19] W. Zhang, F. Jiang, and J. Ou, "Global pesticide consumption and pollution: with China as a focus," *International Academy of Ecology Environment Sciences*, vol. 1, no. 2, pp. 125–144, 2011.

[20] C. Chen, C. Huang, M. M. Wu et al., "Multidrug Resistance 1 Gene Variants, Pesticide Exposure, and Increased Risk of DNA Damage," *BioMed Research International*, vol. 2014, Article ID 965729, 9 pages, 2014.

[21] D. R. Livingstone, "The fate of organic xenobiotics in aquatic ecosystems: Quantitative and qualitative differences in biotransformation by invertebrates and fish," *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, vol. 120, no. 1, pp. 43–49, 1998.

[22] K. Rangasamy, M. Athiappan, N. Devarajan et al., "Pesticide degrading natural multidrug resistance bacterial flora," *Microbial Pathogenesis*, vol. 114, pp. 304–310, 2018.

[23] H. C. Bimboim and J. Doly, "A rapid alkaline extraction procedure for screening recombinant plasmid DNA," *Nucleic Acids Research*, vol. 7, no. 6, pp. 1513–1523, 1979.

[24] L. Zhang, S. Wong, O. King, and F. P. Roth, "Predicting co-complexed protein pairs using genomic and proteomic data integration," *BMC Bioinformatics*, vol. 5, no. 59, pp. 1–9, 2004.

[25] A. Carattoli, E. Zankari, A. Garciá-Fernández et al., "In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 7, pp. 3895–3903, 2014.

[26] A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: a flexible trimmer for Illumina sequence data," *Bioinformatics*, vol. 30, no. 15, pp. 2114–2120, 2014.

[27] S. Nurk, A. Bankevich, D. Antipov et al., "Assembling single-cell genomes and mini-metagenomes from chimeric MDA products," *Journal of Computational Biology*, vol. 20, no. 10, pp. 714–737, 2013.

[28] R. K. Aziz, D. Bartels, A. Best et al., "The RAST Server: rapid annotations using subsystems technology," *BMC Genomics*, vol. 9, no. 75, pp. 1–15, 2008.

[29] C. J. Brown, D. Sen, H. Yano et al., "Diverse broad-host-range plasmids from freshwater carry few accessory genes," *Applied and Environmental Microbiology*, vol. 79, no. 24, pp. 7684–7695, 2013.

[30] M. R. Green and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, vol. 1, Cold Spring Harbor Laboratory Press, 4 edition, 2012.

[31] S. Z. Hussaini, M. Shaker, and M. A. Iqbal, "Isolation of bacterial for degradation of selected pesticides," *Advanced Biomedical Research*, vol. 4, no. 3, pp. 82–85, 2013.

[32] W. L. Jorgensen and J. Tirado-Rives, "The OPLS [optimized potentials for liquid simulations] potential functions for proteins, energy minimizations for crystals of cyclic peptides and crambin," *Journal of the American Chemical Society*, vol. 110, no. 6, pp. 1657–1666, 1988.

[33] W. L. Jorgensen, D. S. Maxwell, and J. Tirado-Rives, "Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids," *Journal of the American Chemical Society*, vol. 118, no. 45, pp. 11225–11236, 1996.

[34] D. Shivakumar, J. Williams, Y. Wu, W. Damm, J. Shelley, and W. Sherman, "Prediction of absolute solvation free energies using molecular dynamics free energy perturbation and the opls force field," *Journal of Chemical Theory and Computation*, vol. 6, no. 5, pp. 1509–1519, 2010.

[35] M. A. Webber, R. N. Whitehead, M. Mount, N. J. Loman, M. J. Pallen, and L. J. V. Piddock, "Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure," *Journal of Antimicrobial Chemotherapy*, vol. 70, no. 8, Article ID dkv109, pp. 2241–2248, 2015.

[36] S. P. Bernier and M. G. Surette, "Concentration-dependent activity of antibiotics in natural environments," *Frontier Microbiology*, vol. 4, no. 20, pp. 1–14, 2013.

[37] L. Walkup, "Junk DNA: Evolutionary Discards or Gods Tools?" *Journal of Creation*, vol. 14, no. 2, pp. 18–30, 2000.

[38] E. Top, I. De Smet, W. Verstraete, R. Dijkmans, and M. Mergeay, "Exogenous isolation of mobilizing plasmids from polluted soils and sludges," *Applied and Environmental Microbiology*, vol. 60, no. 3, pp. 831–839, 1994.

[39] B. G. Spratt, "Resistance to antibiotics mediated by target alterations," *Science*, vol. 264, no. 5157, pp. 388–393, 1994.

[40] C. M. Thomas and K. M. Nielsen, "Mechanisms of, and barriers to, horizontal gene transfer between bacteria," *Nature Reviews Microbiology*, vol. 3, no. 9, pp. 711–721, 2005.

[41] H. Heuer and K. Smalla, "Plasmids foster diversification and adaptation of bacterial populations in soil," *FEMS Microbiology Reviews*, vol. 36, no. 6, pp. 1083–1104, 2012.

[42] S. P. Djordjevic, H. W. Stokes, and P. R. Chowdhury, "Mobile elements, zoonotic pathogens and commensal bacteria: Conduits for the delivery of resistance genes into humans, production animals and soil microbiota," *Frontiers in Microbiology*, vol. 4, no. 86, pp. 1–12, 2013.

[43] C.-H. Gao, M. Yang, and Z.-G. He, "Characterization of a novel ArsR-like regulator encoded by Rv2034 in mycobacterium tuberculosis," *PLoS ONE*, vol. 7, no. 4, Article ID e36255, 2012.

[44] I. R. Montella, R. Schama, and D. Valle, "The classification of esterases: an important gene family involved in insecticide resistance - A review," *Memórias do Instituto Oswaldo Cruz*, vol. 107, no. 4, pp. 437–449, 2012.

[45] A. H. Tang and C. P. Tu, "Biochemical characterization of Drosophila glutathione S-transferases D1 and D21," *The Journal of Biological Chemistry*, vol. 269, no. 45, pp. 27876–27884, 1994.

[46] W. Y. Low, S. C. Feil, H. L. Ng et al., "Recognition and detoxification of the insecticide DDT by drosophila melanogaster glutathione S-transferase D1," *Journal of Molecular Biology*, vol. 399, no. 3, pp. 358–366, 2010.

[47] A. J. Ketterman, C. Saisawang, and J. Wongsantichon, "Insect glutathione transferases," *Drug Metabolism Reviews*, vol. 43, no. 2, pp. 253–265, 2011.

[48] R. H. Ffrench-Constant, "The molecular genetics of insecticide resistance," *Genetics*, vol. 194, no. 4, pp. 807–815, 2013.

[49] J. G. Vontas, G. J. Small, and J. Hemingway, "Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens," *Biochemical Journal*, vol. 357, no. 1, pp. 65–72, 2001.

[50] J. W. Pridgeon and P. H. Klesius, "Major bacterial diseases in aquaculture and their vaccine development," *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, vol. 7, no. 48, pp. 1–16, 2012.

[51] S. Zhao, G. H. Tyson, Y. Chen et al., "Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in *Campylobacter* spp," *Applied and Environmental Microbiology*, vol. 82, no. 2, pp. 459–466, 2016.

[52] N. Eltlbany, Z.-Z. Prokscha, M. P. Castañeda-Ojeda et al., "A new bacterial disease on Mandevilla sanderi, caused by Pseudomonas savastanoi: Lessons learned for bacterial diversity studies," *Applied and Environmental Microbiology*, vol. 78, no. 23, pp. 8492–8497, 2012.

[53] E. Diaz and K. N. Timmis, "Identification of functional residues in a 2-hydroxymuconic semialdehyde hydrolase. A new member of the α/β hydrolase-fold family of enzymes which cleaves carbon-carbon bonds," *The Journal of Biological Chemistry*, vol. 270, no. 11, pp. 6403–6411, 1995.

[54] F. Fischer, S. Künne, and S. Fetzner, "Bacterial 2,4-dioxygenases: New members of the α/β hydrolase-fold superfamily of enzymes functionally related to serine hydrolases," *Journal of Bacteriology*, vol. 181, no. 18, pp. 5725–5733, 1999.

[55] D. Liu, L. Wang, H. Zhai et al., "A Novel α/β-Hydrolase Gene IbMas Enhances Salt Tolerance in Transgenic Sweetpotato," *PLoS ONE*, vol. 9, no. 12, p. e115128, 2014.

[56] B. Kurenbach, D. Marjoshi, C. F. Amábile-Cuevas et al., "Sublethal exposure to commercial formulations of the herbicides dicamba, 2,4-dichlorophenoxyacetic acid, and Glyphosate cause changes in antibiotic susceptibility in Escherichia coli and Salmonella enterica serovar Typhimurium," *mBio*, vol. 6, no. 2, Article ID e00009-15, 2015.

[57] D. H. Nies and S. Silver, "Ion efflux systems involved in bacterial metal resistances," *Journal of Industrial Microbiology and Biotechnology*, vol. 14, no. 2, pp. 186–199, 1995.

[58] N. Neti and V. Zakkula, "In silico structural analysis and binding of organophosphorus hydrolase of Kocuria sp with chlorpyrifos," *International Journal of Computer Applications*, vol. 66, no. 5, pp. 0975–8887, 2013.

[59] K. Rangasamy, M. Athiappan, N. Devarajan, and J. A. Parray, "Emergence of multi drug resistance among soil bacteria exposing to insecticides," *Microbial Pathogenesis*, vol. 105, pp. 153–165, 2017.

[60] C.-L. Qiao, Y.-C. Yan, H. Y. Shang, X. T. Zhou, and Y. Zhang, "Biodegradation of pesticides by immobilized recombinant *Escherichia coli*," *Bulletin of Environmental Contamination and Toxicology*, vol. 71, no. 2, pp. 370–374, 2003.

[61] J. S. Seo, Y. S. Keum, R. M. Harada, and Q. X. Li, "Isolation and Characterization of Bacteria Capable of Degrading Polycyclic Aromatic Hydrocarbons (PAHs) and Organophosphorus Pesticides from PAH-Contaminated Soil in Hilo, Hawaii," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 14, pp. 5383–5389, 2007.

Hindawi
BioMed Research International
Volume 2018, Article ID 2813737, 8 pages
https://doi.org/10.1155/2018/2813737

Research Article

Mannose-Binding Lectin: A Potential Therapeutic Candidate against *Candida* Infection

Noha M. Hammad,[1] **Nissreen E. El Badawy**,[1] **Hamed A. Ghramh**,[2,3] **and Laila M. Al Kady**[1]

[1]*Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Zagazig, Egypt*
[2]*Research Center for Advanced Materials Science (RCAMS), King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*
[3]*Department of Biology, Faculty of Science, King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*

Correspondence should be addressed to Noha M. Hammad; nmhammad@medicine.zu.edu.eg

Received 8 November 2017; Accepted 15 March 2018; Published 2 May 2018

Academic Editor: Mohamed E. Osman

Mannose-binding lectin (MBL) is one of the key players in the innate immune system. It has the ability to identify a broad range of pathogens based on recognition of carbohydrate repeats displayed on microbial surfaces. Since mannans make about 40% of the total polysaccharide content of cell wall of *Candida* species (spp.) and MBL is capable of high-affinity binding to the mannan fraction of their cell wall component, this study has investigated the direct influence of MBL on *Candida in vitro*. *Candida (C.) albicans* and *C. glabrata* were *in vitro* exposed to different doses of recombinant human MBL for various time points to assess MBL influence on the production of hyphae and on the yeast forms. Moreover, the direct effect of MBL on the growth of *C. albicans* was measured by a cell proliferation assay. MBL induced agglutination of yeast forms as well as hyphal forms of *Candida* spp. and significantly reduced proliferation of *C. albicans in vitro*. MBL can be used as a potential antifungal candidate against *Candida* infection.

1. Introduction

Mannose-binding lectin (MBL), synthesized in the liver, is a member of a family of proteins called collectins, which is composed of collagenous domains linked to lectin domains. MBL is a large macromolecule that has a bouquet-like structure. The polypeptide chain of secreted MBL is 228 amino-acid long, not including the 20-residue signal peptide. The basic structural subunit of MBL is a homotrimer of MBL polypeptides, twisted in a triple helix. Each single polypeptide chain has four domains: (1) a 21-amino-acid N-terminal cysteine-rich (containing 3 cysteines) region involved in oligomerization by the formation of intra- and intersubunit disulphide bonds, (2) a 59-amino-acid collagen-like domain consisting of 20 tandem repeats of Glycine-Xaa-Yaa, where Xaa-Yaa indicate any amino acid (except repeat 8, which consists of only Glycine-Glutamine) that account for the long stalk of the molecule, (3) a 30-amino-acid α-helical, hydrophobic coiled-coil neck domain, which is crucial for initiating the oligomerization, and (4) a 188-amino-acid C-terminal carbohydrate recognition domain [1, 2]. Binding of MBL to pathogenic organisms leads to a change in the conformation of MBL multimer with subsequent activation of MBL-associated serine proteases (MASPs) and eventually initiation of complement lectin pathway [3].

Over the past four decades, many functions of MBL have been revealed. It is clear that MBL plays roles in complement activation, promotion of complement-independent opsonophagocytosis, modulation of inflammation, and recognition of altered self-structures and apoptotic cell clearance [4]. Low concentration of MBL is associated with increased susceptibility to infections [5].

The cell wall of *Candida* species (spp.) contains mannoproteins that display mannan in a variety of linkages. The mannan fraction of the cell wall is important for adhesion, cell wall integrity, and immune recognition and comprises up to 40% of the cell wall dry weight [6–8]. All of the major cell wall carbohydrate components of fungal walls serve as pathogen-associated molecular patterns, which are recognized by the innate immune system through pattern recognition receptors

on the surface of immune effector cells [9]. Toll-like receptors and C-type lectin receptors, which are pattern recognition receptors, recognize molecular patterns on *Candida* cell wall, resulting in phagocytosis and killing of the invading fungus [10]. One of the C-type lectin receptors is the soluble, opsonic, multimeric MBL. MBL is able to trigger the complement cascade by recognizing and binding to carbohydrate moieties on the surface of microorganisms in general [11]. Besides, MBL binds with high-affinity to *Candida* spp. [12].

Different *Candida* spp., *C. albicans* and *C. dubliniensis*, are associated with generation of hyphae. Hypha plays vital role in tissue invasion. Morphogenesis observed in *C. albicans* is induced by variations in temperature and pH and presence of serum [13]. Accordingly, most of pathogenicity of *C. albicans* owes to this transition [14]. On the contrary, under most conditions, *C. glabrata* exists only as yeast cells [15].

The aim of this study is to assess the *in vitro* capacity of MBL against yeast cells of *C. albicans* and *C. glabrata* as well as hyphal forms of *C. albicans*.

2. Materials and Methods

This study was conducted at Immunology Research Laboratory in Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. This study was carried out in the period of July 2016 to August 2017.

2.1. Ethical Approval. This study was approved by the institutional review board, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Candida Species. Candida spp. were isolated from vaginal cultures of patients suffering from recurrent vulvovaginal candidiasis and presumptively identified by subculture on chromogenic agar medium *(CHROMagar™ Candida; Paris, France)*. Cultures were examined under light microscope to show the budding yeast cells with or without pseudohyphae, blastospores, and germ tubes [6, 16]. In addition, biochemical tests were studied using *Hi-Candida™ API* identification kit *(Biomereux, France)*. The isolated spp., *Candida. (C.) albicans and C. glabrata*, were preserved on deep SDA and stored at 4°C for subsequent *in vitro* experiments.

Candida spp. maintained on deep SDA were allowed to grow overnight in brain-heart infusion broth *(Brain-Heart Infusion; Oxoid, UK)* at 37°C before use. These conditions allow *Candida* to grow as a >95% pure yeast phase population [17]. Immediately before each experiment, *Candida* spp., harvested by centrifugation, were washed 3 times with phosphate buffer saline (PBS) *(phosphate buffer saline 10x; Electron Microscopy Science, USA)* and resuspended to the appropriate concentration in PBS containing 1 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$ (PBS^{++}). Experiments were done in duplicate and repeated at least three times.

2.2. Influence of MBL on Hyphae Production and on Yeast Forms. Candida spp. (1×10^6 cells/mL counted by hemocytometer) was incubated with MBL *(Recombinant Human MBL, R&D Systems, Minneapolis, USA)* in 100 μL of PBS^{++}, with and without 10% heat-inactivated fetal bovine serum (FBS) *(Fetal Bovine Serum; Sigma-Aldrich)* for *C. albicans* and with only 10% heat-inactivated FBS for *C. glabrata,* in 96-well microtiter plate at 37°C in 5% CO_2 atmosphere *(Heraeus Hera cell)*.

Culturing at 37°C (5% CO_2) in serum-rich medium stimulated *C. albicans* yeasts to germinate. The light microscopic analysis was used to assess MBL influence on yeast cells and on hyphae production. Human recombinant MBL was supplied lyophilized (50 μg). It was reconstituted according to manufacturer's company protocol at a concentration of 100 μg/mL with 0.5 mL sterile PBS. To determine the time-dependent factor for MBL influence, *Candida* yeast cells were incubated with MBL for various time points (30 min, 1 h, and 3 h). Besides, to determine the dose-dependent factor for MBL, *Candida* yeasts were incubated with various doses of MBL (0, 5, and 10 μg/mL), since the average normal human MBL serum level is around 5 μg/mL [17, 18].

2.3. Influence of MBL on the Growth of Candida albicans. Freshly grown *C. albicans* yeasts (2×10^7 cells/mL) were incubated with MBL in a final volume of 100 μL of PBS^{++} in Eppendorf tubes at 37°C in 5% CO_2 atmosphere. To determine the time-dependent factor for MBL influence on *C. albicans* growth, cells were exposed to MBL for various time points (15 min and 30 min). Besides, to determine the dose-dependent factor for MBL, *C. albicans* yeasts were exposed to various doses of MBL (0, 5, and 10 μg/mL) [17].

2.3.1. Assessment of Candida albicans Growth. The yeast cells were washed with 1 mL of PBS followed by resuspension in RPMI (Roswell Park Memorial Institute) medium *(RPMI-1640; Sigma-Aldrich)* at 1×10^6 cells/mL. A final volume of 100 μL was transferred to a 96-well microtiter plate and incubated at 37°C in 5% CO_2 atmosphere for 3 and 6 h. Wells containing RPMI medium alone and those containing 1×10^6 cells/mL without previous exposure to MBL served as negative and positive control, respectively [17].

By using XTT-based cell proliferation assay *(XTT assay; Sigma-Aldrich)*, the growth of *C. albicans* was evaluated after 3 and 6 h of incubation. The XTT-based assay is a spectrophotometric method for estimating cell number based on the mitochondrial dehydrogenase activity in living cells. The key component is the sodium salt of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt). The mitochondrial dehydrogenases of viable cells reduce the tetrazolium ring of XTT, yielding an orange formazan derivative, which is water soluble. The absorbance of the resulting orange solution is measured spectrophotometrically [19]. XTT kit was supplied lyophilized (5 mg). It was reconstituted according to manufacturer's company protocol at a concentration of 1 mg/mL with 5 mL of sterile PBS. Warming the solution in a 56°C water bath helped to dissolve the dye. 20 μL of the XTT solution was added to each well. The microtiter plates were then incubated in the dark at 37°C in 5% CO_2 atmosphere for 2 h. A colorimetric change was then measured at a wavelength of 450 nm by using ELISA reader *(Stat Fax® 303 Plus)*.

2.4. Statistical Analysis. Quantitative data were represented as mean value ± 1 standard deviation (SD). *F* test was used for calculation of the mean difference between different groups. Least significance difference (LSD) test was used for multiple comparisons. Paired *t*-test was used for calculation of the mean difference within the same group at different time points. All analyses were 2-tailed. Results were considered statistically significant when *p* (probability) values were equal to or less than 0.05 at confidence interval (CI) 95%. All analyses were performed using Statistical Package for the Social Sciences software *(SPSS version 20, Inc., Chicago, IL, USA.)*.

3. Results

3.1. Influence of MBL on Hyphae Production and on Yeast Forms. Light microscopic analysis revealed that MBL had no influence on the germination of *C. albicans* yeasts. *C. albicans* yeasts in the presence or absence of MBL germinated and formed hyphae within 3 h incubation. However, in the presence of MBL, agglutination of hyphae was observed. Moreover, in the presence of MBL, agglutination was observed when (1) yeast phase of *C. albicans* was incubated with 10% FBS, (2) yeast forms of *C. albicans* were incubated without FBS, and (3) yeast forms *C. glabrata* were incubated with 10% FBS. When the dose-dependent factor was evaluated, agglutination of both hyphae and yeast cells was markedly observed with 10 μg/mL MBL compared to 5 μg/mL MBL. When the time-dependent factor was evaluated, agglutination of hyphae markedly increased as more hyphae were induced and elongated over different time points: 30 min, 1 h, and 3 h incubation. Besides, agglutination of yeast cells was markedly increased over different time points, 30 min, 1 h, and 3 h incubation, as shown in Figures 1–4.

3.2. Influence of MBL on the Growth of Candida albicans after 3 h Incubation. When the direct influence of MBL on the growth of *C. albicans* by XTT assay was estimated after 3 h incubation period, there was statistically significant reduction in growth when *C. albicans* was exposed to 5 and 10 μg/mL MBL for 15 min and to 5 μg/mL MBL for 30 min compared to positive control (MBL = 0 μg/mL) ($p = 0.013$, $p = 0.005$, and $p = 0.004$, resp.). However, there was no reduction in growth after exposure to 10 μg/mL MBL for 30 min compared to positive control ($p = 0.700$). When the dose-dependent factor was evaluated, there was no statistically significant difference in growth after exposure to 5 and 10 μg/mL MBL for 15 min ($p = 0.631$). However, after 30 min exposure, there was statistically significant reduction in growth with 5 μg/mL MBL compared to 10 μg/mL MBL ($p = 0.002$). When the time-dependent factor was evaluated, there was no statistically significant difference in growth between 15 and 30 min exposure to both 5 and 10 μg/mL MBL ($p = 0.264$ and $p = 0.123$, resp.) as demonstrated by Table 1 and Figure 5.

3.3. Influence of MBL on the Growth of Candida albicans after 6 h Incubation. When the direct influence of MBL on the growth of *C. albicans* by XTT assay was estimated after 6 h incubation period, there was statistically significant reduction in growth when *C. albicans* was exposed to 5 and 10 μg/mL MBL for 15 min and to 5 μg/mL MBL for 30 min compared to positive control (MBL = 0 μg/mL) ($p = 0.007$, $p = 0.036$ and $p = 0.006$, resp.). However, the reduction of growth was not statistically significant when *C. albicans* was exposed to 10 μg/mL MBL for 30 min compared to positive control ($p = 0.934$). When the dose-dependent factor was evaluated, there was statistically significant reduction in growth after 30 min exposure to 5 μg/mL MBL compared to 10 μg/mL MBL; however, such reduction was not statistically significant after 15 min exposure, ($p = 0.007$, $p = 0.346$, resp.). When the time-dependent factor was evaluated, there was no statistically significant difference in growth between 15 and 30 min exposure to both 5 and 10 μg/mL MBL ($p = 0.922$ and $p = 0.198$, resp.) as demonstrated by Table 2 and Figure 5.

