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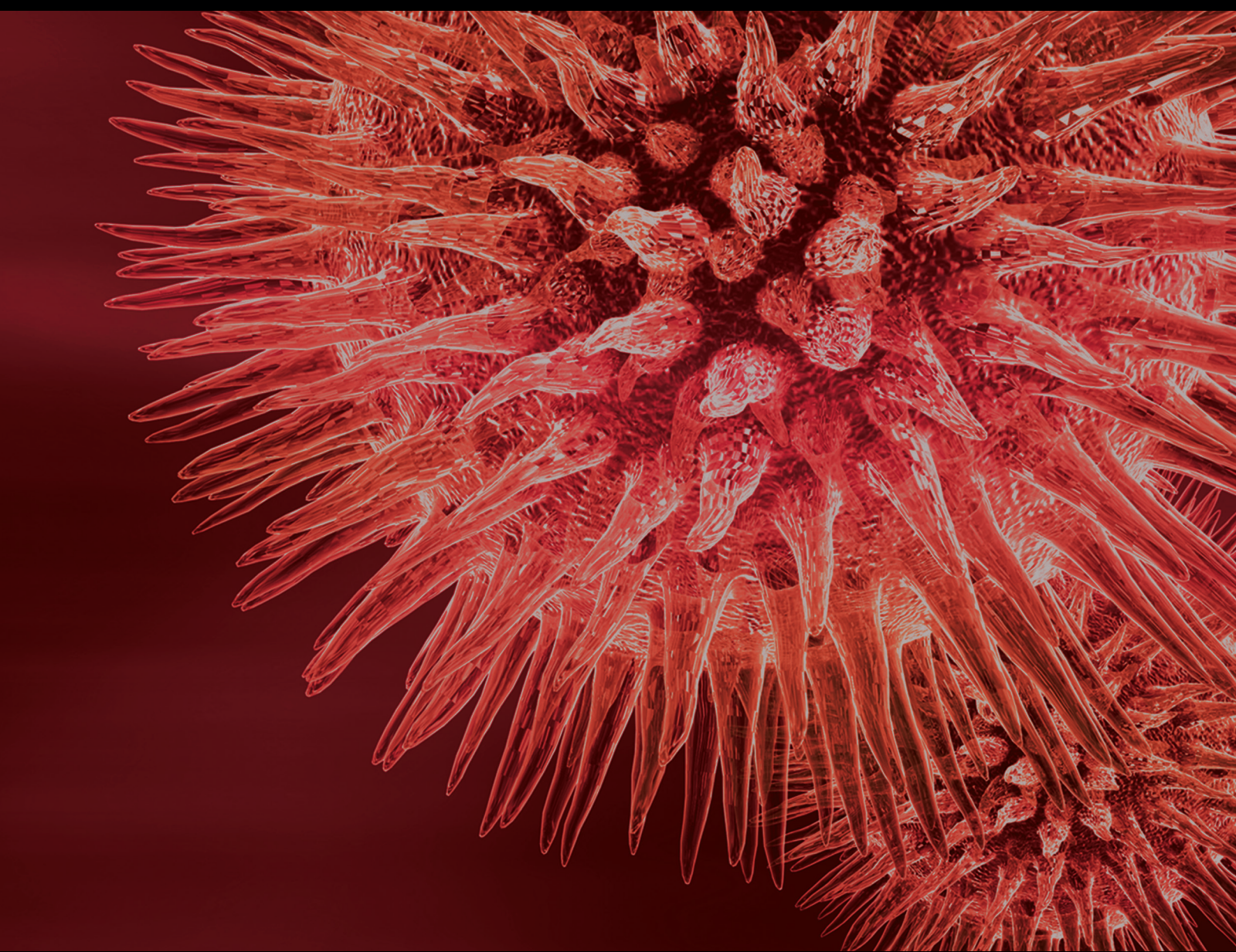

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Contents

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Editorial

New Insights into and Updates on Antimicrobial Agents from Natural Products

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Nowadays, microorganisms play a remarkable role in promotion of science; it has provided a platform for young and established researchers to interact and learn more about the advanced research in microbiology. Worldwide, microorganisms represent a really thoughtful subject on itself; moreover, it can contribute to the science and sustainable development. Therefore, microorganisms are usually considered as a drug-target. The interactions between drugs, their target and their respective direct effects, are generally the way to be explored. In contrast, the microorganism's responses to antimicrobials drug treatments that contribute to cell death are not as well understood and have proven to be quite complex, involving multiple genetic and biochemical pathways.

Furthermore, microorganisms have been used as tools to explore fundamental life processes by researchers. Due to some advantages (e.g., rapid growth, growth manipulation, easy, and quick culture) microbes are frequently used as research models in different fields. Subsequently, microbes play an important role in the research field of enzyme structure and mode of action, drug invention, cellular regulatory mechanism, energy metabolism, protein synthesis, and so on.