4. Discussion

For more than a decade, the potential of MBL as a therapeutic agent has been proposed [20]. MBL replacement therapy was previously investigated in patients with recurrent erythema multiform and severe cystic fibrosis via infusion of fresh frozen plasma containing MBL, resulting in clinical improvement of the patients [21, 22]. In this context, the present study has investigated the direct influence of MBL on *C. albicans* and *C. glabrata in vitro*.

This study demonstrates that MBL recognized *Candida* spp. and induced their agglutination in yeast form as well as upon induction of hyphae. Nevertheless, MBL was unable to inhibit the transition of *C. albicans* from the yeast phase to hyphal phase, since *C. glabrata* grows strictly in pure yeast form under most conditions [15]. Besides, MBL induced agglutination was both time- and dose-dependent. A possible explanation is that long incubations were associated with more hyphal outgrowth with a subsequent increase in MBL-ligand expression on the growing hyphae. Similarly, budding of the yeast cells could enhance the chance of MBL-ligand expression.

These findings agree with the study of Lillegard and his colleagues who found that MBL binds extensively to both hyphae and budding yeast cells [8]. Moreover, the previous report of Ip and Lau suggested that steric hindrance by MBL can prevent the spread of the virulent forms of *Candida* by blocking the receptors. However, Ip and Lau found that MBL induced agglutination only with hyphal forms of *C. albicans* but not with yeast forms [17].

The present study has reported significant suppression in the growth of *C. albicans* during an incubation period for up to 6 hours. This significant suppression was observed with exposure to 5 μg/mL MBL for 15 and 30 min, while 10 μg/mL MBL induced significant suppression with exposure for 15 min but not 30 min. MBL induced growth suppression was not time-dependent; however, it was dose-dependent only with 30 min exposure. The 5 μg/mL MBL is quite close to the average normal human MBL serum level [18]. Therefore, it seems that 5 μg/mL MBL was optimum and more effective than 10 μg/mL MBL. Moreover, MBL-ligand binding, like any macromolecules, is governed by the dissociation constant,

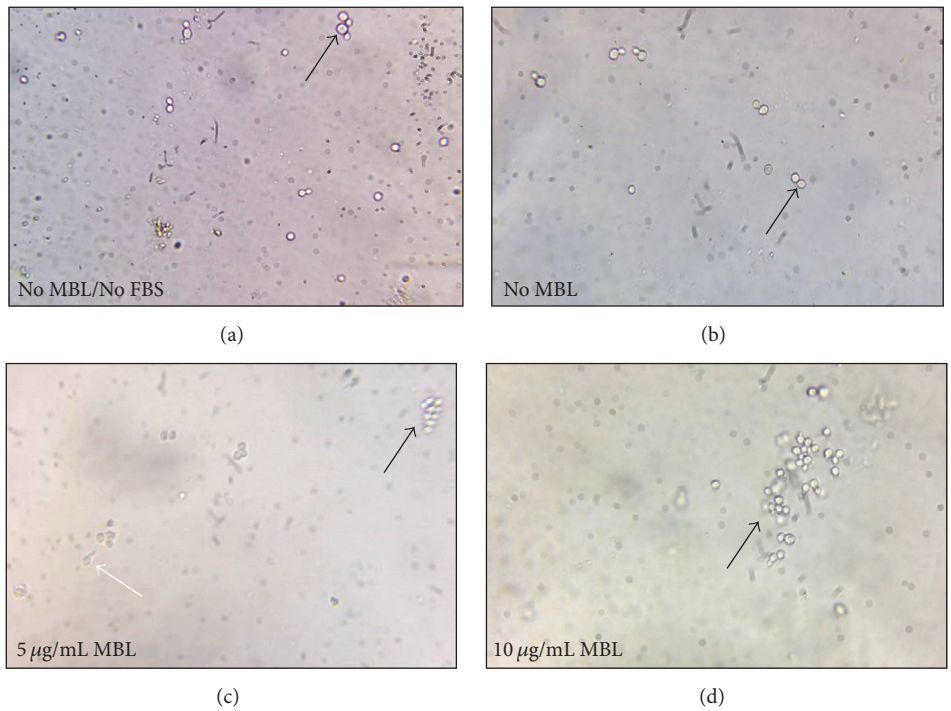


Figure 1: *MBL induced agglutination of Candida albicans after 30 min incubation*. (a) In absence of MBL and FBS, no agglutination is observed with yeast form of *C. albicans (Black arrow)*. (b) In absence of MBL and presence of 10% FBS, no agglutination is observed with yeast phase of *C. albicans (Black arrow)*. (c) and (d) In presence of 5 and 10 μg/mL MBL with 10% FBS, agglutination of yeast phase of *C. albicans* is started to be observed *(Black arrow)*. Germination of hyphae starts to occur *(White arrow)*.

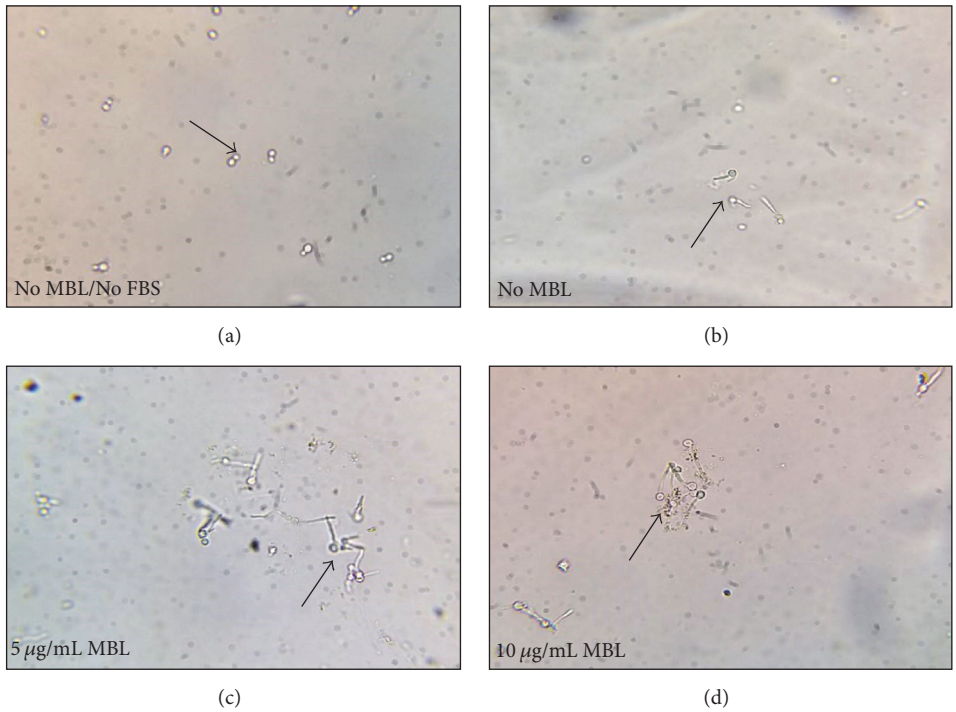


Figure 2: *MBL induced agglutination of Candida albicans after 1 h incubation*. (a) In absence of MBL and FBS, no agglutination is observed with yeast form of *C. albicans (Black arrow)*. (b) In absence of MBL and presence of 10% FBS, no agglutination only germination of hyphae is observed *(Black arrow)*. (c) and (d) In presence of 5 and 10 μg/mL MBL with 10% FBS, agglutination of germinated hyphae of *C. albicans* is started to be observed *(Black arrow)*.

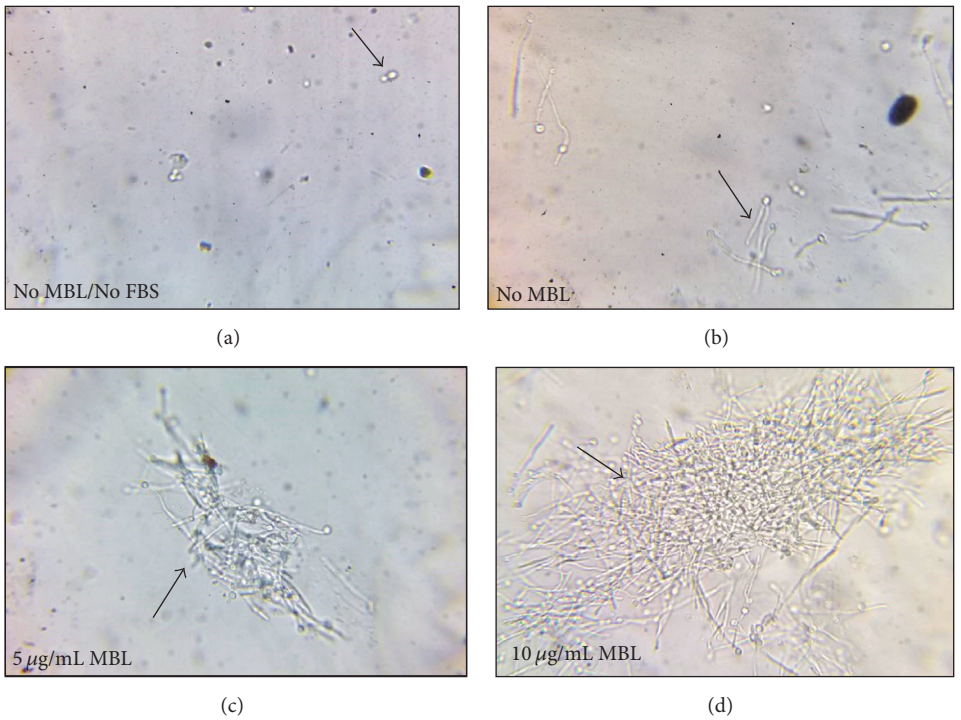


Figure 3: *MBL induced agglutination of Candida albicans after 3 h incubation.* (a) In absence of MBL and FBS, no agglutination is observed with yeast form of *C. albicans (Black arrow)*. (b) In absence of MBL and presence of 10% FBS, no agglutination only elongation of hyphae is observed *(Black arrow)*. (c) and (d) In presence of 5 and 10 μg/mL MBL with 10% FBS, increased agglutination and elongation of hyphae of *C. albicans* are observed *(Black arrow)* The effect is more potentiated with 10 μg/mL MBL when compared to 5 μg/mL.

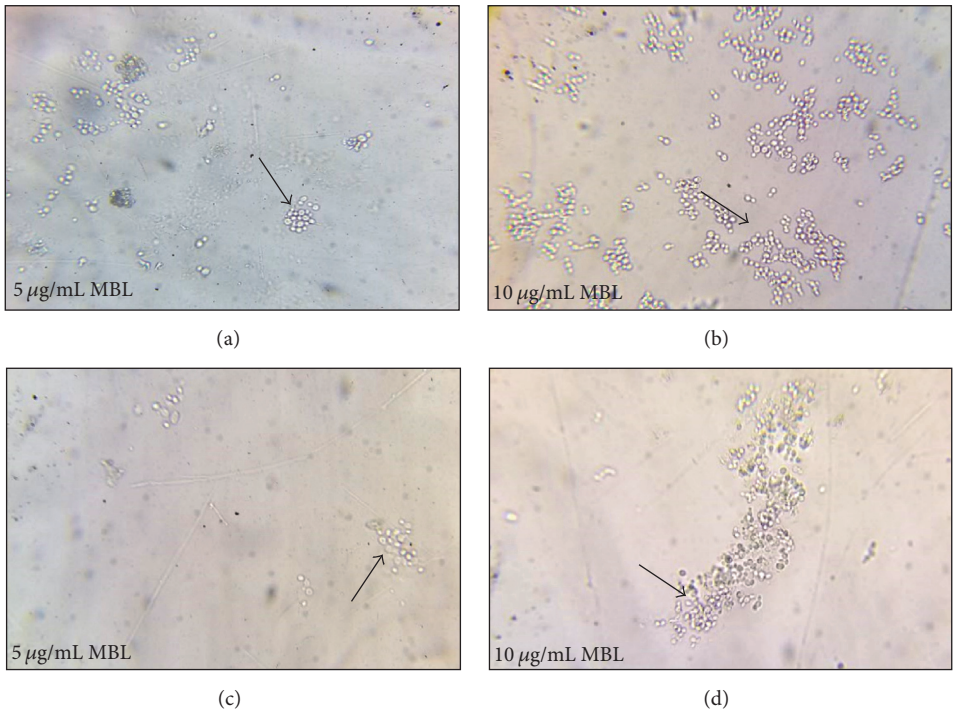


Figure 4: *MBL induced agglutination of yeast cells of Candida species after 3 h incubation.* (a) and (b) In presence of 5 and 10 μg/mL MBL without FBS, agglutination of *C. albicans* is observed. (c) and (d) The same effect of 5 and 10 μg/mL MBL is also observed with *C. glabrata*. The effect is more potentiated with 10 μg/mL MBL *(Black arrows)* when compared to 5 μg/mL MBL *(Black arrows)*.

Table 1: Direct influence of MBL on the growth of *Candida albicans* measured by XTT assay after 3 h incubation period.

MBL concentration	0 μg/mL	5 μg/mL	10 μg/mL	Test of significance	p value	LSD p value
15 min exposure to MBL						
Absorbance Mean ± SD	1.95 ± 0.11	1.37 ± 0.29	1.28 ± 0.38	*F test* **21.398**	<**0.001***	**0.013**^{**1} **0.005**^{**2} **0.631**^{**3}
30 min exposure to MBL						
Absorbance Mean ± SD	1.71 ± 0.23	1.10 ± 0.33	1.78 ± 0.15	*F test* **28.583**	<**0.001***	**0.004**^{**1} **0.700**^{**2} **0.002**^{**3}
Between different MBL exposure time points (15 and 30 min)						
Test of significance		Paired t-test				
		t = 1.370	**t = −2.128**			
p value		**0.264**^{**4}	**0.123**^{**5}			

*Significant difference; $^{**1}p$ value between 0 and 5 μg/mL MBL; $^{**2}p$ value between 0 and 10 μg/mL MBL; $^{**3}p$ value between 5 and 10 μg/mL MBL; $^{**4}p$ value between 15 and 30 min exposures to 5 μg/mL MBL; $^{**5}p$ value between 15 and 30 min exposures to 10 μg/mL MBL.

Table 2: Direct influence of MBL on the growth of *Candida albicans* measured by XTT assay after 6 h incubation period.

MBL concentration	0 μg/mL	5 μg/mL	10 μg/mL	Test of significance	p value	LSD p value
15 min exposure to MBL						
Absorbance Mean ± SD	2.51 ± 0.04	1.59 ± 0.37	1.86 ± 0.59	*F test* **18.197**	<**0.001***	**0.007**^{**1} **0.036**^{**2} **0.346**^{**3}
30 min exposure to MBL						
Absorbance Mean ± SD	2.29 ± 0.17	1.55 ± 0.47	2.27 ± 0.23	*F test* **29.076**	<**0.001***	**0.006**^{**1} **0.934**^{**2} **0.007**^{**3}
Between different MBL exposure time points (15 and 30 min)						
Test of significance		Paired t-test				
		t = 0.106	t **= −1.647**			
p value		**0.922**^{**4}	**0.198**^{**5}			

*Significant difference; $^{**1}p$ value between 0 and 5 μg/mL MBL; $^{**2}p$ value between 0 and 10 μg/mL MBL; $^{**3}p$ value between 5 and 10 μg/mL MBL; $^{**4}p$ value between 15 and 30 min exposures to 5 μg/mL MBL; $^{**5}p$ value between 15 and 30 min exposures to 10 μg/mL MBL.

and the affinity of binding is much influenced by hydrogen bonding, electrostatic interaction, and hydrophobicity of the cell surface clustering of the ligands. In addition, multivalent binding results in clustering of the receptors [23].

This work agrees with the study of Ip and Lau. They observed that MBL induced a significant reduction in the growth of *C. albicans* when added to heat-inactivated MBL-deficient serum, a situation in which complement activation would not occur. This suggested that MBL possesses an intrinsic mechanism through which it inhibits the fungal growth. However, the maximum inhibitory effect was observed with unheated MBL-deficient serum demonstrating the importance of lectin pathway in inhibiting the growth of *C. albicans* [17]. When mice, treated with intravenous MBL, were challenged with *C. albicans*, prolonged survival of mice was significantly observed [8]. It is also possible that MBL induced agglutination of yeast cells could limit the availability of the nutrients to them resulting in growth inhibition. Consistently, intravaginal administration of recombinant human MBL coupled to itraconazole in *MBL* gene knockout mice with *C. albicans* vaginitis resulted in 3-fold clearance of yeast compared to itraconazole alone [24]. Although its clinical efficacy has not been clearly evidenced, no side effects of exogenous MBL administration were identified, and some clinical benefits were apparent [25]. Therefore, the present study strongly recommends the potential usefulness of this approach and its extension to large-scale randomized clinical trials; hence, this would provide a solid evidence concerning

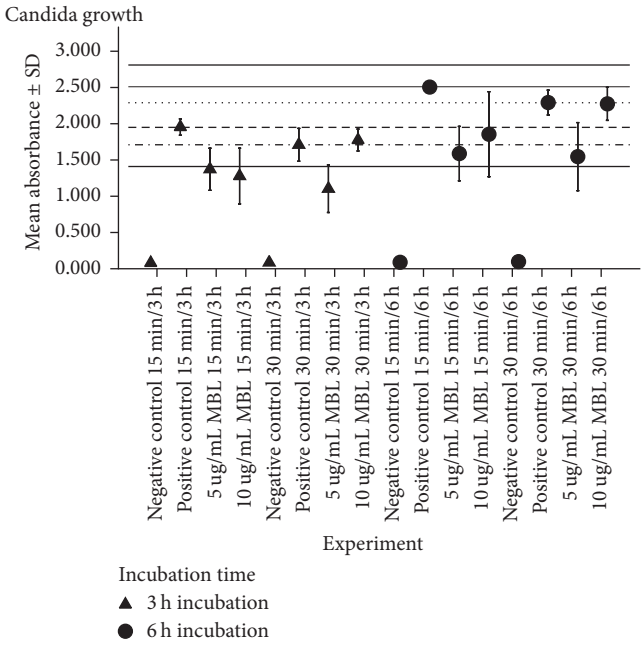


Figure 5: *Summary of direct influence of MBL on Candida albicans growth in vitro*. Dashed line (- - - - - - -) represents mean absorbance of positive control (1.95 ± 0.11) after 15 min exposure to 0 μg/mL MBL and 3 h incubation period. Dot-dash line (· – · – · – · – ·) represents mean absorbance of positive control (1.71 ± 0.23) after 30 min exposure to 0 μg/mL MBL and 3 h incubation period. Continuous line (—) represents mean absorbance of positive control (2.51 ± 0.04) after 15 min exposure to 0 μg/mL MBL and 6 h incubation period. Dotted line (. . . . . .) represents mean absorbance of positive control (2.29 ± 0.17) after 30 min exposure to 0 μg/mL MBL and 6 h incubation period. Data are plotted as mean absorbance ± SD of 3 separate experiments. Absorbance is at 450 nm.

the physiological significance of MBL against resistant or recurrent *Candida* infections, particularly in MBL-deficient subjects.

5. Conclusion

Recombinant human MBL can induce agglutination of *C. albicans* and *C. glabrata* yeast cells and hyphal forms of *C. albicans*. Moreover, it can significantly reduce the growth of *C. albicans in vitro*. These effects could nominate MBL as a potential therapeutic agent against *Candida* infection.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

The authors would like to express their gratitude to Research Center of Advanced Materials, King Khalid University, Saudi Arabia, for support. Also, the authors would like to acknowledge Scientific and Medical Research Center of Zagazig University "ZSMRC", Faculty of Medicine, for support.

References

[1] P. Garred, F. Larsen, J. Seyfarth, R. Fujita, and H. O. Madsen, "Mannose-binding lectin and its genetic variants," *Genes & Immunity*, vol. 7, no. 2, pp. 85–94, 2006.

[2] J. S. Presanis, M. Kojima, and R. B. Sim, "Biochemistry and genetics of mannan-binding lectin (MBL)," *Biochemical Society Transactions*, vol. 31, no. 4, pp. 748–752, 2003.

[3] D. L. Worthley, P. G. Bardy, D. L. Gordon, and C. G. Mullighan, "Mannose-binding lectin and maladies of the bowel and liver," *World Journal of Gastroenterology*, vol. 12, no. 40, pp. 6420–6428, 2006.

[4] Z.-Y. Wang, Z.-R. Sun, and L.-M. Zhang, "The relationship between serum mannose-binding lectin levels and acute ischemic stroke risk," *Neurochemical Research*, vol. 39, no. 2, pp. 248–253, 2014.

[5] E. Kiseljaković, S. Hasić, A. Valjevac et al., "Association of Mannose-Binding Lectin 2 (mbl2) gene heterogeneity and its serum concentration with osteoporosis in postmenopausal women," *Bosnian Journal of Basic Medical Sciences*, vol. 14, no. 1, pp. 9–25, 2014.

[6] T. I. El-Sayed, D. Atef, M. Amer, A. Mahdy, and G. Enan, "Molecular characterization and inhibition by natural agents of multidrug resistant Candida strains causing vaginal candidiasis," *Research Journal of Medical Sciences*, vol. 9, no. 1, pp. 1–7, 2015.

[7] G. Enan, T. I. EL-Sayed, D. Atef, M. Amer, and A. Mahdy, "Causal organisms, pathogenicity, laboratory diagnosis and treatment of candidiasis," *Research Journal of Applied Sciences* , vol. 10, no. 4, pp. 115–135, 2015.

[8] J. B. Lillegard, R. B. Sim, P. Thorkildson, M. A. Gates, and T. R. Kozel, "Recognition of Candida albicans by mannan-binding lectin in vitro and in vivo," *The Journal of Infectious Diseases*, vol. 193, no. 11, pp. 1589–1597, 2006.

[9] R. A. Hall, S. Bates, M. D. Lenardon et al., "The Mnn2 mannosyltransferase family modulates mannoprotein fibril length, immune recognition and virulence of *Candida albicans*," *PLoS Pathogens*, vol. 9, no. 4, Article ID e1003276, 2013.

[10] S. P. Smeekens, F. L. van de Veerdonk, B. J. Kullberg, and M. G. Netea, "Genetic susceptibility to Candida infections," *EMBO Molecular Medicine*, vol. 5, no. 6, pp. 805–813, 2013.

[11] B. Nedovic, B. Posteraro, E. Leoncini et al., "Mannose-binding lectin codon 54 gene polymorphism and vulvovaginal candidiasis: A systematic review and meta-analysis," *BioMed Research International*, vol. 2014, Article ID 738298, 7 pages, 2014.

[12] N. Brouwer, K. M. Dolman, M. van Houdt, M. Sta, D. Roos, and T. W. Kuijpers, "Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding," *The Journal of Immunology*, vol. 180, no. 6, pp. 4124–4132, 2008.

[13] J. F. Fisher, K. Kavanagh, J. D. Sobel, C. A. Kauffman, and C. A. Newman, "Candida urinary tract infection: Pathogenesis," *Clinical Infectious Diseases*, vol. 52, no. 6, pp. S437–S451, 2011.

[14] A. Cassone, "Vulvovaginal Candida albicans infections: Pathogenesis, immunity and vaccine prospects," *BJOG: An International Journal of Obstetrics & Gynaecology*, vol. 122, no. 6, pp. 785–794, 2015.

[15] L. Kasper, K. Seider, and B. Hube, "Intracellular survival of Candida glabrata in macrophages: Immune evasion and persistence," *FEMS Yeast Research*, vol. 15, no. 5, Article ID fov042, 2015.

[16] L. J. R. Milne and T. Mackie, *Mackie & McCartney Practical Medical Microbiology*, Charchil Livingstone, New York, NY, USA, 14th edition, 1998.

[17] W. K. Ip and Y. L. Lau, "Role of mannose-binding lectin in the innate defense against *Candida albicans*: enhancement of complement activation, but lack of opsonic function, in phagocytosis by human dendritic cells," *The Journal of Infectious Diseases*, vol. 190, no. 3, pp. 632–640, 2004.

[18] O. Babula, G. Lazdane, J. Kroica, W. J. Ledger, and S. S. Witkin, "Relation between recurrent vulvovaginal candidiasis, vaginal concentrations of mannose-binding lectin, and a mannose-binding lectin gene polymorphism in latvian women," *Clinical Infectious Diseases*, vol. 37, no. 5, pp. 733–737, 2003.

[19] N. W. Roehm, G. H. Rodgers, S. M. Hatfield, and A. L. Glasebrook, "An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT," *Journal of Immunological Methods*, vol. 142, no. 2, pp. 257–265, 1991.

[20] J. A. Summerfield, "Clinical potential of mannose-binding lectin-replacement therapy," *Biochemical Society Transactions*, vol. 31, no. 4, pp. 770–773, 2003.

[21] H. Valdimarsson, "Infusion of plasma-derived mannan-binding lectin (MBL) into MBL-deficient humans," *Biochemical Society Transactions*, vol. 31, no. 4, pp. 768-769, 2003.

[22] P. Garred, T. Pressler, S. Lanng et al., "Mannose-binding lectin (MBL) therapy in an MBL-deficient patient with severe cystic fibrosis lung disease," *Pediatric Pulmonology*, vol. 33, no. 3, pp. 201–207, 2002.

[23] T. K. Lindhorst, *Oligosaccharides and Glycoconjugates in Recognition Proesses. Carbohydrate-Modifying Biocatalysts*, Pan Stanford Publishing Pte. Ltd., Singapore, 2011.

[24] K. V. Clemons, M. Martinez, M. Axelsen, S. Thiel, and D. A. Stevens, "Efficacy of recombinant human mannose binding lectin alone and in combination with itraconazole against murine Candida albicans vaginitis," *Immunological Investigations*, vol. 40, no. 6, pp. 553–568, 2011.

[25] G. De Pascale, S. L. Cutuli, M. A. Pennisi, and M. Antonelli, "The role of mannose-binding lectin in severe sepsis and septic shock," *Mediators of Inflammation*, vol. 2013, Article ID 625803, 8 pages, 2013.

Hindawi
BioMed Research International
Volume 2018, Article ID 7648152, 8 pages
https://doi.org/10.1155/2018/7648152

Research Article

Mannose-Binding Lectin Gene Polymorphism and Its Association with Susceptibility to Recurrent Vulvovaginal Candidiasis

Noha M. Hammad,[1] **Nissreen E. El Badawy**,[1] **Ashraf M. Nasr**,[2] **Hamed A. Ghramh**,[3,4] **and Laila M. Al Kady**[1]

[1]*Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Zagazig, Egypt*
[2]*Department of Obstetrics and Gynecology, Faculty of Medicine, Zagazig University, Zagazig, Egypt*
[3]*Research Center for Advanced Materials Science (RCAMS), King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*
[4]*Department of Biology, Faculty of Science, King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*

Correspondence should be addressed to Noha M. Hammad; nmhammad@medicine.zu.edu.eg

Received 8 January 2018; Accepted 25 February 2018; Published 4 April 2018

Academic Editor: Mohamed E. Osman

Recurrent vulvovaginal candidiasis (RVVC) is a common illness influencing childbearing women worldwide. Most women suffering from RVVC develop infection without specified risk factors. Mannose-binding lectin (MBL) is an important component of innate immune defense against *Candida* infection. Innate immunity gene mutations and polymorphisms have been suggested to play a role in susceptibility to RVVC. This study aimed to investigate the association between *MBL 2* gene exon 1 codon 54 polymorphism and susceptibility to RVVC in childbearing women. Whole blood and serum samples were obtained from 59 RVVC cases and 59 controls. MBL serum level was measured by enzyme-linked immune-sorbent assay (ELISA). *MBL2* exon 1 codon 54 polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). It was shown that MBL serum level was nonsignificantly different between RVVC cases and controls. The risk of RVVC was 3 times higher in those carrying *MBL2* exon 1 codon 54 variant allele (B). It could be concluded that the carrying of *MBL2* exon 1 codon 54 variant allele (B) was shown to be a risk factor for RVVC in childbearing women.

1. Introduction

Vulvovaginal candidiasis (VVC) is the second most common vaginal infection after bacterial vaginosis [1]. It is diagnosed in up to 40% of women with vaginal complaints in the primary care setting [2–4]. Nevertheless, it was estimated that 3 out of 4 women were likely to endure at least one episode of VVC in their lifetime. Moreover, about 5–10% of those women were suffering from recurrent infections [5]. Recurrent VVC (RVVC) is a multifactorial illness with possible underlying risk factors such as diabetes mellitus, antibiotic use, or pregnancy, but most of women with RVVC develop infection without identifiable risk factors [4]. RVVC can be defined as four episodes or at least three episodes unrelated to antibiotic therapy and occurring within one year [6]. The question about what factors determine which women experience transition from infrequent VVC to RVVC has not been resolved yet [7, 8]. Therefore, different mutations and polymorphisms in innate immune genes can alter the vaginal mucosal defense mechanisms against *Candida* species [9].

Mannose-binding lectin (MBL) is an important component of the innate immune system. MBL is originally synthesized in the liver, circulates throughout the body, and has the ability to identify a broad range of pathogens [10]. MBL is capable of binding to mannan fraction of *Candida* cell wall, activating complement pathway, and hence, might reduce systemic infections by *Candida* spp. and in turn vaginal colonization [11]. Furthermore, macrophages and

dendritic cells express receptors that can recognize MBL, facilitating opsonization of microorganisms with bound MBL on their surface [12, 13].

Mannose-binding lectin is a large macromolecule that has a bouquet-like structure. The basic structural subunit of MBL is a homotrimer of MBL polypeptides, entwined in a triple helix. Each single polypeptide chain has four domains: (1) a 21-amino acid N-terminal cysteine-rich region involved in oligomerization, (2) a 59-amino acid collagen-like domain, (3) a 30-amino acid α-helical, hydrophobic coiled-coil neck domain, which is crucial for initiating the oligomerization, and (4) a 188-amino acid C-terminal carbohydrate recognition domain [14, 15].

Three common structural polymorphisms caused by single point mutations are found in *MBL2* gene and all are present on exon 1: allele "D" at codon 52 which is C to T nucleotide substitution, allele "B" at codon 54, and allele "C" at codon 57, and both are G to A nucleotide substitutions. All three mutations occur within the collagen domain changing the ability of MBL to oligomerize. The wild type is termed allele "A" while the structural variants B, C, and D are often gathered and referred to as zero "0" [16–18]. It has been proposed that polymorphisms in *MBL2* gene are involved in determining susceptibility to RVVC [6]. However, the role of *MBL* genes polymorphism remains controversial and has not been determined by all investigators yet [19]. A similar work was carried out [6, 16–19]. However, none of them discussed Egyptian patients. To fill this gap, this study would give the chance to investigate *MBL* codon 54 polymorphism among childbearing Egyptian women complaining of RVVC.

The aim of this study was to investigate the potential role of MBL serum level and *MBL2* gene exon 1 codon 54 polymorphism in determining susceptibility to RVVC in childbearing Egyptian women. Therefore, it could be possible to explore new potential therapeutic modalities for MBL-deficient women suffering from RVVC.

2. Materials and Methods

This study was conducted at Immunology Research and Molecular Biology Laboratories, Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Zagazig, which is located 100 kilometers northeast of Cairo, the capital of Egypt. This study was carried out in the period of December 2014 to May 2017.

2.1. Ethical Approval. This study was approved by the institutional review board, Faculty of Medicine, Zagazig University, Zagazig, Egypt. The study subjects were informed, preliminary, about the nature and the purpose of the study. The written informed consent was taken from all participants. The study subjects were not exposed to any harm or risk. Participants' data were confidential.

2.2. Subjects. The present study enrolled 59 childbearing women (age, 30–40 years). Three replicates of vaginal swabs were taken from women that were suffering from suspected VVC and were exposed to at least three episodes of VVC in the last 12 months. Control subjects were 59 healthy women (age, 30–40 years) without previous history of *Candida* infection or gynecologic complaints.

All RVVC cases and controls were seen at Zagazig University Hospitals and Qenayat Hospital Gynecology Outpatient Clinics, Qenayat, Egypt (neighbor to Zagazig City). RVVC cases and controls were matched for age, marital status, and socioeconomic status. The sociodemographic variables of the study subjects were collected by a questionnaire. The genital hygiene behavior of the study subjects was assessed by genital hygiene questionnaire. The exclusion criteria for this study were pregnancy, contraception, sexual activity and vaginal douching in the last week, steroid therapy for less than 3 months, diabetes, immunodeficiencies, and immunosuppressive therapy.

2.3. Vaginal Swabs and Identification of Candida Species. Vaginal swabs were obtained using sterile cotton-tipped plastic swabs and cultured onto Sabouraud Dextrose Agar Medium (Sabouraud Dextrose Agar, Oxoid, UK) and then subcultured on chromogenic agar medium (CHROMagar™ Candida, Paris, France) for presumptive identification of species. The vaginal samples were also tested for *Trichomonas vaginalis* by wet mount and for bacterial vaginosis by Amsel criteria [20]. Cultures were examined under light microscope to show the budding yeast cells with or without pseudohyphae, blastospores, and germ tubes [3, 21]. In addition, biochemical tests were studied using Hi-Candida™ API identification kit (Biomereux, France).

2.4. Blood Sampling. Three mL of peripheral blood was obtained from each study participant by venous puncture, collected and divided into 2 (13 × 75 mm) tubes, EDTA containing tube and Wassermann's tube, and stored at −20°C until used. Blood collected in EDTA tube was subjected to subsequent direct blood PCR. The blood collected in Wassermann's tube was centrifuged at 3000 RPM for 10 min and the supernatant serum was collected for subsequent determination of MBL serum level.

2.5. Quantitation of Serum MBL. MBL serum level was measured by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's company protocol (Quantikine® ELISA Human MBL; R&D Systems, Minneapolis, USA).

2.6. Determination of MBL2 Gene Polymorphism. *MBL2* gene exon 1 was amplified by direct blood PCR (Phusion™ Blood Direct PCR Master Mix; Thermo Scientific™, USA). PCR reactions were performed in 50 μL final volume using 0.5 μM of each primer (forward: 5′-TAGGAC AGAGGGCATGCTC-3; reverse: 5′-CAGGCAGTT TCCTCTGGAAGG-3′ PCR product size 349 bp), 25 μL of 2X Phusion Blood Direct PCR Master Mix, and 5 μL whole blood. After an initial lysis of cells for 5 min at 98°C, PCR reactions were run for 40 cycles including 5 s at 98°C, 30 s at 58°C, and 30 s at 72°C with a final extension at 72°C for 1 min. PCR products were loaded directly into pits of 1.5% agarose gel and analyzed by electrophoresis. The obtained PCR products were digested with restriction enzyme, Ban I according to the manufacturer's company protocol (BshNI; Thermo Scientific, USA). The wild allele "A" was cut into two fragments, 260 and 89 bp, while the

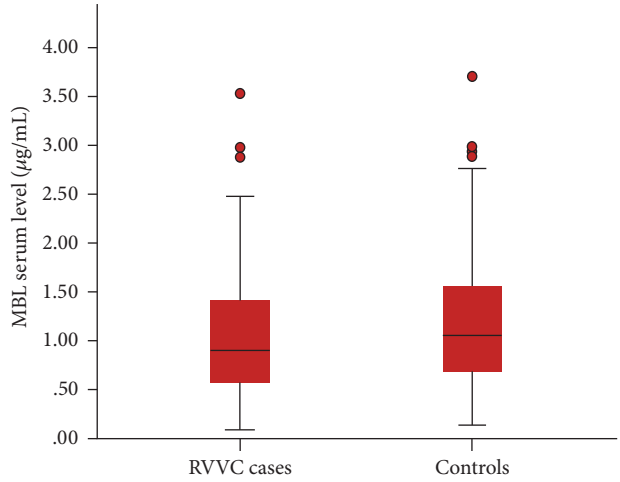


Figure 1: *Box plot showing the difference between RVVC cases* (n = 59) *and controls* (n = 59) *regarding MBL serum level.* The median MBL serum level of RVVC cases was nonsignificantly lower than that of controls (P = 0.145). The upper and lower ends of boxes and inner lines correspond to the upper and lower quartiles and median values, respectively. Whiskers indicate minimum and maximum values, and circles denote outliers.

variant allele "B" remained uncut. The digested products were loaded on 2% agarose gel and analyzed by electrophoresis.

2.7. Statistical Analysis. Quantitative data were represented as mean value ± 1 standard deviation (SD), median and range. Genotype and allele frequencies were determined by direct counting. Associations between MBL genotype or alleles and clinical variables were analyzed by Pearson Chi-Square (χ^2) and Fisher's exact tests. The strength of association of *MBL2* exon 1 codon 54 genotypes and frequency of RVVC was calculated by estimating odds ratios (OR) for matched data at confidence interval (CI) 95%. All tests were 2-tailed. Mann–Whitney U and Kruskal-Wallis tests were used for calculation of median difference between independent groups. Results were considered statistically significant when P (probability) values were equal to or less than 0.05. All analyses were performed using Statistical Package for the Social Sciences software version 24 (SPSS version 24, Inc., Chicago, IL, USA.).

3. Results

All RVVC cases and controls were negative for *Trichomonas vaginalis* and bacterial vaginosis; controls were also negative for *Candida* spp. All the vaginal swabs taken from the 59 RVVC childbearing women showed fungal growth on both Sabouraud Dextrose Agar and chromogenic agar. Further identification of *Candida* spp. by light microscopic examination and API identification kit revealed that 88.1% of the isolates were identified as *C. albicans* while 8.5% and 3.4% of the isolated were identified as *C. glabrata* and *C. tropicalis*, respectively.

No statistically significant difference in MBL serum level was observed between RVVC cases and controls (P = 0.145) (Figure 1). The median MBL serum level in RVVC cases

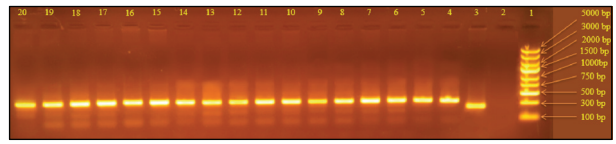


Figure 2: *PCR product of exon 1 of MBL2 gene amplification.* PCR products were electrophoresed on a 1.5% agarose gel and visualized under ultraviolet light by ethidium-bromide staining. Lane 1 is DNA Ladder, lane 2 is master mix as negative control, lane 3 is universal control of amplicon size 237 bp, lane 4 is positive control of purified human genomic DNA, lanes 5 to 12 are controls, and lanes 13 to 20 are RVVC cases. MBL gene amplicon size is of 349 bp.

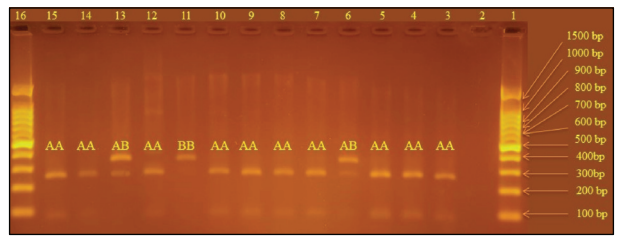


Figure 3: *MBL genotyping by RFLP.* Digested products were electrophoresed on 2% agarose gel and visualized under ultraviolet light by ethidium-bromide staining. Lanes 1 and 16 are DNA Ladder. Lane 2 is negative control. The wild *MBL* genotype (AA) is cut into two fragments with BanI enzyme, 89 and 260 bp, seen in lanes 3–5, 7–10, 12, 14, and 15. The mutant homozygous *MBL* genotype (BB) remains uncut, 349 bp, seen in lane 11. The heterozygous *MBL* genotype (AB) is seen in lanes 6 and 13.

was 0.90 μg/mL (range, 0.09–3.53 μg/mL) compared with 1.05 μg/mL (range, 0.14–3.70 μg/mL) in controls.

As given in Figure 2, the molecular sizes (349 bp) of PCR products from RVVC cases (lanes 13 to 30) were parallel to those from the controls (lanes 5 to 12). This showed a successful process of PCR technique for amplification and detection of exon 1 of *MBL2* gene.

PCR products digested by Ban I restriction enzyme produced two fragments of 260 and 89 bp for wild *MBL* genotypes (AA), three fragments of 349, 260, and 89 bp for heterozygous *MBL* genotypes (AB), and one uncut fragment of 349 bp for mutant homozygous *MBL* genotypes (BB) (Figure 3).

The distribution of MBL genotypes and alleles was significantly different between RVVC cases and controls (P = 0.038 and 0.013, resp.). Allele A (wild allele) was present, respectively, in 83.9% of RVVC cases and in 94.0% of controls, whereas allele B (mutant allele) was present in 16.1% of RVVC cases and in 6% of controls. No homozygous mutant genotype (BB) was found among controls. The risk of RVVC is 3.04 times higher among those who carried variant allele "B" in comparison to those who did not (Table 1).

Table 2 shows that, in the presence of wild *MBL* genotype, the risk of RVVC associated with bad genital hygiene behavior was 3.47 times higher than that associated with good genital hygiene behavior (P = 0.004) while in the presence of

Table 1: MBL genotypes and allelic frequency distribution among RVVC cases and controls.

Variable	RVVC cases $n = (59)$		Controls $n = (59)$		Test of significance	P value	OR (95% CI)
	n	%	n	%			
Genotypes							
AA	42	71.2	52	88.1			
AB	15	25.4	7	11.9	*Fisher's exact*	**0.038***	**2.65****
BB	2	3.4	0	0.0			
Alleles							
A	99	83.9	111	94.0	χ^2 **3.75**	**0.013***	**3.04*****
B	19	16.1	7	6.0			

*Significant difference; **OR for genotypes AB/AA. CI = 0.991–7.104; ***OR for alleles B/A. CI = 1.228–7.545.

Table 2: Risk estimate of bad genital hygiene behaviors in different MBL genotypes among RVVC cases and controls.

MBL genotype	Case $n = (57)$		Control $n = (59)$		Test of significance	P value	OR (95% CI)
	n	%	n	%			
AA							
Bad	28	66.7	19	36.5	χ^2 **8.436**	**0.004***	**3.47****
Good	14	33.3	33	63.5			
AB							
Bad	14	93.3	3	42.9	*Fisher's exact*	**0.021***	**18.67*****
Good	1	6.7	4	57.1			

*Significant difference; **OR for genital hygiene behaviors, bad/good. CI = 1.478–8.146; ***OR for genital hygiene behaviors, bad/good. CI = 1.500–232.291.

mutant *MBL* genotype the risk was increased to 18.67 times ($P = 0.021$).

In RVVC group, the mutant types (AB and BB) MBL serum levels were significantly lower than the wild type (AA) MBL serum level ($P = 0.019$ and 0.033, resp.). However, in control group, no statistically significant difference was found between mutant type and wild type MBL serum levels ($P = 0.23$) (Table 3).

4. Discussion

Vulvovaginal candidiasis is one of the most prevalent vaginal infections and represents approximately 40%–50% of all cases of infectious vulvovaginitis [22]. The prevalence of RVVC among childbearing women and its importance as an Egyptian public health problem make an interest to continue research on such cases to add deep knowledge on RVVC and to understand the behavior of its pathogen and its epidemiology within Egyptian patients [23]. RVVC and its control by MBL were studied previously, but unfortunately none of the published papers discussed such cases in Egyptian patients. In an attempt to fill this gap, this study was designed on 118 childbearing Egyptian women to find out new therapeutic strategy for RVVC.

The suspected *Candida* isolates were identified as described previously [3, 4, 24]. In the present study, *C. albicans* was the most prevalent (88.1%) *Candida* spp. isolated from RVVC cases, followed by *C. glabrata* (8.5%) and *C. tropicalis* (3.4%), respectively. This study agreed with most studies worldwide which have reported that *C. albicans* is the main infectious *Candida* spp. implicated in infections of RVVC (76 to 89%). The overall percentage of non-*albicans Candida* spp. ranges from 11% to 24% [25–28].

Genetic factors often play an important role in primary or idiopathic RVVC occurring in women without any recognizable risk factors. Moreover, the recurrence of acute attacks of VVC triggered by known risk factor is strongly relevant to genetic predisposition [29]. The understanding of such genetic factors that determine susceptibility to RVVC is crucial for future therapeutic modalities in these patients [30]. Variation of MBL concentration in cervicovaginal fluid depends on individual's MBL genotype [12]. The strong binding of *Candida* spp. to MBL suggested the importance of this protein in host defense against VVC [13].

The present study found no statistical significant difference in MBL serum level between RVVC cases and controls. This finding is supported by a previous study that found no significant difference when comparing MBL serum levels between RVVC cases and controls [31]. However, some investigators found that MBL serum level was higher in RVVC cases when compared to controls indicating that MBL might be defensive against RVVC [13]. On the contrary, other studies reported that MBL level was lower in RVVC cases compared to controls [12, 32]. Nevertheless, they depended on measurement of MBL in vaginal fluid while, in the present study, MBL was measured in serum. From practical point

Table 3: Comparison between wild type (AA) and mutant types (AB and BB) MBL serum levels among RVVC cases and controls.