Microorganisms can be used as probiotics sources and are defined as "live microorganisms, which, when administrated in adequate numbers, confer a health benefit to the host". Lactic acid bacteria (LAB), particularly *Lactobacilli*, are widely used in food production and represent the most common microorganisms employed as probiotics in functional foods [1, 2]. The probiotic concept is gaining more attention worldwide, due to the perceived beneficial effects of these bacteria on human and animal health [2]. These microorganisms can produce exopolysaccharides (EPS) which are long-chain polymers that are used industrially as thickeners, stabilizers, and gelling agents in food products. More recently, they were used as depollution agents and there was a growing interest in their biological functions like antitumor, antioxidant, or probiotic activities [3]. *Lactobacillus* strains do not elicit antimicrobial effects because their metabolic production is insufficient or minimal. Considering that *Lactobacillus* have these effects in vitro and that their metabolites may target and play a role in the competitive exclusion of pathogens, this

kind of bacteria produce numerous antimicrobial peptides and acids that are good bio preservatives for pickled products [4].

Additionally, the production of antibiotics by microorganisms are the main way used nowadays; in fact, polymyxin E, also called colistin, is an important old antibiotic known for around six decades for treatment of infection caused by Gram-negative pathogens. Later studies showed that colistin can also kill Gram-positive bacteria [5]. Moreover, colistin can be biosynthesized by a multienzyme nonribosomal peptide synthetase system (NRPS) in *Paenibacillus polymyxa* [6]. Other natural types of antibiotics are the Antimicrobial peptides (AMPs) which are abundant and ubiquitous in nature. Microbial killing result of rapid interaction of the AMP with the microbial membrane is leading to membrane disruption, release of cytoplasmic constituents, and a halt to cellular activities. Little work is ongoing concerning peptides from Ghanaian marine invertebrates, but crude peptides of *Galatea paradoxa* and *Patella rustica* have been reported to possess some antimicrobial activity [7].

Moreover, the biological properties of propolis have been established several years ago and include antifungal, antiatherogenic, antioxidant, and antimicrobial activities. High content of polyphenols in Chilean propolis can inhibit the growth of *Streptococcus mutans* and reduce biofilm formation without bactericidal effect [8]. Polyphenols from Chilean have also been shown to affect the expression of genes involved in *S. mutans* virulence and the capacity for forming a biofilm [9].

Plants are another source of natural products which have been largely used in different domains. Aromatic and medicinal plants have been reported to contain a higher content of bioactive phytochemicals such as substantial number of vitamins, phenolic compounds, and essential oils and thus can be used as important sources of natural antioxidants for food application and pharmaceuticals [10]. Recently, *Pelargonium graveolens* is a herb belonging to the family Geraniaceae that has shown good aromatic properties. It is cultivated worldwide, mainly for its essential oil fraction, which is extensively used in various industries. The essential oil of the fresh plant is widely used in perfume industry [11]. Besides, many studies on active molecules in essential oil and organic extracts of *Pelargonium graveolens* have shown good antioxidant activity and antimicrobial effect, specifically against *Bacillus cereus, B. subtilis,* and *Staphylococcus aureus* [12]. However, because of the toxicity of essential oils and organic extracts, their application in food against spoilage pathogens is limited, and more interest in safety matters should be shown [13].

Currently, natural compounds obtained from vegetables with antibacterial properties could be considered as an alternative to conventional antibiotics [14]. In recent years, the antibacterial properties of some compounds obtained from *Allium* plants such as garlic (*Allium sativum*) and onion (*Allium cepa*) have been described. These can inhibit the growth of a range of Gram-positive and Gram-negative bacteria, including both pathogenic and commensal bacteria in humans and animals [15].

Finally, the textile industry is one of the most polluting industries of clean water; recently microorganisms can be used as wastewater treatment alternative. However, bioprocessing can be considered as a preferred option to overcome these disadvantages because it is cost saving and environmentally friendly. Biological treatments can be used to degrade and/or to adsorb azo dyes contaminants [16]. The most efficient microorganisms to break down colored pollutants so far reported are white-rot fungi. These comprise mostly basidiomycetous fungi, which are capable of extensive aerobic lignin degradation and mineralization. This is possible through several extracellular lignin-degrading enzymes, such as lignin peroxidase, manganese-dependent peroxidase, and laccase [17].

This special issue was dedicated to the First International Congress on Biochemistry and Microbiology Applied Technologies "BMAT-2017" which was held in Tunisia, 03-05 November 2017. In this special issue, several full-length papers related to microorganisms as targets and tools in diverse field of studies were published. Furthermore, the current special issues included collected manuscripts from outside the conference provided that they fit within the scope of the special issue.

Conflicts of Interest

The guest editors declare that there are no conflicts of interest regarding the publication of this special issue.

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Research Article

Effect of *Lonicera caerulea* var. *emphyllocalyx* Extracts on Murine *Streptococcus pyogenes* Infection by Modulating Immune System

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Streptococcus pyogenes (*S. pyogenes*) causes several infectious diseases such as tonsillitis, cellulitis, and streptococcal toxic shock syndrome. The general treatment of *S. pyogenes* infection is by using β-lactam antibiotics; however, the cases of treatment failure were increasing as serious problems. *Lonicera caerulea* var. *emphyllocalyx* (LCE) has been used in the folk medicine in the northern part of Japan, the northern part of China, Korea, and Russia. In this study, we investigated the efficacy of three parts (fruit, stem, and leaf) of *Lonicera caerulea* var. *emphyllocalyx* extract (LCEEs) against murine *S. pyogenes* infection. Oral administration of LCEEs increased the mortality in murine model, and the extracts of its stems and leaves were more effective than the fruit extract significantly. Murine splenocytes and mesenteric lymph nodal cells treated with LCEEs suppressed the excess production of inflammatory cytokine such as TNF-α in comparison to those from untreated cells. LCEEs stimulated the differentiation of pluripotent hematopoietic stem cells in those murine lymph nodal cells. It also activated the proliferative response of murine lymph nodal cells. We also found that the stem and leaf extracts seemed to be more effective than the fruit extract in those phenomena. The concentration of lignins in LCEE prepared from the stems was larger than that from leaves, and that was larger than that from the fruits. Our data suggest that LCE, especially the stems and the leaves, may be useful for the treatment of *S. pyogenes* infection.

1. Introduction

Streptococcus pyogenes (*S. pyogenes*) is a gram-positive pathogenic bacterium. Because it has several virulent factors such as streptolysin O, streptolysin S, NADase, SpeB protease, and streptococcus inhibitory of complement lysis, it causes various infectious diseases such as pharyngitis, tonsillitis, nephritis, cellulitis, and necrotizing fasciitis [1]. As the drug such as macrolide and tetracycline, resistant rate of *S. pyogenes* is gradually increasing worldwide including Japan [2]; novel anti-*S. pyogenes* drug besides popular antibiotics has been desired.

Lonicera caerulea var. *emphyllocalyx* (LCE) belongs to honeysuckle family (Caprifoliaceae) and *Lonicera* genus, which is known as edible berries [3]. LCE lives in the northern temperate zone such as the northern part of Japan (Hokkaido), the northern part of China, Korea, and Russia. It is currently commercially produced in Japan and Russia [3]. The fruits, flowers, leaves branches, and bark of honeysuckle plants were used in the folk medicine in the countries of their origin. For example, branch infusion has been used as a diuretic remedy. As fresh fruit juice has been used as a general strengthening means, they were also recommended for the treatment of some disease of the stomach and tonsillitis for antiseptic effect [4]. Although this mode of action has not been unclear, in recent years, a large number of studies have investigated the therapeutic effects of berries in the prevention of a range of diseases and there is in increasing interest in herbal products [3]. Berries constitute the several important sources of potential health supporting phytochemicals in

the human diet [5]. They contain carbohydrates, lipids, and proteins, organic acids and also ascorbic acid, Vitamin B, magnesium, phosphorus, calcium, and potassium as minor compounds [6, 7]. They have antitumorigenic, antimicrobial, anti-inflammatory, and antimutagenic properties [8–11].

Among the herbal medicines used in Japanese traditional medicine (Kampo medicine) and traditional Chinese medicine, the flower bud, stems, and leaves of *Lonicera japonica* are formulated into a prescription with indications such as the febrile common cold, influenzae infection, and the joint pain [12–14]. However, the scientific report of stem and leaf from LCE about health science had been seldom known.

The gut-associated lymphoreticular tissues (GALT) including mesenteric lymph nodes exist on the intestinal mucosal sites and play an important role in host defence including IgA response in the mucosal immune system [15]. The cytokine network also plays an important role in the inflammatory and immune responses in total immune system [16]. As LCE are taken orally, the digestive mucosal immune system including mesenteric lymph nodes may act as one of the major targets for the expression of pharmacological activity. However, the modulating activity of LCE on GALT system has not been unclear.

Therefore, we hypothesized that LCE which is one of *Lonicera* genus may have anti-infective activity through GALT system. In this study, we tried to clarify whether LCE is novel candidate for anti *S. pyogenes* therapy. Furthermore, we focus on not only the fruits of, which are edible, but also the stems and leaves for exploration of novel drug. In the present study, we compared the anti-inflammatory effects of LCE by the immunostimulatory effects in the total and local immune system by the induction of granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion from murine splenocytes and mesenteric lymph nodes using several parts of LCE.

2. Materials and Methods

2.1. Preparation of Samples. LCE was harvested in the field located in Atsuma-Town, Hokkaido, northern part of Japan. LCE fruit is neither an herbal medicine nor a crude drug. People usually take this fruit as fresh one. Although its leaves and stems are not usually taken, some kind of leaves and stems from plant such as *Lonicera japonica* are used as dried herbal products [12–14]. Now we applied this concept for LCE. Therefore, we used fresh fruit and dried leaves and stems as samples. Thus, the 633 g of the fresh fruit, 5.6 g of the leaves, and 20.9 g of the branches (stems) (fresh weight of fruits, dried weight of leaves, and stems) were soaked in 2 L, 500 mL, and 500 mL of MeOH, respectively, and stood for 72 hours at room temperature. After filtration through filter paper, the same amount of MeOH was again added to the residue, and the mixture was allowed to stand for 72 hours at room temperature. After filtration, each filtrate was evaporated under reduced pressure and finally lyophilized. The weights of the extracts (LCEEs) after lyophilization were 79.8 g for fruits, 1.33 g for leaves, and 0.686 g for stems, respectively. The extraction efficiencies were 12.6% for the fruits, 23.8% for the leaves, and 3.28% for the stems, respectively. Fruits, leaves and stem extracts were dissolved and suspended at 200 mg/mL in water, 40% DMSO, and 20% DMSO, respectively, and stored at −20°C.