Study group	MBL serum level (μg/mL)			Test of significance	P value	
	AA	AB	BB			
RVVC cases ($n = 59$)	($n = 42$)	($n = 15$)	($n = 2$)			
Mean ± SD	1.31 ± 0.88	0.69 ± 0.33	0.29 ± 0.28			**0.019**^{**1}
Median (range)	1.02 (0.09–3.53)	0.67 (0.13–1.05)	0.4 (0.09–0.49)	*Kruskal-Wallis*	**0.011***	**0.033**^{**2} **0.265**^{**3}
Controls ($n = 59$)	($n = 52$)	($n = 7$)				
Mean ± SD	1.35 ± 0.86	1.08 ± 0.96	-	*Mann–Whitney*	**0.23**	
Median (range)	1.15 (0.14–3.70)	0.51 (0.35–2.98)				

*Significant difference; $^{**1}P$ value between AA and AB genotypes MBL concentrations; $^{**2}P$ value between AA and BB genotypes MBL concentrations; $^{**3}P$ value between AB and BB genotypes MBL concentrations.

of view, determination of MBL level in vaginal discharge is technically too difficult to perform and to standardize. Even more, the entity of vaginal fluid is changeable throughout menstrual period [33].

The present study could find an association between *MBL2* exon 1 codon 54 polymorphism and susceptibility to RVVC. Carriage of mutant allele "B" was more frequent in women with RVVC (16.1%) than in controls (6%). In previous studies, *MBL2* codon 54 polymorphism has been associated with increased frequency of RVVC in Latvian, Brazilian, Chinese, and Belgium [12, 32, 34, 35] but not Italian patients [31].

In the present study, analysis of genital hygiene behaviors among wild MBL genotypes estimated that bad genital hygiene behaviors increased the risk of RVVC by 3.47 times while the risk of RVVC has been elevated to 18.67 times in the presence of mutant *MBL* genotypes. These results supported the fact that RVVC is monomicrobial; however, it is multifactorial disease in origin [36]. Moreover, good genital hygiene behaviors might be protective against RVVC in genetically predisposed women [37].

Furthermore, this study has reported that *MBL2* codon 54 polymorphism was significantly associated with reduction in MBL serum levels among RVVC cases; however, such reduction was statistically nonsignificant among controls. This finding was in agreement with Milanese et al. [31] Consistently, *MBL2* codon 54 polymorphism has been associated with reduced vaginal concentrations of MBL [12, 38]. *MBL2* codon 54 polymorphism results in either nonfunctional monomers in homozygotes or low functional serum levels of protein with shorter half-lives that are easily degraded to lower oligomeric forms in heterozygotes [39]. It has been estimated that healthy individuals who are homozygous for the wild type alleles (A/A) have MBL serum levels above 1 μg/mL while heterozygous individuals (A/0) have serum levels ranging from 0.5 to 1 μg/mL. However, homozygous individuals for the variant *MBL2* alleles (0/0) have MBL serum concentration below 0.05 μg/mL [40]. Furthermore, MBL deficiency was defined before by plasmatic protein levels below 0.5 μg/mL or by an MBL function lower than 0.2 U/μL C4 deposition [41].

In addition, other structural and promoter *MBL2* gene polymorphisms have been identified resulting in 7 common secretor haplotypes that eventually determine the serum MBL concentrations. HYPA, LYQA, and LYPA haplotypes are associated with high serum level and LXPA, HYPD, LYPB, and LYQC haplotypes are associated with low serum level. Therefore, it is conceivable that one patient with wild type allele for MBL codon 54 may have a promoter combination, for example, LXP haplotype that downregulates MBL production resulting in low MBL serum level [39]. However, *MBL2* gene structural polymorphisms alone could be responsible for the defective binding of MBL to *Candida* via its lectin domain at early phase of vaginal infection by the fungus [42].

Interestingly, it was previously documented that both wild type and mutant type MBL vaginal levels were significantly higher in acute VVC than in controls but not in RVVC, suggesting that MBL may increase during first episode of VVC, albeit subsequent episodes may make the immune system less sensitive to *Candida* [32]. Moreover, there is an emerging hypothesis that both acute VVC and RVVC stem from different host reaction modulated by *Candida*. Acute VVC associated with high numbers of *Candida* results in immunosuppressive reaction of the host while RVVC being associated with low numbers of *Candida* generates hypersensitivity reaction in genetically predisposed women. The host reactions were linked to modulators resulting from interaction between mast cells and substances such as MBL involved in mycotic infections. That hypothesis introduced a concept of "vaginal cutoff" which is the minimum quantity level of the *Candida* above which it would activate an allergic reaction that justifies the recurrence of episodes of VVC, in vulnerable subjects [43].

Data from previous reports of Babula et al. hypothesized that women's genetic capacity to produce MBL and IL-4 influenced their susceptibility to RVVC. They stated strong inverse relationship between vaginal concentrations of IL-4 and anticandidal compounds, MBL and nitric oxide metabolites. Additionally, the homozygous variant allele IL-4*T was associated with >2-fold increase of IL-4, >3-fold decrease of nitric oxide, and >2-fold decrease of MBL concentrations in vaginal fluid [12, 44].

Since MBL serum levels could be affected by infections, hormone and drug intake, several investigators chose MBL genotyping over MBL serum level. They observed that MBL serum level is strongly associated with *MBL2* gene polymorphisms [45, 46]. Nevertheless, MBL serum levels are not affected by age, circadian rhythm, and physical exercise and, during inflammation, do not increase over 3-4-fold compared to baseline level unlike other acute phase reactants like C-reactive protein which increases sharply from 10-fold to 1000-fold [40, 47]. Despite the fact that MBL serum measurement is widely diffused as a diagnostic test, there are not standard guidelines able to determine which patient is needed to be tested [40, 48].

Taken together, these data suggest that other factors such as cytokine levels, other alleles, or additional polymorphisms are in tight linkage disequilibrium that may affect the level of MBL. Consequently, some observations in this study could be explained, such that some low serum MBL levels were associated with wild genotype (AA) as well as the nonsignificant difference in MBL serum level between RVVC cases and controls and between different genotypes among controls.

Collectively, MBL genotype of a person offers only a general idea of the expected plasma concentration and different combinations of haplotypes are associated with a wide range of MBL concentrations. Genotypes are fairly good indicators of the average MBL concentrations at population level albeit less reliable predictors for plasma MBL at individual level [49, 50].

As RVVC may not be substantially treated; therefore, further deciphering of vaginal host defense mechanisms against *Candida* becomes essential to design novel immunotherapeutic strategies to improve and/or substitute the usual antifungal treatments. Further work in this approach focusing on MBL protein and its direct impact on *Candida spp.* was conducted *in vitro* (results have not been published yet). The efficacy of MBL protein as a potential therapeutic agent against RVVC, particularly among MBL-deficient women, should be clearly investigated.

5. Conclusion

Women carrying the variant allele "B" of *MBL2* codon 54 polymorphism have more risk of developing RVVC. Therefore, MBL genotypic analysis can be used as surrogates for MBL serum levels in order to identify MBL-deficient women for alternative therapeutic options.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

The authors would like to express their gratitude to Research Center of Advanced Materials, King Khalid University, Saudi Arabia, for support. Also, the authors would like to acknowledge Scientific and Medical Research Center of Zagazig University (ZSMRC), Faculty of Medicine, for support.

References

[1] Z. Z. Al-Ahmadey and S. A. Mohamed, "Vulvovaginal candidiasis: agents and its virulence factors," *Microbiology Research International*, vol. 2, no. 3, pp. 28–37, 2014.

[2] D. C. Pereira, L. T. Backes, L. N. Calil, and A. M. Fuentefria, "A six-year epidemiological survey of vulvovaginal candidiasis in cytopathology reports in the state of Rio Grande do Sul, Brazil," *Revista de Patologia Tropical*, vol. 41, no. 2, pp. 163–168, 2012.

[3] T. I. El-Sayed, D. Atef, M. Amer, A. Mahdy, and G. Enan, "Molecular characterization and inhibition by natural agents of multidrug resistant Candida strains causing vaginal candidiasis," *Research Journal of Medical Sciences*, vol. 9, no. 1, pp. 1–7, 2015.

[4] G. Enan, T. I. EL-Sayed, D. Atef, M. Amer, and A. Mahdy, "Causal organisms, pathogenicity, laboratory diagnosis and treatment of candidiasis," *Research Journal of Applied Sciences* , vol. 10, no. 4, pp. 115–135, 2015.

[5] V. M. Bruno, A. C. Shetty, J. Yano, P. L. Fidel, M. C. Noverr, and B. M. Peters, "Transcriptomic Analysis of Vulvovaginal Candidiasis Identifies a Role for the NLRP3 Inflammasome," *mBio*, vol. 6, no. 2, pp. 1–15, 2015.

[6] M. Jaeger, T. S. Plantinga, L. A. B. Joosten, B.-J. Kullberg, and M. G. Netea, "Genetic basis for recurrent vulvo-vaginal candidiasis," *Current Infectious Disease Reports*, vol. 15, no. 2, pp. 136–142, 2013.

[7] A. Cassone, "Vulvovaginal Candida albicans infections: Pathogenesis, immunity and vaccine prospects," *BJOG: An International Journal of Obstetrics & Gynaecology*, vol. 122, no. 6, pp. 785–794, 2015.

[8] A. Puel, S. Cypowyj, L. Maródi, L. Abel, C. Picard, and J. Casanova, "Inborn errors of human IL-17 immunity underlie chronic mucocutaneous candidiasis," *Current Opinion in Allergy and Clinical Immunology*, vol. 12, no. 6, pp. 616–622, 2012.

[9] D. C. Rosentul, C. E. Delsing, M. Jaeger et al., "Gene polymorphisms in pattern recognition receptors and susceptibility to idiopathic recurrent vulvovaginal candidiasis," *Frontiers in Microbiology*, vol. 5, article 483, 2014.

[10] D. L. Worthley, P. G. Bardy, D. L. Gordon, and C. G. Mullighan, "Mannose-binding lectin and maladies of the bowel and liver," *World Journal of Gastroenterology*, vol. 12, no. 40, pp. 6420–6428, 2006.

[11] G. G. G. Donders, G. Bellen, and W. Mendling, "Management of recurrent vulvo-vaginal candidosis as a chronic illness," *Gynecologic and Obstetric Investigation*, vol. 70, no. 4, pp. 306–321, 2010.

[12] O. Babula, G. Lazdane, J. Kroica, W. J. Ledger, and S. S. Witkin, "Relation between recurrent vulvovaginal candidiasis, vaginal concentrations of mannose-binding lectin, and a mannose-binding lectin gene polymorphism in latvian women," *Clinical Infectious Diseases*, vol. 37, no. 5, pp. 733–737, 2003.

[13] E. Henić, S. Thiel, and P.-A. Mårdh, "Mannan-binding lectin in women with a history of recurrent vulvovaginal candidiasis,"

European Journal of Obstetrics & Gynecology and Reproductive Biology, vol. 148, no. 2, pp. 163–165, 2010.

[14] P. Garred, F. Larsen, J. Seyfarth, R. Fujita, and H. O. Madsen, "Mannose-binding lectin and its genetic variants," *Genes & Immunity*, vol. 7, no. 2, pp. 85–94, 2006.

[15] J. S. Presanis, M. Kojima, and R. B. Sim, "Biochemistry and genetics of mannan-binding lectin (MBL)," *Biochemical Society Transactions*, vol. 31, no. 4, pp. 748–752, 2003.

[16] H. O. Madsen, P. Garred, J. A. L. Kurtzhals et al., "A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein," *Immunogenetics*, vol. 40, no. 1, pp. 37–44, 1994.

[17] M. Sumiya, M. Super, P. Tabona et al., "Molecular basis of opsonic defect in immunodeficient children," *The Lancet*, vol. 337, no. 8757, pp. 1569-1570, 1991.

[18] R. J. Lipscombe, M. Sumiya, A. V. S. Hill et al., "High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene," *Human Molecular Genetics*, vol. 1, no. 9, pp. 709–715, 1992.

[19] B. Wächtler, F. Citiulo, N. Jablonowski et al., "*Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process," *PLoS ONE*, vol. 7, no. 5, Article ID e36952, 2012.

[20] R. Amsel, P. A. Totten, C. A. Spiegel, K. C. Chen, D. Eschenbach, and K. K. Holmes, "Nonspecific vaginitis," *American Journal of Medicine*, vol. 74, no. 1, pp. 14–22, 1983.

[21] L. J. R. Milne and T. Mackie, *Mackie & McCartney Practical medical microbiology*, Charchil Livingstone, New York , USA, 14th edition, 1998.

[22] J. Li, S.-R. Fan, X.-P. Liu et al., "Biased genotype distributions of *Candida albicans* strains associated with vulvovaginal candidosis and candidal balanoposthitis in China," *Clinical Infectious Diseases*, vol. 47, no. 9, pp. 1119–1125, 2008.

[23] I. M. Hayat, S. S. Nagat, N. Nermine, and A. B. Zeinab, "Prevalence of Vaginal Infection and Associated Risk Health Behaviors Among Married Women in Ismailia City," *International Journal of Current Microbiology and Applied Sciences*, vol. 4, no. 5, pp. 555–567, 2015.

[24] P. Kamara, T. Hylton-Kong, A. Brathwaite et al., "Vaginal infections in pregnant women in Jamaica: Prevalence and risk factors," *International Journal of STD & AIDS*, vol. 11, no. 8, pp. 516–520, 2000.

[25] S. A. Corsello, A. Spinillo, G. Osnengo et al., "An epidemiological survey of vulvovaginal candidiasis in Italy," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 110, no. 1, pp. 66–72, 2003.

[26] J. Holland, M. L. Young, O. Lee, and S. C. A. Chen, "Vulvovaginal carriage of yeasts other than Candida albicans," *Sexually Transmitted Infections*, vol. 79, no. 3, pp. 249-250, 2003.

[27] J. M. Achkar and B. C. Fries, "Candida infections of the genitourinary tract," *Clinical Microbiology Reviews*, vol. 23, no. 2, pp. 253–273, 2010.

[28] J. O. Isibor, S. O. Samuel, C. I. Nwaham, I. N. Amanre, O. Igbinovia, and A. O. Akhile, "Prevalence of bacterial and Candida albicans infection amongst women attending Irrua specialist teaching hospital, Irrua, Nigeria," *African Journal of Microbiology Research*, vol. 5, no. 20, pp. 3126–3130, 2011.

[29] J. Sobel, "Vaginitis, vulvitis, cervicitis, and cutaneous vulval lesions," in *Infectious diseases*, vol. 1, pp. 483–491, St, Louis, Mosby, 4th edition, 2017.

[30] S. P. Smeekens, F. L. van de Veerdonk, B. J. Kullberg, and M. G. Netea, "Genetic susceptibility to Candida infections," *EMBO Molecular Medicine*, vol. 5, no. 6, pp. 805–813, 2013.

[31] M. Milanese, L. Segat, F. De Seta et al., "MBL2 genetic screening in patients with recurrent vaginal infections," *American Journal of Reproductive Immunology*, vol. 59, no. 2, pp. 146–151, 2008.

[32] F. Liu, Q. Liao, and Z. Liu, "Mannose-binding lectin and vulvovaginal candidiasis," *International Journal of Gynecology and Obstetrics*, vol. 92, no. 1, pp. 43–47, 2006.

[33] B. J. Schlosser and G. W. Mirowski, "Approach to the patient with vulvovaginal complaints," *Dermatologic Therapy*, vol. 23, no. 5, pp. 438–448, 2010.

[34] P. C. Giraldo, O. Babula, A. K. S. Gonçalves et al., "Mannose-binding lectin gene polymorphism, vulvovaginal candidiasis, and bacterial vaginosis," *Obstetrics & Gynecology*, vol. 109, no. 5, pp. 1123–1128, 2007.

[35] G. G. G. Donders, O. Babula, G. Bellen, I. M. Linhares, and S. S. Witkin, "Mannose-binding lectin gene polymorphism and resistance to therapy in women with recurrent vulvovaginal candidiasis," *BJOG: An International Journal of Obstetrics & Gynaecology*, vol. 115, no. 10, pp. 1225–1231, 2008.

[36] S. N. Astill, G. Shankland, and A. Winter, "Practical management of recurrent vulvovaginal candidiasis," in *Trends in Urology*, vol. 14, pp. 18–22, Gynaecology Sexual Health, 2009.

[37] M. E. Ocaktan, E. Baran, and R. Akdur, "Evaluation of habitual behavior related to genital hygiene in women living in a health care center area," *Saudi Medical Journal*, vol. 31, no. 11, pp. 1251–1256, 2010.

[38] M. D. K. H. Wojitani, L. M. De Aguiar, E. C. Baracat, and I. M. Linhares, "Association between mannose-binding lectin and interleukin-1 receptor antagonist gene polymorphisms and recurrent vulvovaginal candidiasis," *Archives of Gynecology and Obstetrics*, vol. 285, no. 1, pp. 149–153, 2012.

[39] B. Nedovic, B. Posteraro, E. Leoncini et al., "Mannose-binding lectin codon 54 gene polymorphism and vulvovaginal candidiasis: A systematic review and meta-analysis," *BioMed Research International*, vol. 2014, Article ID 738298, 2014.

[40] G. De Pascale, S. L. Cutuli, M. A. Pennisi, and M. Antonelli, "The role of mannose-binding lectin in severe sepsis and septic shock," *Mediators of Inflammation*, vol. 2013, Article ID 625803, 8 pages, 2013.

[41] M. M. Dean, R. M. Minchinton, S. Heatley, and D. P. Eisen, "Mannose binding lectin acute phase activity in patients with severe infection," *Journal of Clinical Immunology*, vol. 25, no. 4, pp. 346–352, 2005.

[42] W. K. Ip and Y. L. Lau, "Role of mannose-binding lectin in the innate defense against *Candida albicans*: enhancement of complement activation, but lack of opsonic function, in phagocytosis by human dendritic cells," *The Journal of Infectious Diseases*, vol. 190, no. 3, pp. 632–640, 2004.

[43] F. Murina, A. Graziottin, R. Felice, G. L. Radici, and S. Di Francesco, "The Recurrent Vulvovaginal Candidiasis: Proposal of a Personalized Therapeutic Protocol," *ISRN Obstetrics and Gynecology*, vol. 2011, pp. 1–4, 2011.

[44] O. Babula, G. Lazdane, J. Kroiča, I. M. Linhares, W. J. Ledger, and S. S. Witkin, "Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis," *Clinical Infectious Diseases*, vol. 40, no. 9, pp. 1258–1262, 2005.

[45] R. M. Shawky, S. M. Abd El-Fattah, T. M. Kamal, M. A. Esa, and G. H. El Nady, "Genotyping of mannose-binding lectin (MBL2)

codon 54 and promoter alleles in Egyptian infants with acute respiratory tract infections," *Egyptian Journal of Medical Human Genetics*, vol. 15, no. 1, pp. 31–38, 2014.

[46] C. L. Lin, L. K. Siu, J. C. Lin et al., "Mannose-binding lectin gene polymorphism contributes to recurrence of infective exacerbation in patients with COPD," *CHEST*, vol. 139, no. 1, pp. 43–51, 2011.

[47] W. K. Eddie Ip, K. Takahashi, R. Alan Ezekowitz, and L. M. Stuart, "Mannose-binding lectin and innate immunity," *Immunological Reviews*, vol. 230, no. 1, pp. 9–21, 2009.

[48] J. W. Huh, K. Song, J.-S. Yum, S.-B. Hong, C.-M. Lim, and Y. Koh, "Association of mannose-binding lectin-2 genotype and serum levels with prognosis of sepsis.," *Critical Care (London, England)*, vol. 13, no. 6, p. R176, 2009.

[49] D. C. Kilpatrick, "Mannan-binding lectin: clinical significance and applications," *Biochimica et Biophysica Acta*, vol. 1572, no. 2-3, pp. 401–413, 2002.

[50] S. Esmat, D. Omran, G. A. Sleem, and L. Rashed, "Serum Mannan-Binding lectin in Egyptian patients with chronic hepatitis C: Its relation to disease progression and response to treatment," *Hepatitis Monthly*, vol. 12, no. 4, pp. 259–264, 2012.

Hindawi
BioMed Research International
Volume 2018, Article ID 5657085, 10 pages
https://doi.org/10.1155/2018/5657085

Research Article

*Lra*I from *Lactococcus raffinolactis* BGTRK10-1, an Isoschizomer of *Eco*RI, Exhibits Ion Concentration-Dependent Specific Star Activity

Marija Miljkovic,[1] Milka Malesevic,[1] Brankica Filipic,[1,2] Goran Vukotic,[1,3] and Milan Kojic[1]

[1]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia*
[2]*Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia*
[3]*Faculty of Biology, University of Belgrade, Belgrade, Serbia*

Correspondence should be addressed to Milan Kojic; mkojic@imgge.bg.ac.rs

Received 22 November 2017; Accepted 2 January 2018; Published 29 January 2018

Academic Editor: Gamal Enan

Restriction enzymes are the main defence system against foreign DNA, in charge of preserving genome integrity. *Lactococcus raffinolactis* BGTRK10-1 expresses *Lra*I Type II restriction-modification enzyme, whose activity is similar to that shown for *Eco*RI; *Lra*I methyltransferase protects DNA from *Eco*RI cleavage. The gene encoding *Lra*I endonuclease was cloned and overexpressed in *E. coli*. Purified enzyme showed the highest specific activity at lower temperatures (between 13°C and 37°C) and was stable after storage at −20°C in 50% glycerol. The concentration of monovalent ions in the reaction buffer required for optimal activity of *Lra*I restriction enzyme was 100 mM or higher. The recognition and cleavage sequence for *Lra*I restriction enzyme was determined as 5′-G/AATTC-3′, indicating that *Lra*I restriction enzyme is an isoschizomer of *Eco*RI. In the reaction buffer with a lower salt concentration, *Lra*I exhibits star activity and specifically recognizes and cuts another alternative sequence 5′-A/AATTC-3′, leaving the same sticky ends on fragments as *Eco*RI, which makes them clonable into a linearized vector. Phylogenetic analysis based on sequence alignment pointed out the common origin of *Lra*I restriction-modification system with previously described *Eco*RI-like restriction-modification systems.

1. Introduction

Restriction endonucleases are generally accompanied by a cognate methyltransferase [1]. Both enzymes working together form a restriction-modification system (RM system). RM systems are important for the maintenance of the genome integrity of prokaryotic organisms. The range of biological processes that utilize RM system also includes involvement in DNA transposition [2] and recombination [3]. In addition, there is evidence that the genes for restriction and modification enzymes may act together as selfish elements [4, 5].

Restriction endonucleases exhibit high sequence specificity in substrate binding and use versatile DNA cleavage mechanisms and thus are excellent model systems for understanding DNA recognition and phosphodiester bond hydrolysis. Restriction endonucleases are classified according to their subunit composition, cofactor requirement, recognition site, cleavage site, and mode of action to define the different types (I, II, III, and IV). Restriction endonucleases Type II are essential tools for recombinant DNA technology. It seems unlikely that today's modern molecular biology and the biotechnology industry would have developed without Type II restriction enzymes. Because of their great importance in gene analysis and cloning there is a constant need to discover new ones. According to data from the REBASE [6, http://rebase.neb.com] which summarizes all information known about every restriction enzyme and any associated protein, there are more than 3945 biochemically or genetically characterized restriction enzymes and, out of 3834 Type II restriction enzymes, 299 distinct specificities are known. By 2010, six hundred and forty-one restriction enzymes were commercially available, including 235 distinct specificities [7].