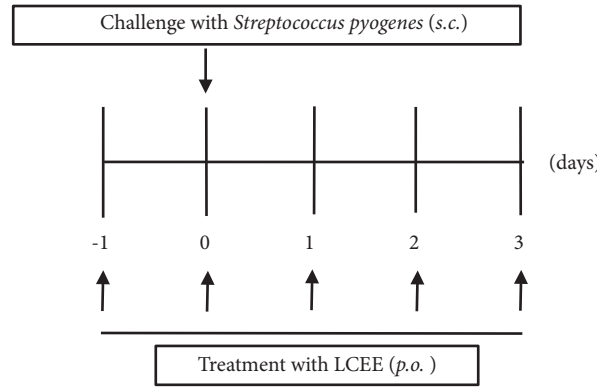

Figure 1: Protocols for the experiments of *S. pyogenes*-induced murine model. In infected groups, 1×10^6 CFU bacteria were injected subcutaneously using a 27-gauge needle at day 0. In the LCEE-treated groups, mice were administrated with each LCEE prepared from fruits, leaves, or stems (1 g/kg body weight/day) orally.

2.2. Evaluation of Animal Challenge Assay. The ability of *S. pyogenes* to cause cellulitis in mice after subcutaneous inoculation was assessed using a procedure described elsewhere [17]. In brief, *S. pyogenes* 1529, which was clinical isolates from severe invasive disease in Japan [17], was harvested after 16-hour growth on brain heart infusion agar (Eiken Chemical, Tokyo, Japan) containing 0.3% yeast extract (BHY agar) mixed in 1 mL of phosphate buffered saline (0.15 M, pH 7.2, PBS) and then centrifuged at 2,000 × *g* for 2 min. The pellets were diluted in 1 ml PBS to 1×10^8 CFU and then injected 1×10^6 CFU under the skin surface of inbred 3-week-old female Slc:ICR mice (Japan SLC, Shizuoka, Japan) using a 27-gauge needle. The number of CFU injected was verified for each experiment by plating the bacteria on BHY agar and counting CFU. The general status of mice was observed daily. In the LCEEs-treated groups, mice were gavaged with each LCEE of the fruits, leaves, or stems (1 g/kg/day) on days −1, 0, 1, and 2 after *S. pyogenes* inoculation, respectively. Mice in the control group were given an equal volume of PBS and were infected using the same method (Figure 1). The experimental procedures were conducted according to Nagoya City University Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the local Animal Ethics Committee of Nagoya City University (H24-M11).

2.3. Evaluation of Anti-Inflammatory Action in Splenocytes and Mesenteric Lymph Nodal Cells In Vitro. Evaluation of anti-inflammatory action in spleen and mesenteric lymph nodes was performed as described elsewhere [18]. Briefly, splenocytes and mesenteric lymph nodal cells prepared from ICR mice (3-week-old, female) were treated with 2% fatal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100

U/mL penicillin (Wako Pure Chemical, Osaka, Japan), 100 μg/mL streptomycin (Wako), and 10 μg/mL of lipopolysaccharide (LPS) from *E. coli* serotype 026: B6, (Sigma-Aldrich) and LCEEs for 24 hours at 37°C, cultured at 5% CO_2, and the concentrations of tumor necrosis factor- (TNF-) α and interferon- (INF-) γ in the subsequent culture medium were measured with ELISA kits (BioLegend, San Diego, CA, USA).

2.4. Determination of Proliferative Response of Splenocytes and Mesenteric Lymph Nodal Cells. Determination of proliferative response of spleen and mesenteric lymph nodal cells was described elsewhere [18]. After the mice were sacrificed by CO_2 inhalation, spleen and mesenteric lymph nodes were removed aseptically and those tissues were filtered and cultured in RPMI1640 medium (Wako) with 5% FBS. The 10 μg/mL of LPS from *E. coli* serotype 026:B6 was added according study. At 20 hours before the end of the splenocyte culture, ^{3}H-thymidine (2.0 Ci/mmol; PerkinElmer, MA, USA) was added to the medium. When the culture was finished, the cells were adsorbed on 0.45 μm membrane filters (Advantec Japan, Tokyo, Japan), washed with distilled water, and then dried. The filters were transferred to vials filled with liquid scintillator cocktail, and the radioactivity was measured with a liquid scintillation counter (LSC-6100, Hitachi Aloka Medical, Tokyo, Japan). Results are given as DPM (Disintegration per minute).

2.5. Evaluation of Differentiation of Pluripotent Hematopoietic Stem Cells in Splenocytes and Mesenteric Lymph Nodal Cells In Vitro. Evaluation of differentiation of pluripotent hematopoietic stem cells in spleen and mesenteric lymph nodes were performed by modified assay [19, 20]. Splenocytes and mesenteric lymph nodal cells prepared from ICR mice (3 weeks old, female) were treated with 2% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and LCEEs for 24 hours at 37°C, cultured at 5% CO_2, and the concentration of GM-CSF in the subsequent culture medium was measured with an ELISA kit (BioLegend).

2.6. Measurement of Lignins. The concentrations of lignins in LCEEs were performed by sulfuric acid method [21]. After the LCEEs (5 mg sample) were taken in beakers, the 72% of sulfuric acid was added, and the mixtures were stirred and allowed to stand at room temperature for 4 hours. The contents of the beakers were transferred to flasks containing distilled water and covered with aluminum foil. This was heated in a high-pressure steam sterilizer at 121°C and 0.08 MPa for 2 hours. At this point, the carbohydrate in the sample was hydrolyzed. After cooling, the black precipitates in the flasks were suction-filtered using a glass filter. The recovered precipitates were washed with hot water, washed with cold water, dried in a dryer at 105°C, and cooled in a desiccator and weighed. As acid soluble lignins, the filtrates were diluted 10 times with 3% sulfuric acid, and the optical densities (340 nm) were measured.

2.7. Statistical Analysis. The statistical analysis was conducted using Bonferroni-Dunnett's multiple comparison *t*-test for the differences among multiple groups. Survival data were assessed by Kaplan–Meier survival analysis and tested for significance using the log-rank test. *P*-values less than 0.01 were considered statistically significant (EZR version 1.36).

3. Results

3.1. Evaluation of Animal Challenge In Vivo. ICR mice were infected subcutaneously with *S. pyogenes* 1529 strain. The following survival curves were monitored for 3 days while orally administering LCEEs. No significant difference of survival rate was observed in the fruit LCEE group (Figure 2(a)). However, the groups treated with LCEEs of leaves and stems observed significantly extended survival rate (Figures 2(b) and 2(c)).

3.2. Evaluation of Anti-Inflammatory Action in Splenocytes and Mesenteric Lymph Nodal Cells In Vitro. Splenocytes and mesenteric lymph nodal cells isolated from ICR mice were incubated with both LPS and LCEEs from fruits, leaves, and stems for 24 hours, respectively. The concentrations of TNF-α and INF-γ in the culture medium were measured. LPS significantly induced the productions of TNF-α and INF-γ in splenocytes and mesenteric lymph nodal cells. And each of LCEEs showed a significant anti-inflammatory effect at a concentration of 500 μg/mL in both splenocytes (Figures 3 and 4) and mesenteric lymph nodal cells (Figures 5 and 6), and the inhibitory activities of LCEEs prepared from the leaves and stems seemed to be significant higher than that of LCEE from the fruits.

3.3. Evaluation of Proliferative Response of Splenocytes and Mesenteric Lymph Nodal Cells with LPS. We focused on the activity of splenocytes and mesenteric lymph nodal cells, because these cells play one of major roles in immune system. To determine whether splenocytes treated with LCEEs showed elevated activities, we performed ^{3}H-thymidine uptake analysis. As shown in Figure 7, the uptakes of ^{3}H-thymidine into splenocytes treated with LPS were significantly higher than the group without the treatment, and the groups treated with LCEEs (500 μg/mL) were significantly higher than that of untreated groups. We also confirmed the similar results in mesenteric lymph nodal cells treated with LPS and LCEEs. LCEEs (500 μg/mL) significantly induced the proliferation than that of the group treated with LPS (Figure 8).

3.4. Evaluation of Differentiation of Pluripotent Hematopoietic Stem Cells in Splenocytes and Mesenteric Lymph Nodal Cells In Vitro. Next, splenocytes and mesenteric lymph nodes cells isolated from ICR mice were incubated with each LCEEs for 24 hours, respectively. The concentrations of GM-CSF in the culture solution were measured. As a result, each LCEEs showed significant differentiated effect as dose-dependent manner in both splenocytes (Figure 9) and mesenteric lymph nodes cells (Figure 10). The activities of LCEEs from leaves and stems seemed to be higher than that of LCEEs from fruits.