Because of the large number of sequenced genomes, rate of discovery of new putative restriction and modification genes is rising rapidly. In contrast, the number of restriction enzymes that are biochemically characterized has actually dropped down to the level that was three decades ago.

Restriction endonucleases Type II are homodimeric or tetrameric enzymes that cleave DNA at defined sites of 4–8 bp in length and require Mg^{2+} ions for catalysis [8]. For many of restriction endonucleases Type II, it was found that modified conditions (lower ionic strength, higher pH, presence of different metallic cofactors, and organic solvents) could decrease their substrate specificity [9–13]. Under nonoptimal restriction conditions, these endonucleases can usually cleave degenerate sequences, which differ from standard recognition sites at only one nucleotide. This alteration in digestion specificity causing cleavage of DNA at novel, similar but not identical sequences is defined as enzyme star activity. Modified specificity of restriction enzyme (star) activity could be exploited to facilitate recombinant DNA techniques since the same enzyme in controlled conditions could recognize different DNA sequences and cleave at additional positions [9].

Restriction endonucleases with identical recognition sites isolated from different organisms are termed isoschizomers [7, 14]. The *Rsr*I endonuclease found in *Rhodobacter sphaeroides* is an isoschizomer of the *Eco*RI. Both enzymes recognize the sequence GAATTC and cleave it at the same position (G/AATTC) and are sharing 50% amino acid sequence identity [15]. Interesting, *Mun*I recognizes the sequence CAATTG, which differs from the recognition sequence of *Eco*RI (and *Rsr*I) only in the external base pairs. Comparison of the *Mun*I amino acid sequence with that of *EcoR*I and *Rsr*I revealed only a low level of overall similarity wherefore sequence homology between *Eco*RI and *Rsr*I has a stronger significance [16].

This work describes for the first time the occurrence of *Eco*RI-like restriction-modification genes in lactococci. The objective was to clone, purify, and biochemically and genetically characterize novel lactococcal *Lra*I restriction enzyme. *Lra*I restriction enzyme was overexpressed and purified to the homogeneity from *E. coli* using pMAL expression and purification system. Results demonstrate that *Lra*I restriction enzyme, although an isoschizomer of *Eco*RI, shows different characteristics. One of characteristics that could be further exploited is star activity of *Lra*I that is limited to one variant of the recognition site, which after cleavage leaves identical cohesive ends as *Eco*RI and *Lra*I restriction enzymes, so that the fragments obtained after digestion could be cloned without additional processing.

2. Material and Methods

2.1. Bacterial Strains and Culture Conditions. *Lactococcus raffinolactis* BGTRK10-1 was isolated from autochthonous sweet kajmak produced from sheep milk without the use of starter cultures in a household of the Vlašić mountain region, central Bosnia and Herzegovina [17] (Table 1). Preliminary strain classification was done according to its fermentation ability using API 50CHL (Api System SA; Bio-Merieux, Montelieu-Vercieu, France), temperature of growth (30°C, 37°C, and 45°C), growth in the presence of salt (4% and 6.5%), and pH tolerance. Final taxonomic classification of BGTRK10-1 was performed by sequencing of amplified 16S rDNA using primers previously described [18]. The strain was grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at 30°C. *Escherichia coli* DH5α, HB101, and ER2523 strains were grown aerobically in Luria-Bertani (LB) broth at 37°C, unless otherwise specified. Solid medium was made by adding 1.75% (w/v) agar (Torlak, Belgrade, Serbia), to the liquid media. Antibiotics were used at the following concentrations: erythromycin 300 μg/ml and ampicillin 100 μg/ml for selection and maintaining of transformants. The 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added to LB medium plates for blue/white colour screening of colonies with cloned fragments at final concentration of 40 μg/ml.

2.2. Construction of Cosmid Library of L. raffinolactis BGTRK10-1. Total DNA isolated from the *L. raffinolactis* BGTRK10-1 was partially digested with *Xba*I restriction enzyme. Incubation was carried out during 1 h and was stopped in different time intervals by adding EDTA. Optimally digested DNA, giving fragments 30–40 kb, was purified and ligated overnight at 16°C with the pAZILcos vector [19] predigested with *Xba*I restriction enzyme and dephosphorylated. Ligation for formed concatemers of high molecular weight was checked on agarose gel and encapsulated into phage particles using packaging kit (Agilent Technologies). Encapsulated cosmids were transfected into *E. coli* HB101 magnesium cells and selection of clones was done on LA plates containing erythromycin 300 μg/ml. Constructed cosmid library in *E. coli* was stored in LB containing 15% (v/v) glycerol at −80°C.

2.3. DNA Manipulations. Total DNA from *L. raffinolactis* BGTRK10-1 was isolated by modified method described by Hopwood et al. [20]; the logarithmic phase cells were pretreated with lysozyme (4 mg/ml, for 15 min at 37°C) prior to treatment with SDS. For plasmid isolation from *E. coli* the QIAprep Spin Miniprep kit was used according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Standard heat-shock transformation was used for plasmid transfer into *E. coli* [21]. Digestion with restriction enzymes was conducted according to the supplier's instructions (Thermo Fisher Scientific). The DNA fragments from agarose gels were purified using QIAqick Gel extraction kit as described by the manufacturer (Qiagen, Hilden, Germany). DNA was ligated with T4 DNA ligase (Agilent technologies, USA) according to the manufacturer's recommendations. Platinum™ *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA) was used to amplify DNA fragments by PCR in GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with QiAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA sequencing was done by the Macrogen Sequencing Service (Macrogen Europe, The Netherlands).

Table 1: Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Lactococcus raffinolactis		
BGTRK10-1	Natural isolate from autochthonous sweet kajmak	[17]
Escherichia coli		
DH5α	*supE44* Δ*lacU169 (ø80 lacZ*Δ*M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[21]
HB101	F^- *hsdS20* (r_B^- m_B^-) *supE44 recA13 ara-14 proA2 lacY1 rpsL20*(Sm^R) *xyl-5 mtl-1 galK2 lacY1* λ^-	[24]
ER2523	*fhuA2 [lon] ompT gal sulA11 R(mcr- 73::miniTn10- -Tet^S) 2 [dcm] R(zgb-210::Tn10- -Tet^S) endA1* Δ*(mcrC-mrr)114::IS10*	New England Biolabs, Ltd. UK
ER2523/pAZIL-LraRM	Competent cells obtained by transformation of ER2523 cells with pAZIL-LraRM	This study
Plasmids		
pAZIL	7109 bp, Em^r, shuttle cloning vector	[19]
pAZIL-LraRM	PCR fragments of LraI operon from BGTRK10-1 cloned into pAZIL vector predigested with *Sma*I	This study
pAZIL-LraI∗672pBS	DNA fragment of 672 bp obtained after digestion of pBluescipt SK+ with *Lra*I∗ activity cloned into pAZIL vector predigested with *Lra*I	This study
pAZILcos	8194 bp, Em^r, shuttle cosmid vector	[19]
pAZILcosLra	Cosmid selected from total *Xba*I cosmid library of BGTRK10-1	This study
pBluescript SK+	2958 bp, Amp^r, cloning vector	Stratagene
pBSLraCla	*Cla*I fragment of 4239 bp obtained from pAZILcosLra cloned into pBluescript SK+ vector	This study
pMAL-c5X	5677 bp, pMB1 origin, *lacI*, *malE*, *bla*, Factor Xa cleavage site;	New England Biolabs, Ltd. UK
pMAL-cX5LraI-29	PCR fragment of *Lra*I restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with *Xmn*I and *Hin*dIII restriction enzymes	This study
pMAL-cX5LraI-31	PCR fragment of *Lra*I restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with *Xmn*I and *Hin*dIII restriction enzymes	This study
pMAL-cX5LraI-42	PCR fragment of *Lra*I restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with *Xmn*I and *Hin*dIII restriction enzymes	This study

The primers used in PCR for amplification of LraI operon (*lraIR* and *lraIM* genes) were as follows: LraRM-Fw (5′-GTATAGAAAGAAGAATCG-3′) and LraRM-Rev (5′-GCAGGGTAATGTTCCTCAC-3′), while following primers were used for overexpression of *Lra*I restriction enzyme (*lraIR* gene) in pMALc5X vector: LraI-Fw (5′-ATGTCGAGAAAAAATCAGTCG-3′) and LraI-Rev (5′-CTCAAGCTTTCTAATTAATCCTTTTTTGC-3′; *Hin*dIII restriction site is underlined). Total DNA (1 ng) was mixed with 17.9 μl of bidistilled water, 2.5 μl of 10x PCR buffer (Thermo Fisher Scientific), 1 μl dNTP mix (10 mM), 1.5 μl of $MgCl_2$ (25 mM), 1 μl (10 pmol) of each primer, and 0.1 μl of Platinum™ *Taq* DNA Polymerase High Fidelity. Performed using the GeneAmp 2700 PCR Cycler (Applied Biosystems), the PCR programs consisted of initial denaturation (5 min at 96°C), 30 cycles of denaturation (30 s at 96°C), annealing (30 s at 40°C) and polymerization (2 or 1 min at 68°C), and an additional extension step of 5 min at 68°C. PCR fragments of LraI operon amplified using Platinum™ *Taq* DNA Polymerase High Fidelity were cloned into pAZIL vector predigested with *Sma*I.

2.4. Recombinant LraI Restriction Endonuclease Overexpression in E. coli and Purification. PCR fragment consisted of *lraIR* gene (from ATG to stop codon) obtained by LraI-Fw/LraI-Rev primers and Platinum™ *Taq* DNA Polymerase High Fidelity was purified, digested with *Hin*dIII and cloned into pMAL-c5X vector digested with *Xmn*I and *Hin*dIII restriction enzymes and transformed into ER2523 competent cells (New England Biolabs, Ltd. UK) previously transformed with pAZIL-LraRM construct (Table 1). Transformants were selected on LA Petri dishes containing 2% glucose, ampicillin 100 μg/ml, and erythromycin 300 μg/ml at 23°C. Confirmation of fragment presence in adequate orientation was obtained by restriction enzyme analysis (with *Sac*I and *Hin*dIII digestion) and sequencing. Expression of recombinant protein was carried out at 23°C by induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Purification (cell lysis, affinity chromatography, and cleavage of fusion protein with Xa protease) was performed according to manufacturer instruction (pMAL Protein Fusion & Purification System; New England Biolabs, Ltd., UK). Purified recombinant LraI restriction endonuclease was stored at −20°C in CM

buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT) containing 50% glycerol.

2.5. Endonuclease Assays. Endonuclease activity was assayed by incubating various amounts of purified *Lra*I enzyme in buffer recommended for use with *Eco*RI (50 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 100 mM sodium chloride, 0.02% Triton X-100, supplemented with 100 μg/ml BSA; Thermo Fisher Scientific) containing 1 μg of pBluescipt SK+ plasmid DNA per 50 μl reaction mixture for 1 h at 37°C. One unit of enzyme activity was defined as amount of purified *Lra*I enzyme, which was able to completely cut 1 μg of plasmid DNA for 1 h.

Influence of reaction buffer composition on *Lra*I enzyme activity was assayed in different commercial buffers (Buffer B (blue; 10 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 100 μg/ml BSA), Buffer G (green; 10 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 50 mM sodium chloride, 100 μg/ml BSA), Buffer O (orange; 50 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 100 mM sodium chloride, 100 μg/ml BSA), Buffer R (red: 10 mM Tris-HCl pH 8.5, 10 mM magnesium chloride, 100 mM potassium chloride, 100 μg/ml BSA), Buffer Tango (yellow: 33 mM Tris–acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 100 μg/ml BSA), and buffer recommended for use with *Eco*RI; Thermo Fisher Scientific) used for reaction with 1 U of purified *Lra*I enzyme and 1 μg of plasmid DNA for 1 h at 37°C. To measure the activity of purified *Lra*I enzyme at different temperatures 1 U of purified *Lra*I enzyme was incubated with 1 μg of plasmid DNA for 1 h at different temperatures (13°C, 23°C, 30°C, 37°C, 45°C, 60°C, and 80°C). In all endonuclease activity assays commercial *Eco*RI restriction enzyme (Thermo Fisher Scientific) was used as control. Reactions were stopped by addition 1/10 volume of stop solution (50 mM EDTA pH 8, 50% glycerol, 0.02% orange G) and products were analyzed by electrophoresis in 1% agarose gels.

2.6. Determination of LraI Cleavage Site. To determine the precise positions and nucleotide sequence of cleavage sites within double stranded DNA for the *Lra*I restriction enzyme, pBluescript SK+ was used as template. Plasmid was digested with recombinant enzyme *Lra*I; complete digestion was confirmed by agarose gel electrophoresis and digest was sequenced with M13F and M13R primers (Macrogen Europe, The Netherlands). Simultaneously as control, the whole experiment was conducted with commercial *Eco*RI restriction enzyme (Thermo Fisher Scientific).

2.7. Bioinformatic Analysis of LraI Homologs. Sequence searches on the NCBI nucleotide and protein databases were conducted with BLAST [22] using *lraIR*/LraIR and *lraIM*/LraIM sequences. The phylogenetic inferences between restriction and methylase enzymes were obtained by MEGA version 6.0 (http://www.megasoftware.net/). The first 30 protein reference sequences of *Eco*RI-like endonuclease or methyltransferase enzymes chosen according to results of BLASTP search and LraI restrictase and LraI methylase sequences separately were trimmed and aligned using Clustal W [23] with default parameters. The phylogenetic trees were constructed by the maximum-likelihood (ML) method using a Tamura-Nei model. Bootstrapping of 1000 replicates was used to infer confidence levels of ML trees.

The nucleotide sequences of DNA fragments carrying genes encoding *Lra*I restriction-modification system and 16S rRNA from *L. raffinolactis* BGTRK10-1 were submitted to ENA GenBank under accession numbers LT222052 and LT854837, respectively.

3. Results and Discussion

3.1. Identification of LraI (EcoRI-Like) Methylase Activity in L. raffinolactis BGTRK10-1. The mesophilic lactic acid bacterium *L. raffinolactis* is prevalent in dairy foods, such as raw milks, natural dairy starter cultures, and a great variety of cheeses. *L. raffinolactis* BGTRK10-1 is a natural isolate from autochthonous young sweet kajmak produced in the Vlašić mountain region of central Bosnia and Herzegovina [17]. Strain BGTRK10-1 was selected because of its strong autoaggregation phenotype. In order to construct cosmid library of strain BGTRK10-1 to clone aggregation ability coding gene(s), total DNA of the strain was digested with several restriction enzymes (including *Eco*RI). It has been observed that the *Eco*RI did not cut isolated DNA, in several attempts, unlike the other used restriction enzymes. It was suspected that the strain possesses RM system (named Lra *L. raffinolactis*) that recognizes the same DNA sequence as *Eco*RI RM system. Hence, the methylase activity of the strain *L. raffinolactis* BGTRK10-1, which protects its DNA from the digestion by *Eco*RI restriction enzyme, was quite accidentally discovered during routine laboratory work.

Restriction endonucleases, commonly known as restriction enzymes, are ubiquitously present in prokaryotes. The main function of restriction enzymes is the protection against foreign genetic material, especially against bacteriophage DNA. Several restriction-modification systems have been identified in lactococci. Most of them are plasmid encoded and function as phage-resistance mechanism, which is very important for the strains used in the dairy industry in terms of preventing phage infection and cell lysis [25–29].

3.2. Selection of Clone Carrying LraI RM Operon from Cosmid Library. Cosmid DNA was isolated from total *Xba*I cosmid library in *E. coli* HB101 and 1 μg of DNA mix from total cosmid clones was subjected to digestion with *Eco*RI restriction enzyme and after that directly transformed into DH5α competent cells. Cosmid DNA isolated from obtained transformants was rechecked for resistance to *Eco*RI restriction enzyme digestion. One cosmid, named pAZILcosLra, providing resistance to *Eco*RI restriction enzyme digestion was selected for further analyses: subcloning and DNA sequencing (Table 1).

3.3. LraI Operon for RM System Provides Resistance to EcoRI Restriction Enzyme Digestion. To localize the minimum genetic unit on the cosmid pAZILcosLra that is responsible for the resistance to digestion with *Eco*RI restriction enzyme, the cosmid pAZILcosLra was digested with several restriction enzymes (*Xba*I-generated four fragments, *Hin*dIII-three

fragments, *Cla*I-four fragments, and *Eco*RV-three fragments) and then subcloned into pBluescript SK+ vector digested with corresponding restriction enzymes. Only one construct, pBSLraCla (obtained with *Cla*I), was able to reestablish the resistance to *Eco*RI restriction enzyme digestion (Table 1). The *Cla*I DNA fragment of 4239 bp carrying complete information for resistance to *Eco*RI digestion was completely sequenced by primer walking. Four complete (*Eco*RI-like endonuclease, *Eco*RI-like methylase, hypothetical protein, and site specific integrase), one truncated (pentapeptide repeat containing protein), and one partial (N(5)-(carboxyethyl) ornithine synthase) open reading frame (ORFs) were revealed on *Cla*I DNA fragment (Figure 1). Position of LraI RM operon in genome of strain BGTRK10-1 indicates the possibility that the operon was acquired by horizontal gene transfer; the conserved lactococcal gene for pentapeptide repeat containing protein is interrupted in the middle by LraI RM operon and immediately after methylase gene is located gene for site specific integrase. This event that occurred in the distant past is indicated by the fact that additional mutations were accumulated within the first part of the gene for pentapeptide repeat containing protein, most probably due to its nonfunctionality. The distance between the restrictase and the methylase genes is 10 nucleotides without promoter and ribosomal binding site and, in other EcoRI-like operons, strongly indicates polycistronic RNA transcription from upstream promoter and translation from consensus RBS (AGGAGA) 4 nucleotides distant from ATG codon of restrictase gene.

To confirm the functionality of *Lra*I restriction-modification operon, a region that includes both (*lraIR* and *lraIM*) genes was amplified using LraRM-Fwand LraRM-Rev primers (for details see Section 2.2) and cloned into pAZIL vectors giving construct pAZIL-LraRM. Construct carrying only these two genes was completely sequenced while resistance to *Eco*RI restriction enzyme digestion was confirmed *in vitro*.

3.4. Cloning, Overexpression, and Purification of LraI Restriction Endonuclease. Plasmid clone pAZIL-LraRM was used as matrix for amplification of the open reading frame encoding *Lra*I restriction endonuclease with primers LraI-Fw and LraI-Rev. Since *Hin*dIII restriction site has been integrated into LraI-Rev primer, obtained amplified fragment was first treated with *Hin*dIII to provide directed cloning of PCR fragment into expression vector pMAL-c5X, which was digested with *Xmn*I and *Hin*dIII restriction enzymes. Ligation mix was transformed into ER2523 cells which were previously transformed with a pAZIL-LraRM vector expressing *Lra*I methylase, in order to protect transformed cells from the nuclease activity of *Lra*I towards their own. Transformants of ER2523/pAZIL-LraRM with pMAL-cX5LraI were successfully obtained when selection was carried out at 23°C on LA selective plates (erythromycin 300 μg/ml and ampicillin 100 μg/ml) containing 2% glucose in order to minimise expression of enzymes. Three clones (named pMAL-cX5LraI-29, pMAL-cX5LraI-31, and pMAL-cX5LraI-42, Table 1) were selected for restriction enzyme analysis, complete sequencing, and overexpression of enzyme. *Lra*I restriction nuclease was successfully overexpressed in all three clones by overnight induction with 0.1 mM IPTG at 23°C and purified using amylose resins and cleaved by Xa protease (which cleaves fusion protein between maltose binding protein and clone providing release of exactly the same protein as natural). The overexpression of *Lra*I restriction enzyme under aforementioned conditions (overnight induction with 0.1 mM IPTG at 23°C) represents the result that is similar to results observed by other researchers [30]. The possible explanation for this could be the expression of restriction enzymes is toxic at higher temperatures.

Purified *Lra*I restriction enzymes from all three clones were stored at −20°C in CM buffer with 50% glycerol.

3.5. Functional Analysis, Determination of Ionic Strength, and Temperature Optimum of the Purified LraI Endonuclease Activity. Considering that *Lra*I RM system provided complete protection against digestion of *Eco*RI endonuclease, it was assumed that it recognizes and cleaves the identical nucleotide sequence. Since plasmid pBluescipt SK+ contains one *Eco*RI restriction site in polycloning region it was used for functional analysis of purified *Lra*I restriction enzyme. Specific activity (1 U) of purified *Lra*I restriction enzyme was determined in *Eco*RI reaction buffer (Thermo Fisher Scientific) at 37°C. Different levels of *Lra*I restriction enzyme expression were observed in selected clones, but specific activities (U/μg of purified proteins) were almost the same (1 U/50 ± 5 ng) among the clones pMAL-cX5LraI-29, pMAL-cX5LraI-31, and pMAL-cX5LraI-42 (Figure 2).

To determine optimal temperature for *Lra*I activity, purified enzyme was incubated with pBluescipt SK+ vector at temperatures ranging from 13°C to 80°C. *Lra*I enzyme showed the highest activity at lower temperatures (between 13°C and 37°C), while at 45°C and higher temperatures it partially cut DNA (Figure 3). However, this is in agreement with the optimal growth temperature of strain BGTRK1-10 (30°C). Briefly, since the first description of *Eco*RI restriction enzyme in 1970 [31], more than 500 isoschizomers have been reported or predicted with very high levels of identity (50–70%) pointing to the widespread distribution among species of different Phyla and indicating possible common origin. It seems that some specific characteristics, such as optimum working temperature, diverged depending on the optimum growth temperature of the enzyme producing bacteria, which is why we think that *Lra*I exhibits better activity at lower temperatures. It was found that commercial *Eco*RI (used as control) showed high activity at 45°C in contrast to *Lra*I enzyme pointing to the difference between these two enzymes.

In addition, stability of *Lra*I enzyme was tested after different period of storage at −20°C; *Lra*I enzyme did not lose activity after storage for more than six months at −20°C in CM buffer with 50% glycerol.