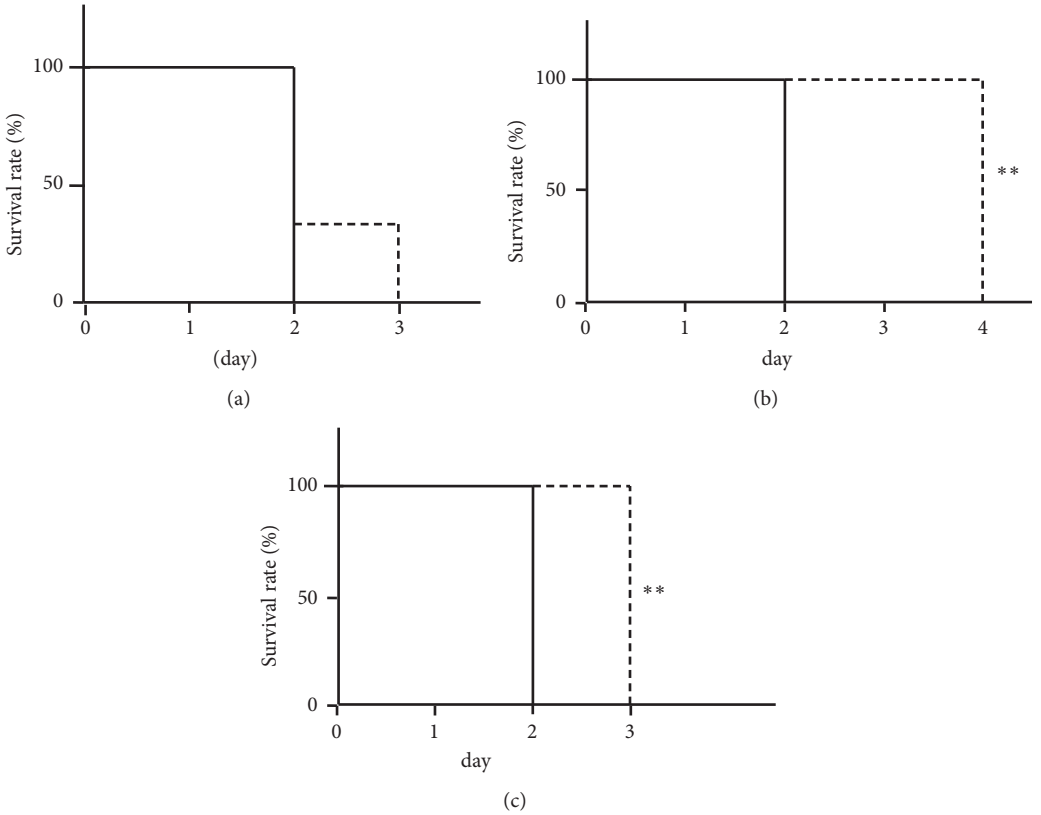

Figure 2: Administration of LCEEs increased the survival rate of *S. pyogenes*-infected murine models. Three-week-old ICR mice were gavaged with LCEEs ((a) fruits, (b) stems, and (c) leaves) for 4 consecutive days (day −1, 0, 1, and 2) and inoculated with 1×10^8 CFU of *S. pyogenes* 1529 at day 0. Mortality was monitored for 7 days. Survival data were assessed by Kaplan–Meier survival analysis and tested for significance using the log-rank test ($n = 6$). $**p< 0.01$ by Kaplan–Meier survival analysis.

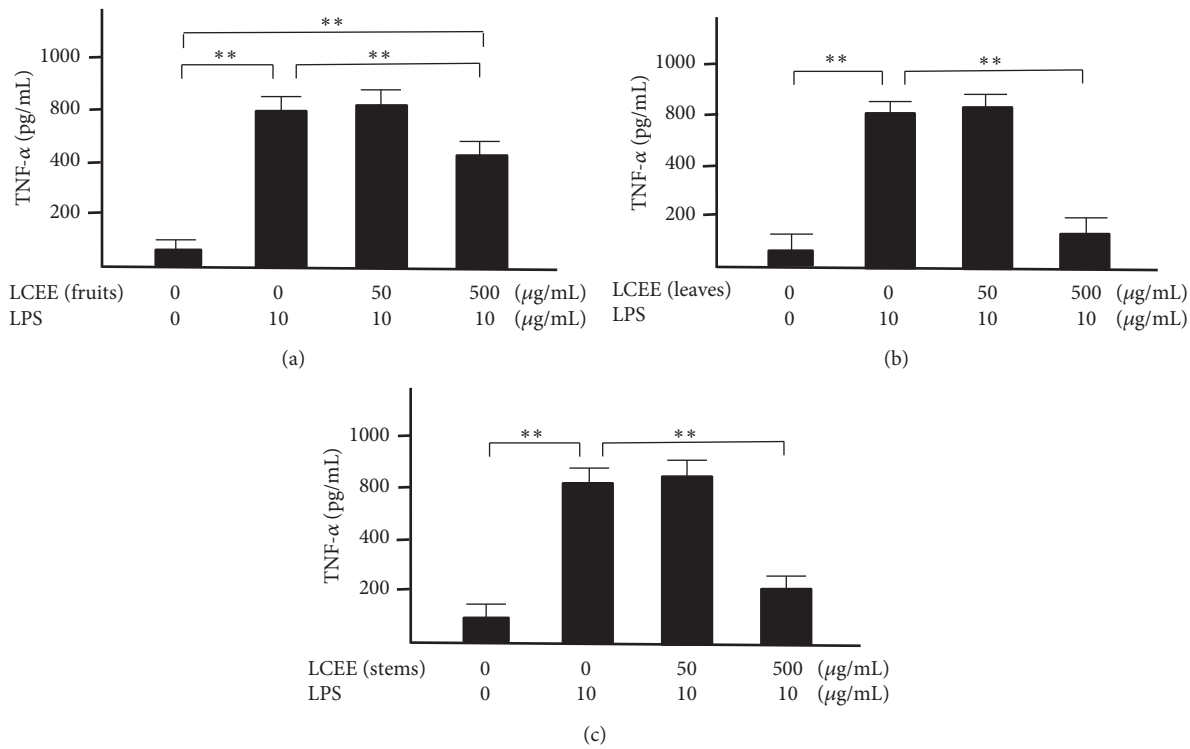

Figure 3: TNF-α levels in culture medium of splenocytes. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. TNF-α was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p< 0.01$ by Bonferroni-Dunnett's t-test.

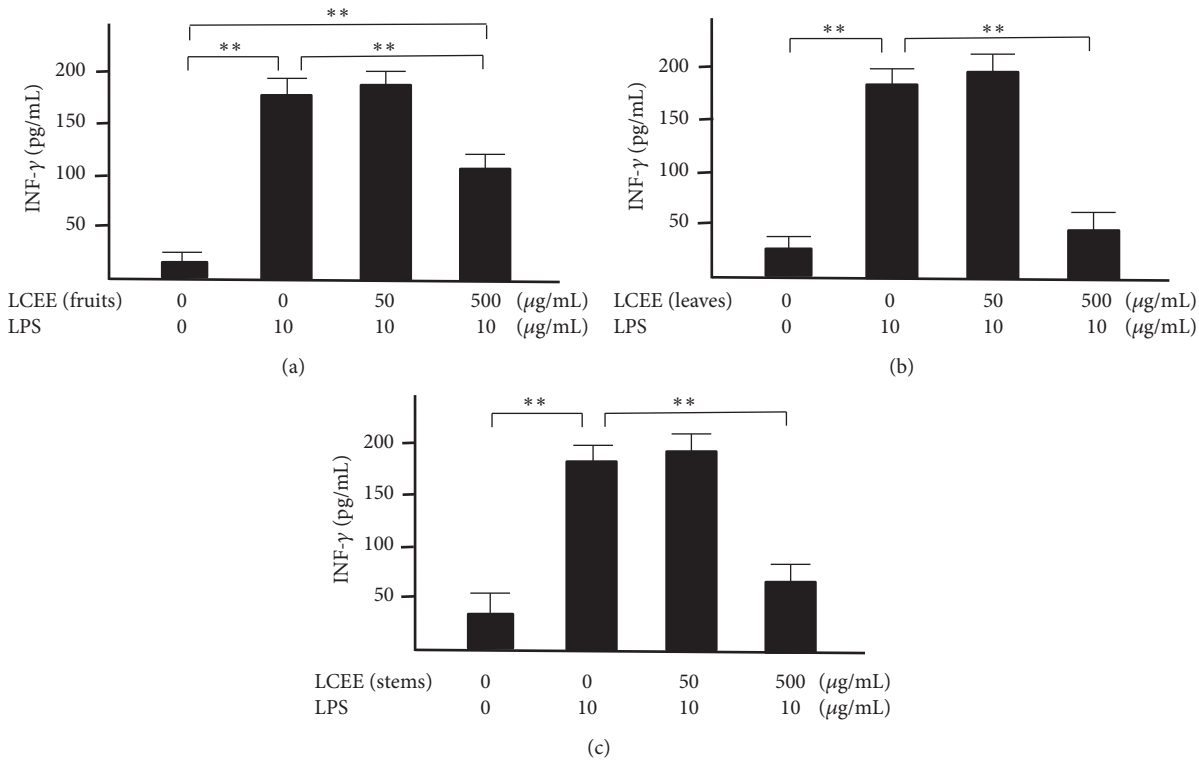

Figure 4: INF-γ levels in culture medium of splenocytes. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. INF-γ was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