To establish the optimal salt concentration in the reaction buffer for *Lra*I enzyme activity different commercial buffers, Buffer B, Buffer G, Buffer O, Buffer R, Buffer Tango, and buffer recommended for use with *Eco*RI (Thermo Fisher Scientific) were used. *Lra*I enzyme exhibited high and specific activity in buffers with 100 mM and higher salt concentrations (Figure 4, buffer recommended for use with *Eco*RI, Buffer 2x Tango

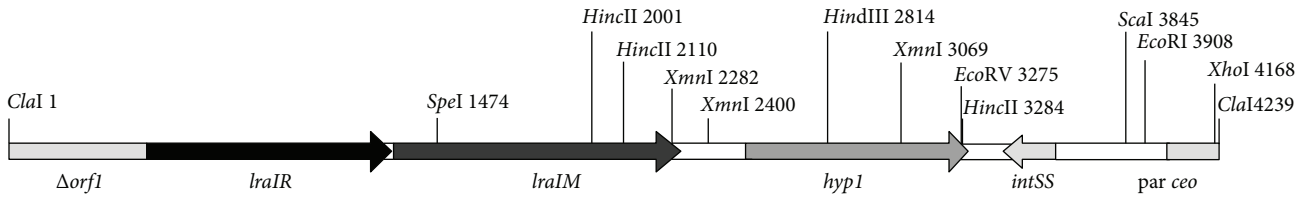


Figure 1: Schematic presentation of *Cla*I DNA fragment of 4239 bp carrying complete information for providing resistance to *Eco*RI digestion. *Cla*I DNA fragment containing following ORFs: truncated gene for pentapeptide repeat containing protein (Δ*orf1*), *Eco*RI-like endonuclease *(lraIR)*, *Eco*RI-like methylase *(lraIM)*, hypothetical protein *(hyp1)*, site specific integrase *(intSS)*, and partial gene for N(5)-(carboxyethyl) ornithine synthase (par *ceo*).

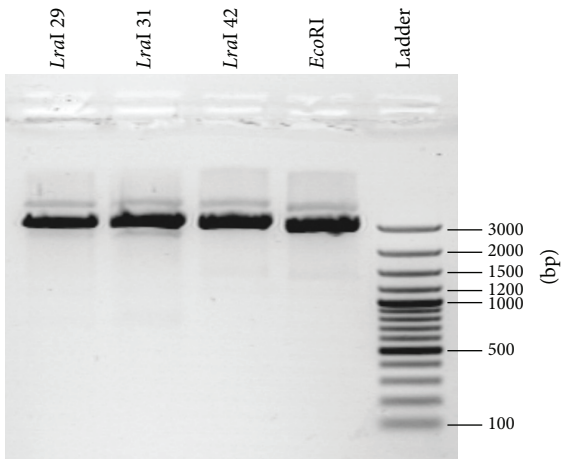


Figure 2: *Lra*I activity assay. Digestion of pBluescipt SK+ by purified *Lra*I restriction enzyme *Lra*I 29, *Lra*I 31, and *Lra*I 42 from clones pMAL-cX5LraI-29, pMAL-cX5LraI-31, and pMAL-cX5LraI-42, respectively.

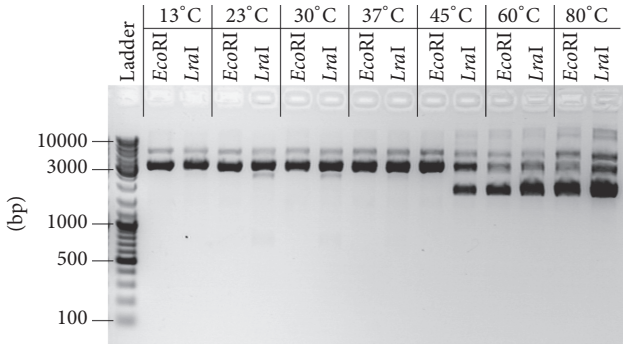


Figure 3: Determination of temperature optimum of the purified *Lra*I restriction enzyme activity. Commercial *Eco*RI restriction enzyme was used in control reactions.

and Buffer O), except in Buffer R (red) (10 mM Tris-HCl pH 8.5, 10 mM magnesium chloride, 100 mM potassium chloride, and 100 μg/ml BSA) (Figure 4; Buffer R). It was noticed that, in buffers with lower ionic strengths, the *Lra*I enzyme exhibited a specific star activity, cutting the vector at another position (Figure 4; Buffer 1x Tango, Buffer B, Buffer G).

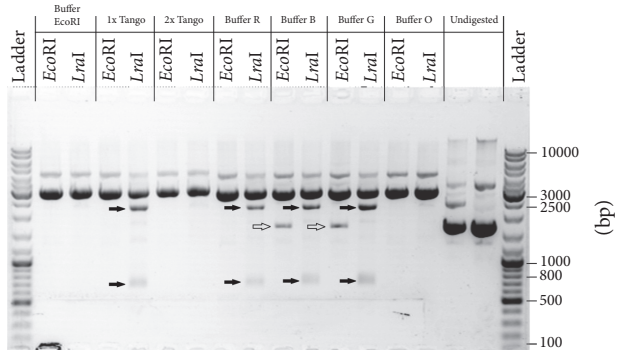


Figure 4: Determination of influence of ionic strength in different reaction buffers on the *Lra*I restriction enzyme activity. Commercial *Eco*RI restriction enzyme was used in control reactions. L: ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific). Black arrows present fragment obtained by *Lra*I∗ activity; white arrows present undigested plasmid DNA in *Eco*RI (Thermo Fisher Scientific) digestion.

It is interesting that in Buffer B and Buffer G commercial *Eco*RI restriction enzyme exhibited weaker activity, partial digestion.

3.6. LraI Is an Isoschizomer of EcoRI. Sequencing of double stranded cleaved DNA by the *Lra*I enzyme has shown that the *Lra*I enzyme recognizes the identical nucleotide sequence (5′-G/AATTC-3′), as expected, and cuts it at the same position (between G and A) like *Eco*RI enzyme (Figure 5) leaving identical sticky ends. The same cleavage results were obtained with *Lra*I enzymes purified from all three clones which supported our conclusion that *Lra*I enzyme is an isoschizomer of *Eco*RI.

3.7. Determination of the Cleavage Site of LraI∗ Activity. One of the important characteristics of restriction enzymes is their high sequence specificity in order to adequately provide the function of protecting the genome integrity. In addition, it was established that restriction enzymes in nonoptimal conditions could exhibit a modified specificity so that the same restriction enzyme could recognize and cleave DNA at additional positions to canonical one [32]. For *Eco*RI restriction enzyme, it was detected that in at low ionic strength

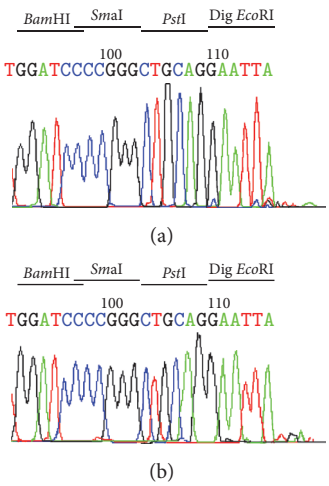


Figure 5: Determination of the *Lra*I cleavage site 5′-G/AATTC-3′ of pBluescipt SK+ by DNA sequencing. (a) Sequence of pBluescipt SK+ predigested with *Lra*I restriction enzyme and (b) with *Eco*RI obtained using M13R primer. The colour-coded sequence traces are A (green), T (red), C (blue), and G (black). The extra A base was added at the end of the cleaved template by the *Taq* DNA polymerase used for sequencing due to template-independent terminal nucleotide transferase activity of *Taq* DNA polymerase.

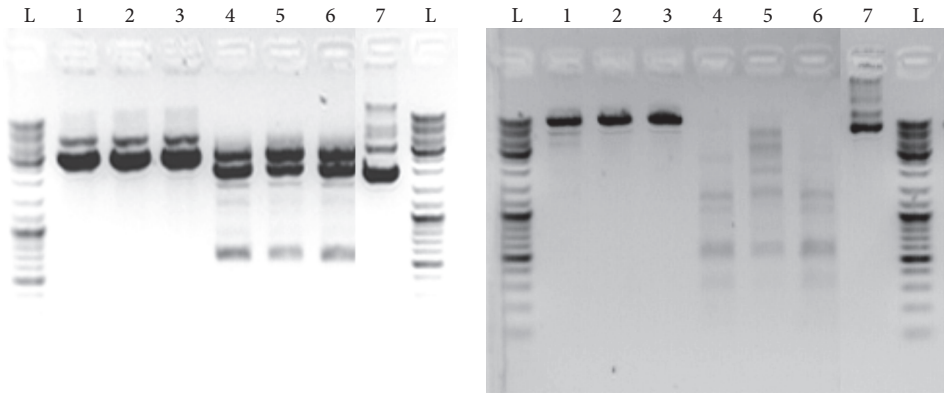
and high pH enzyme is transformed so that it recognizes and digests the shortened tetranucleotide sequence 5′-/AATT-3′ [32]. This activity is termed star activity and is usually labeled as "∗" adjacent to the enzyme name and was also detected for other restriction enzymes and could be used to cleave DNA at additional sites for cloning purposes. In order to be used for cloning star restriction enzyme activity should (i) be restricted to limited number of sequence variants, (ii) provide the same sticky ends for ligation into the vector, and (iii) be controlled by changeable conditions.

To determine the cleavage site of *Lra*I∗ activity, the fragment of 672 bp obtained after digestion of pBluescipt SK+ in buffer with low ionic strength (1x Tango; Thermo Fisher Scientific) (Figure 6(a)) was cloned into pAZIL vector predigested with *Lra*I. Isolated plasmids (named pAZIL-LraI∗672pBS; Table 1) from selected white colonies were first checked for the presence of DNA fragment of 672 bp by restriction analysis with *Lra*I enzyme and then sequenced. The sequence of the entire fragment as well as the adjacent regions of the vector showed that the cloned 672 bp fragment originating from pBluescipt SK+ was cut out by *Lra*I∗ enzyme activity at positions (701 *Eco*RI) and at additional position (29) in which the 5′-AAATTC-3′ sequence is present. The same sequencing results were obtained for three independent clones confirming that additional recognition and cleavage site for *Lra*I∗ activity is 5′-A/AATTC-3′ sequence. To be sure that only AAATTC sequence is recognized and cleaved by *Lra*I∗ activity, but not other sequences with alternative changes within the GAATTC site, a search analysis for the presence of other alternatives in plasmid sequences was performed. Since all alternatives with one nucleotide change of recognition sequence exist in pBluescipt SK+ (in addition to AAATTC, at position 29, the following recognition sequences GAATTG, at position 647; CAATTC, at position 850; TAATTC, at position 2824 were found), but were not cleaved, we conclude that *Lra*I∗ activity specifically recognizes and cleaves only one variant of degenerate recognition sequence (5′-A/AATTC-3′). To further test the conclusion obtained on pBluescipt SK+, the plasmid pAZIL sequence was analyzed and subjected to digestion by *Lra*I enzyme under conditions that induce star activity. The obtained digestion results were completely in correlation with the predicted expectations (nine positions/fragments) (Figure 6(b)), so we could conclude that *Lra*I∗ activity is limited to only one variant of the recognition sequence giving identical cohesive ends as in optimal conditions making the resulting fragments after *Lra*I∗ activity clonable without further processing into *Lra*I or *Eco*RI treated vectors.

*Lra*I restriction enzyme star activity meets all the given requirements: it recognizes only one variant of the sequence which can enable more precise restriction mapping and cloning, provides the same cohesive ends compatible with *Eco*RI (contained by most cloning vectors), and is completely controlled by low ion concentration and/or high pH. Both factors which induce star activity of *Lra*I restriction enzyme, low ionic strength (Buffer 1x Tango) and buffer with higher pH (Buffer R, pH 8.5), also influence on *Eco*RI, but, in contrast, *Lra*I∗ recognizes only one additional sequence expressing more specific star activity.

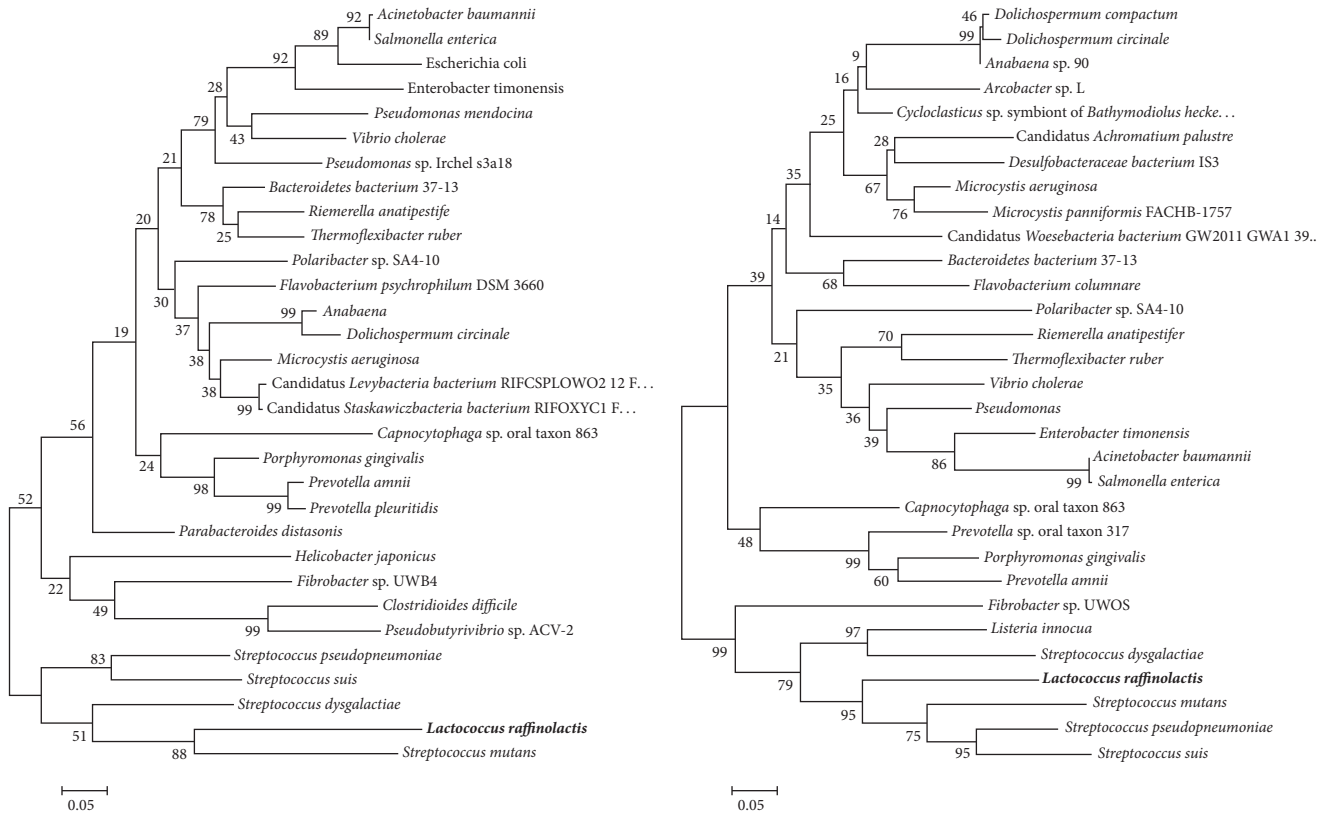
3.8. Phylogenetic Similarity of LraI Restriction-Modification Enzymes with Others Belonging to EcoRI-Like Group. A search for number of restriction enzymes recognizing 5′-GAATTC-3′ sequence present in REBASE revealed 526 putative *Eco*RI-like proteins. Protein BLAST analysis showed that 196 restriction enzymes in NCBI database (from various microorganisms) share more than 50% identity on at least 50% protein coverage with *Lra*I enzyme. Highest identity was observed with restriction endonucleases from streptococci (*Streptococcus suis* 68%, *Streptococcus dysgalactiae* 64%, and *Streptococcus mutans* 64%). It is interesting that similar but higher identity was observed for *Lra*I methylase, again with streptococci (*Streptococcus pseudopneumoniae* 74%, *Streptococcus suis* 73%, *Streptococcus mutans* 73%, and *Streptococcus dysgalactiae* 68%). Similar percentage of identity was observed also at nucleotide level (about 70%) for both *Lra*I restrictase and methylase genes. The fact that most *Eco*RI isoschizomers, unlike other restriction enzymes, share a high level of identity, indicates their common origin [7].

Phylogenetic trees were constructed for both *Lra*I restrictase (Figure 7(a)) and methylase (Figure 7(b)) enzymes. Phylogenetic analysis showed that *Lra*I homologs (restriction endonucleases and methylases) can be divided into two main branches, one (that could be additionally subdivided, comprising close homologs (Gram-negative bacteria and Cyanophyta) and the other comprising homologs from species belonging to Firmicutes phylum. The position of genus *Fibrobacter* (phylum: Fibrobacteres) which consists of only two species is interesting; its restrictase enzyme belongs to one branch, while methylase protein belongs to the other (Figure 7).



(a) (b)

Figure 6: Determination of the cleavage site of *Lra*I∗ activity. (a) Digestion of pBluescipt SK+ vector by *Lra*I 29 (1 and 4), *Lra*I 31 (2 and 5), and *Lra*I 42 (3 and 6) in buffer recommended for use with *Eco*RI (1, 2, and 3) and in 1x Tango Buffer (4, 5, and 6); 7: undigested pBluescipt SK+; digestion of pAZIL vector by *Lra*I 29 (1 and 4), *Lra*I 31 (2 and 5), and *Lra*I 42 (3 and 6) in buffer recommended for use with *Eco*RI (1, 2, and 3) and in 1x Tango Buffer (4, 5, and 6); 7: undigested pAZIL vector; L: ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific).



(a) (b)

Figure 7: Phylogenetic similarity of *Lra*I RM system with other belonging to *Eco*RI-like group constructed using a Tamura-Nei model. (a) Phylogenetic tree for *Lra*I restrictase; (b) phylogenetic tree for *Lra*I methylase enzymes.

4. Conclusions

We identified a potent Type II restriction endonuclease in *L. raffinolactis* BGTRK10-1, named *Lra*I. The recognition and cleavage sequence for *Lra*I restriction enzyme was determined as 5′-G/AATTC-3′, indicating that *Lra*I restriction enzyme is an isoschizomer of *Eco*RI but with different characteristics. One of characteristics that has been thoroughly studied is star activity of *Lra*I restriction enzyme that is limited to one variant of the recognition site and cuts another alternative sequence 5′-A/AATTC-3′ leaving the same sticky ends on fragments as *Eco*RI, making the fragments obtained after digestion easy to clone without additional processing.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The Ministry of Education, Science and Technological Development of the Republic of Serbia, Serbia, supported this work (Grant No. 173019).

References

[1] T. A. Bickle and D. H. Kruger, "Biology of DNA restriction," *Microbiology and Molecular Biology Reviews*, vol. 57, no. 2, pp. 434–450, 1993.

[2] A. B. Hickman, Y. Li, S. V. Mathew, E. W. May, N. L. Craig, and F. Dyda, "Unexpected structural diversity in DNA recombination: The restriction endonuclease connection," *Molecular Cell*, vol. 5, no. 6, pp. 1025–1034, 2000.

[3] M. Mücke, G. Grelle, J. Behlke, R. Kraft, D. H. Krüger, and M. Reuter, "EcoRII: A restriction enzyme evolving recombination functions?" *EMBO Journal*, vol. 21, no. 19, pp. 5262–5268, 2002.

[4] T. Naito, K. Kusano, and I. Kobayashi, "Selfish behavior of restriction-modification systems," *Science*, vol. 267, no. 5199, pp. 897–899, 1995.

[5] I. Kobayashi, "Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution," *Nucleic Acids Research*, vol. 29, no. 18, pp. 3742–3756, 2001.

[6] R. J. Roberts, T. Vincze, J. Posfai, and D. Macelis, "REBASE—a database for DNA restriction and modification: enzymes, genes and genomes," *Nucleic Acids Research*, vol. 38, pp. D234–D236, 2010.

[7] A. Pingoud, G. G. Wilson, and W. Wende, "Type II restriction endonucleases - A historical perspective and more," *Nucleic Acids Research*, vol. 42, no. 12, pp. 7489–7527, 2014.

[8] A. Pingoud and A. Jeltsch, "Structure and function of type II restriction endonucleases," *Nucleic Acids Research*, vol. 29, no. 18, pp. 3705–3727, 2001.

[9] T. I. Tikchonenko, E. V. Karamov, B. A. Zavizion, and B. S. Naroditsky, "EcoRI* activity: Enzyme modification or activation of accompanying endonuclease?" *Gene*, vol. 4, no. 3, pp. 195–212, 1978.

[10] M. Nasri and D. Thomas, "Relaxation of recognition sequence of specific endonuclease HlndIII," *Nucleic Acids Research*, vol. 14, no. 2, pp. 811–821, 1986.

[11] M. Nasri and D. Thomas, "Alteration of the specificity of pvuII restriction endonuclease," *Nucleic Acids Research*, vol. 15, no. 19, pp. 7677–7687, 1987.

[12] F. Barany, "The TaqI 'star' reaction: strand preferences reveal hydrogen-bond donor and acceptor sites in canonical sequence recognition," *Gene*, vol. 65, no. 2, pp. 149–165, 1988.

[13] J. Bitinaite and I. Schildkraut, "Self-generated DNA termini relax the specificity of SgrAI restriction endonuclease," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 99, no. 3, pp. 1164–1169, 2002.

[14] R. J. Roberts and K. Murray, "Restriction endonuclease," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 4, no. 2, pp. 123–164, 1976.

[15] F. H. Stephenson, B. T. Ballard, H. W. Boyer, J. M. Rosenberg, and P. J. Greene, "Comparison of the nucleotide and amino acid sequences of the RsrI and EcoRI restriction endonucleases," *Gene*, vol. 85, no. 1, pp. 1–13, 1989.

[16] T. Chuluunbaatar, T. Ivanenko-Johnston, M. Fuxreiter et al., "An EcoRI-RsrI chimeric restriction endonuclease retains parental sequence specificity," *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, vol. 1774, no. 5, pp. 583–594, 2007.

[17] A. Terzic-Vidojevic, S. Mihajlovic, G. Uzelac et al., "Characterization of lactic acid bacteria isolated from artisanal Travnik young cheeses, sweet creams and sweet kajmaks over four seasons," *Food Microbiology*, vol. 39, pp. 27–38, 2014.

[18] B. Jovčić, J. Begović, J. Lozo, L. Topisirović, and M. Kojić, "Dynamics of sodium dodecyl sulfate utilization and antibiotic susceptibility of strain Pseudomonas sp. ATCC19151," *Archives of Biological Sciences*, vol. 61, no. 2, pp. 159–164, 2009.

[19] M. Kojic, B. Jovcic, I. Strahinic et al., "Cloning and expression of a novel lactococcal aggregation factor from Lactococcus lactis subsp. lactis BGKP1," *BMC Microbiology*, vol. 11, article 265, 2011.

[20] D. A. Hopwood, J. M. Bib, J. F. Chater et al., *Genetic Manipulation of* Streptomyces*: A Laboratory Manual*, 1985.

[21] D. Hanahan, "Studies on transformation of *Escherichia coli* with plasmids," *Journal of Molecular Biology*, vol. 166, no. 4, pp. 557–580, 1983.

[22] S. F. Altschul, T. L. Madden, A. A. Schäffer et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.

[23] M. A. Larkin, G. Blackshields, N. P. Brown et al., "Clustal W and clustal X version 2.0," *Bioinformatics*, vol. 23, no. 21, pp. 2947-2948, 2007.

[24] H. W. Boyer and D. Roulland-dussoix, "A complementation analysis of the restriction and modification of DNA in Escherichia coli," *Journal of Molecular Biology*, vol. 41, no. 3, pp. 459–472, 1969.

[25] C. Hill, K. Pierce, and T. R. Klaenhammer, "The conjugative plasmid pTR2030 encodes two bacteriophage defense mechanisms in lactococci, restriction modification (R+/M+) and abortive infection (Hsp+)," *Applied and Environmental Microbiology*, vol. 55, no. 9, pp. 2416–2419, 1989.

[26] S. Moineau, S. Pandian, and T. R. Klaenhammer, "Restriction/modification systems and restriction endonucleases are more effective on lactococcal bacteriophages that have emerged recently in the dairy industry," *Applied and Environmental Microbiology*, vol. 59, no. 1, pp. 197–202, 1993.

[27] D. J. O'Sullivan, K. Zagula, and T. R. Klaenhammer, "In vivo restriction by LlaI is encoded by three genes, arranged in an operon with llaIM, on the conjugative Lactococcus plasmid

pTR2030," *Journal of Bacteriology*, vol. 177, no. 1, pp. 134–143, 1995.

[28] N. Nyengaard, F. K. Vogensen, and J. Josephsen, "Restriction-modification systems in lactococcus lactis," *Gene*, vol. 157, no. 1-2, pp. 13–18, 1995.

[29] A. Madsen and J. Josephsen, "Cloning and characterization of the lactococcal plasmid-encoded type II restriction/modification system, LlaDII," *Applied and Environmental Microbiology*, vol. 64, no. 7, pp. 2424–2431, 1998.

[30] D.-H. Chung, J. R. Huddleston, J. Farkas, and J. Westpheling, "Identification and characterization of CbeI, a novel thermostable restriction enzyme from Caldicellulosiruptor bescii DSM 6725 and a member of a new subfamily of HaeIII-like enzymes," *Journal of Industrial Microbiology and Biotechnology*, vol. 38, no. 11, pp. 1867–1877, 2011.

[31] R. J. Roberts, "How restriction enzymes became the workhorses of molecular biology," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 102, no. 17, pp. 5905–5908, 2005.

[32] B. Polisky, P. Greene, D. E. Garfin, B. J. McCarthy, H. M. Goodman, and H. W. Boyer, "Specificity of substrate recognition by the EcoRI restriction endonuclease," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 72, no. 9, pp. 3310–3314, 1975.

Hindawi
BioMed Research International
Volume 2018, Article ID 6870139, 8 pages
https://doi.org/10.1155/2018/6870139

Research Article

Manipulation of Plant Growth Regulators on Phytochemical Constituents and DNA Protection Potential of the Medicinal Plant *Arnebia benthamii*

Javid A. Parray,[1] Azra N. Kamili,[2] Sumira Jan,[3] Mohammad Yaseen Mir,[2] Nowsheen Shameem,[2] Bashir A. Ganai,[2] Elsayed Fathi Abd_Allah,[4] Abeer Hashem,[5] and Abdulaziz A. Alqarawi[4]

[1]*Department of Environmental Science, Govt. SAM Degree College Budgam, Jammu and Kashmir 191111, India*
[2]*Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir 190006, India*
[3]*ICAR-CITH, Rangreth, Srinagar, Jammu and Kashmir 190003, India*
[4]*Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia*
[5]*Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia*

Correspondence should be addressed to Elsayed Fathi Abd_Allah; eabdallah@ksu.edu.sa

Received 18 August 2017; Accepted 28 November 2017; Published 4 January 2018

Academic Editor: Gamal Enan

Arnebia benthamii of the family Boraginaceae is a critically endangered nonendemic plant of the Kashmir Himalayas and is used to treat a number of human diseases. The current study was based on developing an *in vitro* micropropagation protocol vis-à-vis induction of various secondary metabolites under *in vitro* conditions for the possible biological activity. A tissue culture protocol was developed for *A. benthamii* for the first time in the Himalayan region using varied combinations and proper media formulations, including various adjuvants: Murashige and Skoog (MS) media, growth hormones, sugars, agar, and so forth. The influence of different media combinations was estimated, and the MS + thidiazuron (TDZ) + indole 3-acetic acid (IAA) combination favors a higher regeneration potential. The higher amounts of chemical constituents were also recorded on the same treatment. The *in vitro* plant samples also showed a noteworthy effect of scavenging of hydroxyl radicals vis-à-vis protection from oxidative DNA damage. The *in vitro* raised plants are good candidates for the development of antioxidant molecules.

1. Introduction

Medicinal plants are vital curative agents for curing human ailments. The overexploitation of natural plant resources encouraged various programs such as conservation, micropropagation, and incremental plant architecture [1, 2]. Looking at the present scenario, a significant proportion of the Northwest Himalayan (NWH) medicinal herbs, particularly *Arnebia benthamii* (Wall. ex G. Don) Johnston [syn. *Macrotomia benthamii* (Wall.) DC.], have been rendered rare and threatened over the past few years because the demand for various plant products has increased. Even though different initiatives such as habitat protection as well as Seed and Field gene banks have been started, these techniques may still prove inadequate. The tissue culture technique has become established as a very valuable methodology among the different conservation and multiplication practices presently being employed for increasing the number and improving the development of numerous economically important and threatened medicinal plants [2–5]. *A. benthamii* (Wall. ex G. Don) Johnston [syn. *Macrotomia benthamii* (Wall.) DC.] of the family Boraginaceae, locally known as Kahzaban, is a perennial medicinal herb growing in the subalpine and alpine zones of the Northwest Himalayas [6]. It is an important medicinal plant, prominent upon the list of 59 medicinal herbs that have been prioritized for conservation in NWH [6] due to their high extinction threat [7]. It is an alpine herb occurring at an altitude of 3500–4000 m, from Kashmir

to Western Nepal, normally on open slopes with stony or rocky substrates. In Kashmir Valley, it is confined to certain areas such as Sinthon Top, Duksum, Karnah, Gurez, Lolab, Sonamarg, and Kargil and is rarely found [8, 9]. It is classified as a critically endangered nonendemic plant of Kashmir [10, 11]. *A. benthamii* has been listed in the Indian Red Data Book due to its overexploitation for various purposes [12, 13]. Numerous secondary metabolites (i.e., shikonin and its intermediates, alkannins, other naphthoquinones, etc.) have been reported from *Arnebia* spp. However, "Gule Khazaban," a very expensive medicine, is derived from *A. benthamii* [11, 13, 14]. *Arnebia* spp. are reported to be traditional ayurvedic medicines for the treatment of diseases of the throat and tongue, fevers, and cardiac symptoms. The flowers are known to have a comforting effect on heart patients [11], while its roots have antiseptic and antibiotic properties [9]. Conservation of the existing germplasm of such a useful and threatened species is, therefore, a strategic need. Harborne and Baxter [15] have documented the anticancer properties of Arnebin 1 and Arnebin 3 isolated from the *Arnebia* species, and anti-HIV activity has been reported in *Arnebia euchroma* extracts [16].

Due to the higher incidence of microbial pathogens in foods and to control the antibiotic resistant bacterial strains by novel plant extracts [17, 18], search should be continued to propagate medicinal plants by tissue culture biotechnology and to purify their medicinally active compounds for development of a novel treatment of pathogens [19]. Consequently, the study employed herein was undertaken to develop a tissue culture protocol for micropropagation of the depleted germplasm of *A. benthamii*. The study also endeavors to establish the effect of plant growth hormones on the induction of chemical constituents and antioxidant potential of *in vitro* raised plants.

2. Materials and Methods

2.1. Chemicals Required. MS medium, plant growth hormones (BAP/IAA/TDZ/IAA/NAA), mercuric chloride, agar, and solvents such as methanol (HPLC), water (HPLC), and ethyl acetate (HPLC) were purchased from *HiMedia*, *Merck,* and others.

2.2. Plant Collection and Authentication. The whole plants with roots and seeds of *A. benthamii* were collected from Sinthon Top of the Kashmir region (India) (3748 m a.s.l.). The plants were collected in the months from July to September 2013. The plants were authenticated at Kashmir University Herbarium, Centre of Plant Taxonomy, with accession number 1748.

2.3. Sterilization of Seeds and Media Selection. Seeds were used as experimental material. Seeds soaked overnight were washed with a few drops of laboratory detergent (Labolene) and 2-3 drops of Tween 20 (surfactant) after washing under running tap water. Chemical sterilization of the seeds was achieved by treating them with 0.1% streptomycin (20 min) followed by 0.1% $HgCl_2$ (5 min) and 70% ethanol (45 sec). The seeds were washed 3-4 times with autoclaved double-distilled water to remove all traces of sterilant before inoculating on Murashige and Skoog (MS) basal medium [20] containing 3% (w/v) sucrose *(HiMedia)* and 0.8% (w/v) Difco Bacto Agar *(HiMedia)*. Different plant growth hormones such as thidiazuron (TDZ), indole 3-acetic acid (IAA), kinetin (Kn), 6-benzylamino purine (BAP), and indole 3-butyric acid (IBA) were augmented with MS medium for various morphogenetic responses. The media were sterilized at 121°C for 20 min, and the pH of the medium was adjusted to approximately 5.5. The cultures were incubated in a culture room maintained at 25 ± 3°C with relative humidity of 60–70%. The whole plantlets after cleaning with sterile distilled water were transplanted into pots containing peat-vermiculite-sand-soil mixture (ratio 1 : 1 : 1 : 1, v/v). All the experiments were done in RBD manner. The number of shoots and their length per plant were recorded.

2.4. Analysis of Various Chemical Constituents. In this trial, the four best plant hormonal combinations were selected based on higher shootlet formation. The four selected treatments were assigned as T1, T2, T3, and T4 and were compared with plants (T0) collected from the natural habitat. Different metabolites were analyzed.

2.5. Alkaloids. The plant samples obtained from different cultural conditions were dried and macerated in a grinder to a powder form and 2.5 g of each plant sample was extracted with ethanol (200 ml) with 20% acetic acid added for 4 h. The filtrate was concentrated to approximately 25 ml and conc. NH_4OH was added for precipitate formation. The precipitate was further washed with dilute NH_4OH. The precipitate was dried and weighed, and the amount of alkaloids was determined. The filtrate was dried and weighed [21, 22].

2.6. Phenolics. The plant sample powder (1 g) was extracted with 80% ethanol (20 ml). The supernatant obtained was evaporated to dryness and was resuspended in 5 ml of H_2O. Folin's reagent (0.5 ml) was added to different aliquots (0.1–1 ml), followed by Na_2CO_3 (2 ml). A final volume up to 5 ml was obtained with dist. water. The tubes were kept in a water bath for 1 min and slightly vortexed. The reading was taken at 650 nm with standard catechol (2 mg%) [23].

2.7. Tannins. The plant sample (2 g) of plant powder was extracted 3 times with acetone (acetone 70%). The supernatant taken was diluted to 3 ml by dist. water. Then, different components in 0.1 M HCl (i.e., 0.016 M K_3 $[Fe(CN)_6]$ (1 ml) and 0.02 M $FeCl_3$ (1 ml)) were added. The tubes were kept for 15 min after vortexing. The stabilizer solution (5 ml water, H_3PO_4, and 1% gum arabic [3 : 1 : 1]) was added, and the solution was revortexed. The reading was taken at 700 nm, and the concentration was determined against gallic acid (1.9 mg%) [24].

2.8. Total Flavonoid Content. 0.5 ml of the supernatant from the plant sample solution (2 mg/2 ml) was mixed with water (2 ml) and then 5% $NaNO_2$ solution (0.15 ml) was added. Then, 10% $AlCl_3$ solution (0.15 ml) was added and allowed to stand for 6 min followed by 4% NaOH solution (2 ml), and

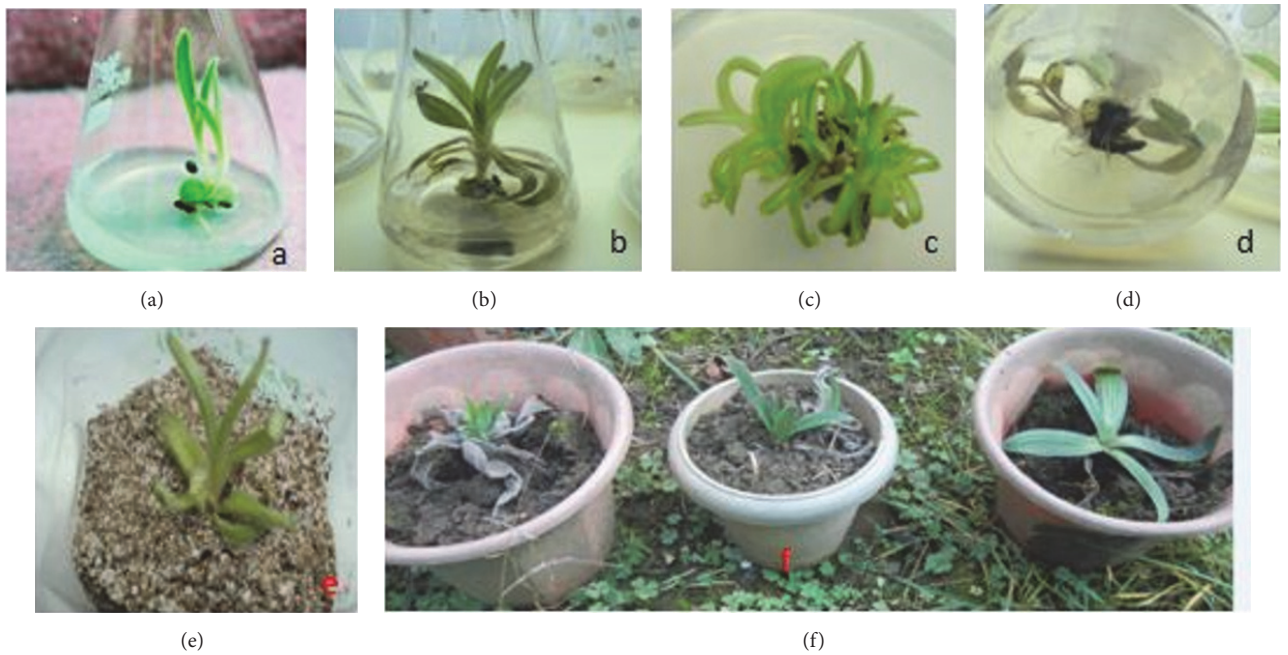


Figure 1: *In vitro* culture of *Arnebia benthamii* Wall. (a) seed germination on 1/2-MS + 5 μM BA; (b) shootlet elongation on 20 μM BA; (c) shootlet formation on 4 μM BA + 1 μM IBA; (d) root formation on 7 μM BA; (e) plantlets in a 1 : 1 mixture of autoclaved sand : soil; (f) plantlets in net house.

the final volume was made up to 5 ml with water and allowed to stand for 15 min. Absorbance was checked at 510 nm versus a water blank [25].

2.9. DNA Damage Assay. The antioxidant potential was determined by observing the DNA protection ability of the *in vitro* plant samples [14]. 15 μl of calf thymus DNA (25 mg%) was dissolved in 20.0 mM phosphate buffer saline (pH 7.4), and to this solution, different concentrations of plant extracts were added and the mixture was incubated for 15 min at room temperature. Then, the oxidative substances (i.e., 1 μl of 30 mM H_2O, 1 μl of 20 mM ferric nitrate, and 1 μl of 100 mM ascorbic acid) were added, and the whole reaction was held for 1 h at 37°C. The reaction was stopped by the addition of a loading dye (40% sucrose and 0.25% bromophenol blue). Gel electrophoresis (0.7% agarose/TAE buffer) was run at 100 V for observing changes in DNA and was visualized by the Gel Doc system (Labnet, Germany).

2.10. Statistical Analysis. The whole data set was subjected to ANOVA by SPSS software, and significance was set at $P < 0.005$.

3. Results and Discussion

3.1. Seed Germination and Shootlet Formation. The sterilized seeds were inoculated on MS full- and half-strength medium supplemented with gibberellic acid GA_3 (1.5–20 μM). Complete germination with elongated shoots was observed on 1/2 MS + GA_3 (15 μM). The minimal time period required for seed germination initiation was recorded as 20 days with 78% seed germination response to the same treatment (Table 1 and Figure 1(a)). Embryonic shoot tips were excised and further cultured on a multiplication medium using auxin/cytokinin combinations. The cytokinins BAP and TDZ (1.5–10.5 μM) were used separately in combination with either IAA or NAA (in the conc. range of 1–10 μM) on MS ×1/2 medium for the multiplication of shoots. The concentration of BAP (4.5 μM) and IAA (4 μM) resulted in the average number of multiple shoots (9.1) exhibiting slightly blackish basal portion. With a further increase in concentration, the number of shoots showed a decreasing trend and the lowest number of shoots was noticed. In another trial, the higher average number of multiple shoots (11) with broad and healthy shoots was obtained on MS 1/2 + BAP (5.5 μM) + NAA (5 μM) (Figures 1(b) and 1(c)). Shoot tips of *A. benthamii* cultured on MS ×1/2 basal medium supplemented with different concentrations of TDZ and IAA/NAA resulted in multiple shoot regeneration with a friable brownish callus at the basal zone of the plant tissues, which was quite significant compared with previous trials. The best response was scored with shoot tips on 3 μM of TDZ and 1.5 μM of IAA which resulted in an average of 20.1 multiple shoots with slight friable callus formation, and with a further increase in concentrations up to 10 μM, the shoot number was found to decrease (Table 2). In another trial, shoot tips cultured with different concentrations of TDZ were used in combination with NAA (0.5–10.0 μM) for multiplication of shoots and the maximum average number of shoots (15.3) with 90% shooting response on MS 1/2 + TDZ (5.0) + NAA (2.5) with low friable brownish callus formation

Table 1: Effect of GA_3 enriched MS medium on seed germination of *Arnebia benthamii* Wall.

Growth medium	Germination response	Time period (days) for seed germination initiation	Percentage seed germination response (%)
MS	NR	-	-
MS ×1/2	NR	-	-
MS ×1/2 + GA_3 (1.5 μM)	NR	-	-
MS ×1/2 + GA_3 (2.5 μM)	NR	-	-
MS ×1/2 + GA_3 (5 μM)	Seed germination	45	35
MS ×1/2 + GA_3 (7.5 μM)	Seed germination	40	35
MS ×1/2 + GA_3 (10 μM)	Seed germination	40	48
MS ×1/2 + GA_3 (12.5 μM)	Complete seedling formation	34	53
MS ×1/2 + GA_3 (15 μM)	Complete seedling formation with elongated shoots	20	78
MS ×1/2 + GA_3 (17.5 μM)	Complete seedling formation with elongated shoots	30	54
MS ×1/2 + GA_3 (20 μM)	Seed germination with stunted shoot formation	45	39

Note. Data scored after 10 weeks of culture period. NR: no response.

at the basal end. However, the response to the multiple shootlet formation was lower compared to the TDZ/IAA combination.

Different concentrations of IAA/NAA used along with BAP/TDZ resulted in multiple shoot regeneration and further increase in concentration, resulting in a decreased shoot number. Shoot tips of *A. benthamii* cultured on MS ×1/2 basal medium supplemented with different concentrations of TDZ and IAA/NAA resulted in 20.1 times the average number of multiple shoots. Such results are quite similar to the earlier reports on *Crocus sativus* [2], *Hyoscyamus niger* [26], and *Cichorium intybus* [4, 5], where TDZ was found to be effective in enhancing the shootlet formation. However, further increase in both auxin/cytokinin concentrations was not found to be favorable. Similarly, organogenesis was also noticed from the leaf-derived callus of *Arnebia euchroma* [27] and *Bergenia ciliata* [28]. The additive effect of IAA and TDZ has been seen in numerous plants such as *Santolina canescens* [29] and *Bupleurum fruticosum* [30], which confirms that even the least auxin concentrations in combination with a cytokinin positively tailored the frequency of shoot induction and plant growth. In the present study, the combined interaction of BAP and IBA resulted in maximum multiple shoot regeneration, suggesting that their interaction perhaps resulted in shifts in the endogenous synthesis of TDZ and IAA, making it either suboptimal or supraoptimal and resulting in fulfilling varying needs in the exogenous supply of both hormones [31]. Therefore, in *A. benthamii,* the optimum level of exogenously supplied phytohormones to maximum shoot multiplication has been registered to be 4 μM TDZ and 4.5 μM IAA.

3.2. Root Hardening and Field Trial. The elongated and semielongated shoots recorded from the multiplication phase were transferred to MS ×1/2 medium supplemented with different concentrations of IBA/IAA (1.0–10.0 μM) with TDZ (0.5 μM–5.0 μM) for induction and elongation of roots (Table 3). Elongation as well as root initiation was noticed after a 6–8-week culture period. The basal zone of the elongated shoots first turned black and apparently became hard. Lower concentrations of IBA (1–2.0 μM) failed to promote any desired response. However, when the concentration was raised, the basal zone of the shoots became blacker, hard, and elongated, thus giving a tap root-like appearance, followed by the initiation of lateral root initials, and a maximum of 5.3 multiple black short, thick rootlets were recorded from the main black hard tap root such as structures on 8 μM of IBA and TDZ (4 μM). Further increase in concentration again had no effect on rooting. When IBA was replaced with IAA (1.0–10.0 μM), the basal zone of the shoots again became hard, resembling a tap root-like structure in different concentrations of IAA. This combination was fruitful for the root generation with a maximum average number of roots (8.3) noticed on 3 μM of IAA and TDZ (1.5 μM) (Table 3 and Figure 1(d)). However, when the concentration was raised from 3 μM to 5.0 μM, no response was noticed. In some treatments, a friable light brownish callus of various degrees was also recorded at different concentrations. A protocol for the complete plantlets formation under *in vitro* conditions and their successful transfer into the greenhouse has been developed. The cytokinins used alone as well in combination with auxins resulted in certain desirable responses, but the most suitable plant growth regulator was found to be the combination of BAP and IBA and TDZ and IAA where maximum multiple shoots were recorded.

The *in vitro* raised plantlets were successfully transferred and kept in a growth chamber present in the laboratory at the requisite temperature and humidity. Initially, caps of the culture vials were removed and culture vials containing *in vitro* born plantlets of *A. benthamii* were kept in the incubation room for 1 week to slowly reduce the high humidity conditions within the culture vials. After

Table 2: Effect of different plant growth regulators on shoot multiplication from *in vitro* raised shoot tips of *A. benthamii* on MS ×1/2 medium.

BAP (μM)	TDZ (μM)	IAA (μM)	NAA (μM)	Shoot number	Shoot length (cm)	Callusing (%)	Number of days for minimum shoot formation ($n = 3$)	Minimum shooting response (%)
-	-	-	-	-	-		-	-
1.5		1.0		3.1 ± 1.07^{a}	2.0 ± 0.97^{a}	+++	55	80
2.5		2.0	-	5.3 ± 0.87^{b}	2.3 ± 1.32^{a}	+	53	80
3.5		3.0	-	8.3 ± 2.9^{c}	3.5 ± 1.1^{b}	+	44	75
4.5		4.0	-	9.1 ± 2.13^{c}	3.4 ± 0.76^{b}	+	40	70
5.5		5.0	-	3.2 ± 0.56^{ab}	2.1 ± 0.63^{a}	+	50	80
6.5		6.0	-	1.1 ± 0.16^{a}	2.0 ± 0.87^{a}	+++	-	-
3.5			3.0	4.5 ± 1.3^{b}	2.9 ± 0.13^{ab}	+++	50	90
4.5			4.0	8.5 ± 3.2^{c}	3.3 ± 1.3^{b}	++	45	90
5.5			5.0	11.0 ± 3.1^{d}	3.6 ± 0.76^{b}	++	45	85
6.5			6.0	12.4 ± 2.6^{d}	4.4 ± 1.98^{b}	++	35	85
7.5			7.0	11.2 ± 2.1^{d}	3.65 ± 2.1^{a}	++	30	80
8.5			8.0	9.7 ± 1.4^{c}	3.0 ± 1.5^{b}	++	46	80
9.5			9.0	5.6 ± 1.65^{b}	1.3 ± 0.33^{a}	+++	52	90
10.5			10.0	2.2 ± 0.8^{a}	1.25 ± 0.78^{a}	+++	-	-
	1.0	0.5		9.0 ± 1.8^{c}	3.13 ± 1.8^{b}	+	40	90
	2.0	1.0		14.2 ± 2.6^{d}	3.32 ± 1.32^{b}	++	37	100
	3.0	1.5		20.1 ± 3.9^{e}	5.6 ± 1.6^{c}	+	28	100
	4.0	2.0		16.0 ± 2.0^{de}	5.3 ± 0.5^{c}	+	27	100
	5.0	2.5		12.03 ± 1.4^{d}	4.8 ± 0.32^{b}	+	35	90
	6.0	3.0		8.9 ± 2.3^{c}	3.0 ± 0.97^{b}	+	37	90
	7.0	3.5		5.2 ± 1.2^{b}	3.0 ± 1.0^{b}	+	40	90
	8.0	4.0		3.2 ± 1.6^{ab}	2.7 ± 1.43^{a}	++	40	90
	3.0		1.5	10.5 ± 2.0^{cd}	3.0 ± 1.3^{b}	++	44	80
	4.0		2.0	13.0 ± 1.5^{d}	3.5 ± 1.9^{b}	++	35	80
	5.0		2.5	15.3 ± 1.09^{d}	4.8 ± 1.5^{b}	+	30	90
	6.0		3.0	9.9 ± 2.76^{c}	4.4 ± 1.2^{b}	++	30	95
	7.0	-	3.5	5.3 ± 1.23^{b}	3.1 ± 0.56^{b}	++	38	85

Note. Data scored after 12 weeks of culture period. Data represented as mean ± SD ($n = 10$); + = low intensity callus formation; ++ = moderate intensity callus formation; +++ = high intensity callus formation. Data was statically analyzed using Duncan multiple range test by SPSS 17.0 software. The values followed by different superscripts are statically significant with each other at $P < 0.05$. TDZ: thidiazuron; IAA: indole 3-acetic acid; Kn: kinetin; BAP: 6-benzylamino purine; IBA: indole 3-butyric acid.

these plantlets were transferred from incubation to normal room conditions where *in vitro* plantlets were deflasked, agar was washed under tap water with the help of mild brushing. The plantlets were then potted in small plastic cups containing an autoclaved sand : soil : peat : vermiculite (1 : 1 : 1 : 1) mixture (Figure 1(e)). The potted plantlets were placed in a growth chamber at a temperature of 20–25°C with humidity of 60–70% for one to two weeks followed by their transfer to a greenhouse chamber. These plantlets were then shifted to the greenhouse and were kept there for more than one month. Then, the plantlets were transferred to a net house. The plantlets were continuously monitored, and they showed a response in their growth and development. The plants started showing growth, which indicated their acclimatization behavior. The number of plants transferred to the greenhouse and then into the field was 35 and 64 in 2013-14 and 2014-15, respectively. The survival percentage of plants in the greenhouse was recorded as 75 and 83%, while in the field the number was approximately 60 and 67%, respectively (Table 4 and Figure 1(f)). The tissue culture technique is being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable germplasm threatened by extinction. An efficient procedure for *in vitro* multiplication is an essential prerequisite for employing *in vitro* techniques for germplasm conservation. The present investigation carried out on shoot tips of *A. benthamii* offers a potentially efficient protocol for mass propagation and conservation of this medicinal herb. There is only one published report on *in vitro* studies of *A. benthamii* [32], and the current study uses extensive trials pertaining to the use of growth hormones in

Table 3: Effect of different concentrations of auxins (IBA/IAA) with TDZ on root regeneration of *Arnebia benthamii* from multiple shoots.

TDZ (μM)	IBA (μM)	IAA (μM)	Root number	Callusing (%)	Rooting response (%)
1.0	0.5	-	-		-
2.0	1.0	-	-		-
3.0	1.5	-	1.0 ± 0.12^{a}	++	70
4.0	2.0	-	1.8 ± 0.55^{a}	+++	80
5.0	2.5	-	2.5 ± 0.76^{b}	++	80
6.0	3.0	-	2.6 ± 0.3^{a}	++	85
7.0	3.5	-	3.7 ± 0.2^{bc}	++	85
8.0	4.0	-	5.3 ± 0.0^{c}	+	65
1.0	-	0.5	1.9 ± 0.6^{ab}	++	80
2.0	-	1.0	3.2 ± 0.5^{b}	+++	80
3.0	-	1.5	8.3 ± 0.0^{d}	-	85
4.0	-	2.0	7.4 ± 0.0^{cd}	-	70
5.0	-	2.5	6.0 ± 0.0^{c}	-	80
6.0	-	3.0	3.3 ± 0.0^{b}	+	70
7.0	-	3.5	2.12 ± 0.5^{b}	+	65
8.0	-	4.0	0.5 ± 0.04^{a}	+++	75

Data scored after 12 weeks of culture period. Data represented as mean ± SD ($n = 10$); + = low intensity callus formation; ++ = moderate intensity callus formation; +++ = high intensity callus formation. Data was statically analyzed using Duncan multiple range test by SPSS 17.0 software. The values followed by different superscripts are statically significant with each other at $P < 0.05$. TDZ: thidiazuron; IAA: indol 3-acetic acid; IBA: indole 3-butyric acid.

Table 4: Survival percentage of *in vitro* raised plants in greenhouse and field.

Year	Number of plants	Greenhouse (% survival)	*Field (% survival)
2013-14	35	75	60
**2014-15	64	83	67

*Values are % of greenhouse survival (%); **values are cumulative of 2013 and 2015.

addition to successful hardening of plantlets as well. Rooting was observed from multiple shoots and initiation of lateral roots and maximum 8.3 multiple black short thick rootlets was recorded from the main black hard tap root-like structure on 3 μM of IBA and TDZ (4 μM). In earlier reports on *A. euchroma*, rooting was noticed under the influence of IBA [27, 32, 33], and hence our results are in accordance with these. In the present case, IAA was found to be most effective for rooting compared to IBA and NAA. The efficacy of IAA in rooting may be due to its faster uptake than NAA in the present studies.

In this experimental line, the four best plant hormonal combinations were selected based on higher shootlet formation. The four selected treatments were assigned as T1 = BAP (4 μM) + IAA (4 μM), T2 = BAP (6.5 μM) + NAA (6 μM), T3 = TDZ (3 μM) + IAA (1.5 μM), and T4 = BAP (5 μM) + NAA (2.5 μM) and were compared with plants

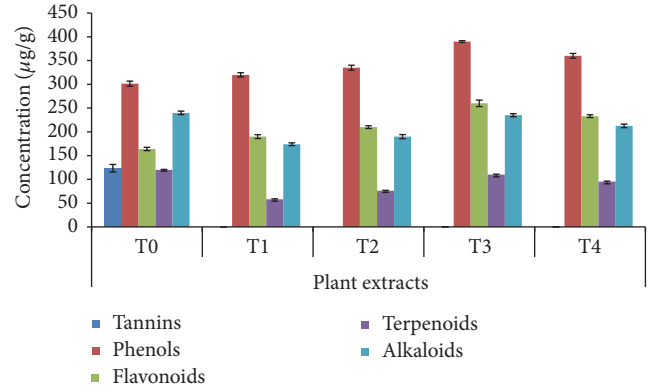


Figure 2: Comparative quantitative estimation of major metabolites present in the *in vitro* plants obtained from various media compositions.

(T0 = wild plants) collected from the natural habitat. The results of phytochemical analysis of different types of shoots resulting from application of varieties of media containing various combinations of plant growth regulators (PGRs) are depicted in Figure 2. In the current study, the highest induction of secondary metabolites was observed in T3, obtaining plants with higher amounts of phenols (390 μg/g) followed by flavonoids (260 μg/g) and alkaloids (235 μg/g), which may be due to the influence of the specific plant growth regulator used in the media [34]. PGR has a significant impact on regulation of biosynthetic pathways in plants for synthesis of various metabolites. In addition, the *in vitro* raised plants were recorded to have a higher content of some essential compounds such as phenolic substances, alkaloids, and flavonoids. The results are in line with the findings of various researchers who also report that the PGRs may also influence the production of secondary metabolites under *in vitro* conditions [35, 36]. For example, the accumulation of alkamide and CADs in *Echinacea angustifolia* increased upon addition of cytokines to the culture medium. The amplified biosynthesis of metabolites is allied with tissue differentiation, that is, a clump formation in cell suspensions or the formation of more complex structures [34], which may explain the accumulation of high amounts of metabolites in cultured shoots in our study. However, tannins (123.5 μg/g) and terpenoids (120 μg/g) were found to be higher in T0 plants (Figure 2). The overall discrepancy between the field and *in vitro* plants may be due to a number of factors such as seasonal variation [32], plant-to-plant variation in chemical content, and variation in agroclimatic conditions [37]. It appears to be pertinent to accept the *in vitro* system which may serve as an alternative source of metabolites and thus may be exploited for efficient generation of such substances throughout the year, which are pharmacologically promising but are severely limited in production [14, 38].

3.3. Biological Activity. The oxidative substances completely cause DNA degradation (Figure 3). The scavenging of radicals in different ethyl acetate extracts of *in vitro* raised plants of *Arnebia benthamii* protecting the DNA against damage by ascorbic acid (standard) was determined. In the current

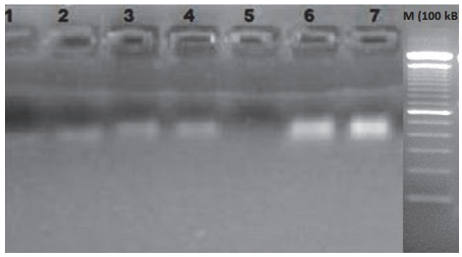


Figure 3: Protective effect of DNA through scavenging of radicals by *A. benthamii* extracts. Lane 1: native calf thymus (ct) DNA + reaction mixture + T1 plant samples (500 μg/ml). Lane 2: native calf thymus (ct) DNA + T2 plant samples (500 μg/ml). Lane 3: native calf thymus (ct) DNA + reaction mixture + T3 plant samples (500 μg/ml). Lane 4: native calf thymus (ct) DNA + reaction mixture + T4 plant samples (500 μg/ml). Lane 5: native calf thymus (ct) DNA + reaction mixture. Lane 6: native calf thymus DNA. Lane 7: native calf thymus DNA + ascorbic acid (500 μg/ml) + reaction mixture.

study, methanol extracts of all samples obtained from various media combinations under *in vitro* conditions showed significant scavenging of the OH radicals and vis-à-vis protection from DNA damage in ct DNA (Lanes 1–4). The current findings are in continuation of the previous reports on the DNA protective effect of field grown plants of *A. benthamii* [14, 39] suggesting the role of some bioactive compounds in scavenging of hydroxyl radicals [13]. This is the first report depicting the DNA damage protection ability of *in vitro* raised plants, which might be due to the presence of various secondary metabolites as determined above.

4. Conclusions

In this study, the first ever-complete protocol for the *in vitro* regeneration and acclimatization under field conditions of *A. benthamii*, a critically endangered medicinal plant of NWH, was developed, leading to a conservation plan for the endangered species. The *in vitro* raised plants, particularly the PGR specific growth, had a significant impact on the presence of volatile/nonvolatile metabolites. The biological efficacy of plant extracts allows their use in drug formulations against possible oxidative DNA damage.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was funded by DST, GoI, New Delhi, with Sanction Letter no. DST/SSTP/J&K/11/146(G) Dtd. 26/04/2012. Their assistance is deeply appreciated. The first author is also thankful to the assistance of SERB-DST GoI for providing financial support under Grant no. YSS/2014/000590. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research group NO (RGP-271).

References

[1] J. Purkayastha, T. Sugla, A. Paul et al., "Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in Jatropha curcas," *Biologia Plantarum*, vol. 54, no. 1, pp. 13–20, 2010.

[2] J. A. Parray, A. N. Kamili, R. Hamid, and A. M. Husaini, "*In vitro* cormlet production of saffron (*Crocus sativus* L. Kashmirianus) and their flowering response under greenhouse," *GM Crops & Food*, vol. 3, no. 4, pp. 289–295, 2012.

[3] J. A. Parray, A. N. Kamili, Z. A. Reshi, R. A. Qadri, and S. Jan, "Interaction of rhizobacterial strains for growth improvement of *Crocus sativus* L. under tissue culture conditions," *Plant Cell, Tissue and Organ Culture*, vol. 121, no. 2, pp. 325–334, 2015.

[4] R. Hamid, A. N. Kamili, M. Zaffar, J. A. T. da Silva, A. Mujib, and J. A. Parray, "Callus mediated shoot organogenesis from shoot tips of *Cichorium intybus*," *Med Arom Plant Sci Biotechnol*, vol. 4, no. 1, pp. 84–86, 2010.

[5] R. Hamid, A. N. Kamili, Mahmooduzzafar, S. Gücel, M. Öztürk, and P. Ahmad, "Analysis of physiobiochemical attributes, some key antioxidants and esculin content through HPLC in *in vitro* grown *Cichorium intybus* L. treated with ethylmethane sulfonate," *Plant Growth Regulation*, vol. 76, no. 3, pp. 233–241, 2015.

[6] A. R. K. Sastry and S. Chatterjee, "Prioritization of medicinal plants of India," in *Setting Biodiversity Conservation Priorities for India*, S. Singh, A. R. K. Sastry, R. Mehta, and V. Uppal, Eds., pp. 467–473, World Wide Fund for Nature, India, 2000.

[7] S. Manjkhola and U. Dhar, "Conservation and utilization of *Arnebia benthamii* (Wall, ex G. Don) Johnston—A high value Himalayan medicinal plant," *Current Science*, vol. 83, no. 4, pp. 484–488, 2002.

[8] G. H. Dar and A. A. Khuroo, "Floristic diversity in the Kashmir Himalaya: progress, problems and prospects," *Sains Malaysiana*, vol. 42, no. 10, pp. 1377–1386, 2013.

[9] N. Shameem, A. N. Kamili, J. A. Parray, R. Hamid, and S. A. Bandh, "Antimicrobial and antioxidant activity of methanol extracts of *Arnebia benthamii* (Wall ex. G. Don) Johnston—a critically endangered medicinal plant of North western Himalaya," *Journal of Analytical Science and Technology*, vol. 6, no. 1, pp. 1–8, 2015.

[10] IUCN, "Guidelines for using the IUCN red list categories and criteria. version 8.1," Standards and Petitions Subcommittee, 2010.

[11] G. D. Dar, R. C. Bhagat, and I. A. Khan, *Biodiversity of the Kashmir Himalaya*, Biodiversity of the Kashmir Himalaya, Srinagar, India, 2002.

[12] S. K. Jain and A. R. K. Shastry, *The Indian Plant Red Data Book*, Botanical Survey of India, Howrah, India, 1984.

[13] S. A. Ganie, A. Jan, S. Muzaffar, B. A. Zargar, R. Hamid, and M. A. Zargar, "Radical scavenging and antibacterial activity of *Arnebia benthamii* methanol extract," *Asian Pacific Journal of Tropical Medicine*, vol. 5, no. 10, pp. 766–772, 2012.

[14] J. A. Parray, A. N. Kamili, R. Hamid, Z. A. Reshi, and R. A. Qadri, "Antibacterial and antioxidant activity of methanol extracts of *Crocus sativus* L. c.v. Kashmirianus," *Frontiers in Life Science*, vol. 8, no. 1, pp. 40–46, 2015.

[15] S. B. Harborne and H. Baxter, "Phytochemical dictionary: a handbook of bioactive compounds from plants," *Economic Botany*, vol. 48, no. 3, pp. 258-258, 1993.

[16] Y. Kashiwada, M. Nishizawa, T. Yamagishi et al., "Anti-AIDS agents, 18. Sodium and potassium salts of caffeic acid tetramers

from *Arnebia euchroma* as anti-HIV agents," *Journal of Natural Products (Lloydia)*, vol. 58, no. 3, pp. 392–400, 1995.

[17] A. A.-R. Ismaiel, A. E.-S. Ali, and G. Enan, "Incidence of listeria in egyptian meat and dairy samples," *Food Science and Biotechnology*, vol. 23, no. 1, pp. 179–185, 2014.

[18] T. I. El-Sayed, D. Atef, M. Amer, A. Mahdy, and G. Enan, "Molecular characterization and inhibition by natural agents of multidrug resistant Candida strains causing vaginal candidiasis," *Research Journal of Medical Sciences*, vol. 9, no. 1, pp. 1–7, 2015.

[19] G. Enan, S. Hamdy, S. Abdel-Shafi, and A. R. Al-Mohammadi, "Biological characteristics and inhibition by both natural agents and antibiotics of *Streptococcus pyogenes*," *Research Journal of Medical Sciences*, vol. 10, pp. 573–586, 2016.

[20] T. Murashige and F. Skoog, "A revised medium for rapid growth and bioassay with tobacco tissue culture," *Plant Physiology*, vol. 15, pp. 473–497, 1962.

[21] A. J. Harborne, *Phytochemical Methods- A Guide to Modern Techniques of Plant Analysis*, Chapman and Hall, London, UK, 2nd edition, 1984.

[22] D. E. Okwu and C. Josiah, "Evaluation of the chemical composition of bryophyllum pinnatum," *Journal of Science*, vol. 6, pp. 30–37, 2006.

[23] C. P. Mallick and M. B. Singh, *PlanT Enzymology And Histoenzymology*, vol. 286, Kalyani publishers, New Delhi, India, 1980.

[24] H. D. Graham, "Stabilization of the Prussian blue color in the determination of polyphenols," *Journal of Agricultural and Food Chemistry*, vol. 40, no. 5, pp. 801–805, 1992.

[25] C. Chang, M. Yang, H. Wen, and J. Chern, "Estimation of total flavonoid content in propolis by two complementary colorimetric methods," *Journal of Food Drug Analaysis*, vol. 10, pp. 178–182, 2002.

[26] R. R. Quadri, A. N. Kamili, A. M. Shah, and J. A. Da Silva, "Effect of benzyl adenine, kinetin and thidiazuron on *in vitro* shoot proliferation of Hyoscyamus niger L," *Med Aroma Plant Sci Biotechnol*, vol. 6, no. 1, pp. 81–83, 2012.

[27] S. Manjkhola, U. Dhar, and M. Joshi, "Organogenesis, embryogenesis, and synthetic seed production in *Arnebia euchroma*—A critically endangered medicinal plant of the Himalaya," *In Vitro Cellular & Developmental Biology - Plant*, vol. 41, no. 3, pp. 244–248, 2005.

[28] S. Rafi, A. N. Kamili, B. A. Ganai, M. Y. Mir, and J. A. Parray, "Morpho-biochemical evaluation of EMS regenerated mutants of Bergenia ciliata (Haw.) Sternb. under *in vitro* conditions," *Journal of Nature and Natural Sciences*, vol. 1, no. 01, pp. 1–4, 2016.

[29] J. P. Casado, M. C. Navarro, M. P. Utrilla, A. Martínez, and J. Jiménez, "Micropropagation of *Santolina canescens* Lagasca and *in vitro* volatiles production by shoot explants," *Plant Cell, Tissue and Organ Culture*, vol. 69, no. 2, pp. 147–153, 2002.

[30] D. Fraternale, L. Giampe, D. Ricci, and B. L. Rocchi, "Micropropagatioo of Bupleurum fruttcosum: the effect of rriacontanol," *Plant Cell, Tissue and Organ Culture*, vol. 69, pp. 134–140, 2002.

[31] S. Bhan, *Tissue Culture*, Minai Publications, New Delhi, India, 1998.

[32] R. R. Quadri, A. N. Kamili, A. M. Shah, and A. J. Da Silva, "In vitro multiplication of *Arnebia benthamii* Wall. A critically endangered medicinal herb of the Western Himalaya," *Functional Plant Science and Biotechnology*, vol. 6, no. 1, pp. 54–57, 2012 (Chinese).

[33] J. Bo, Y.-G. Yang, Y.-M. Guo, Z.-C. Guo, and Y.-Z. Chen, "Thidiazuron-induced *in vitro* shoot organogenesis of the medicinal plant Arnebia euchroma (Royle) Johnst," *In Vitro Cellular & Developmental Biology—Plant*, vol. 41, no. 5, pp. 677–681, 2005.

[34] N. Chakraborty, D. Banerjee, M. Ghosh et al., "Influence of plant growth regulators on callus mediated regeneration and secondary metabolites synthesis in *Withania somnifera* (L.) Dunal," *Physiology and Molecular Biology of Plants*, vol. 19, no. 1, pp. 117–125, 2013.

[35] C. Kiferle, M. Lucchesini, A. Mensuali-Sodi, R. Maggini, A. Raffaelli, and A. Pardossi, "Rosmarinic acid content in basil plants grown *in vitro* and in hydroponics," *Central European Journal of Biology*, vol. 6, no. 6, pp. 946–957, 2011.

[36] A. Matkowski, "Plant *in vitro* culture for the production of antioxidants—a review," *Biotechnology Advances*, vol. 26, no. 6, pp. 548–560, 2008.

[37] V. Ciddi, "Withaferin A from cell cultures of *Withania somnifera*," *Indian Journal of Pharmaceutical Sciences*, vol. 68, no. 4, pp. 490–492, 2006.

[38] S. Jan, A. N. Kamili, J. A. Parray, and Y. S. Bedi, "Differential response of terpenes and anthraquinones derivatives in *Rumex dentatus* and *Lavandula officinalis* to harsh winters across north-western Himalaya," *Natural Product Research (Formerly Natural Product Letters)*, vol. 30, no. 5, pp. 608–612, 2016.

[39] S. A. Ganie, T. A. Dar, R. Hamid et al., "*In vitro* antioxidant and cytotoxic activities of *Arnebia benthamii* (Wall ex. G. Don): a critically endangered medicinal plant of Kashmir valley," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 792574, 8 pages, 2014.