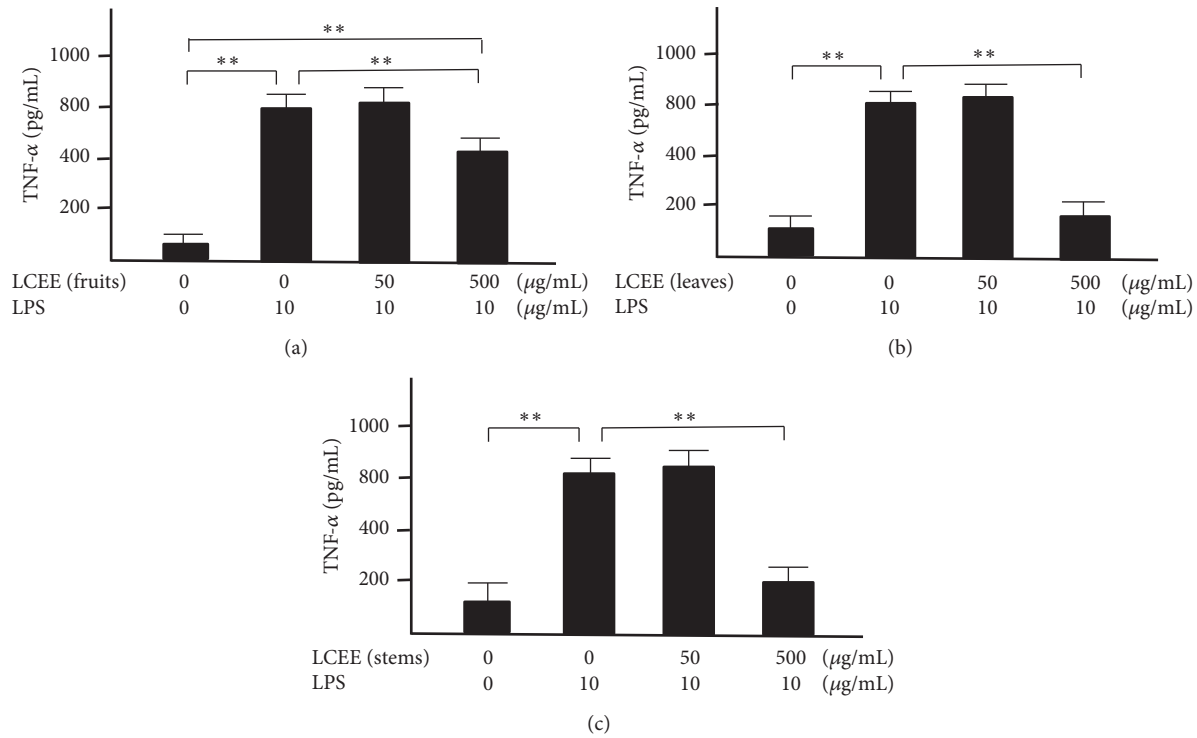

Figure 5: TNF-α levels in the culture medium of mesenteric lymph nodal cells. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. TNF-α was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

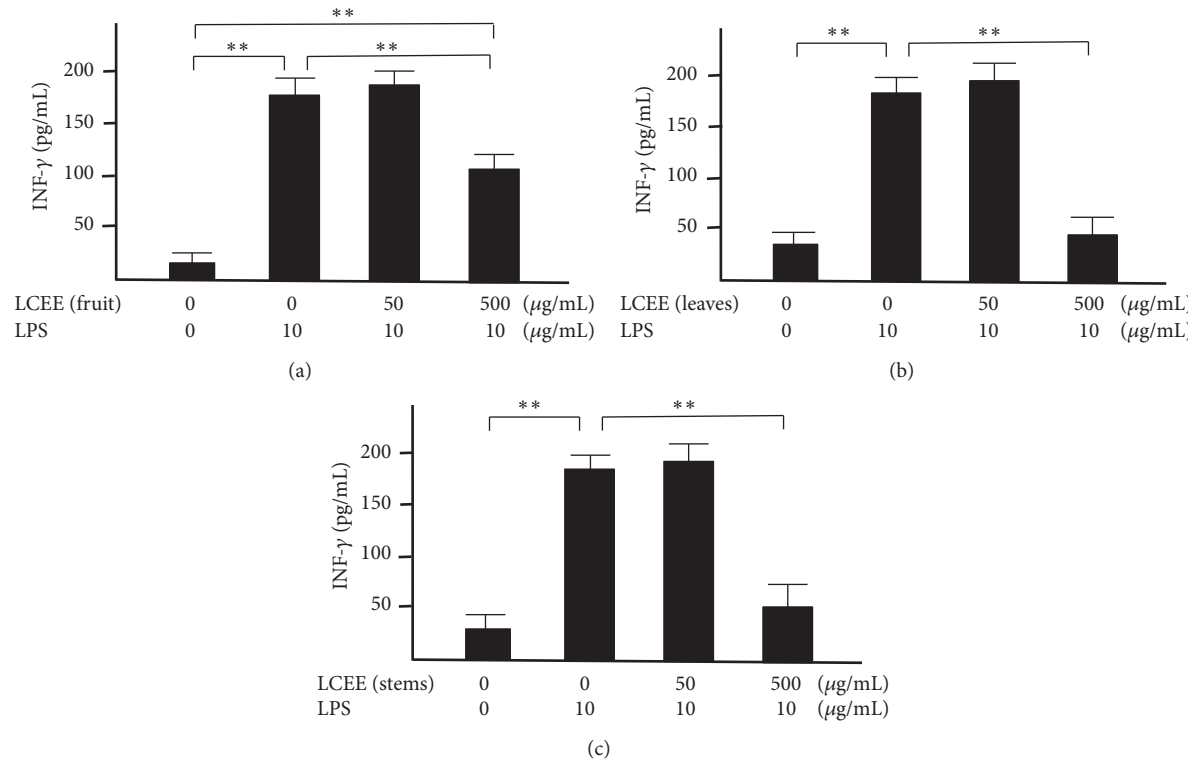

Figure 6: INF-γ levels in the culture medium of mesenteric lymph nodal cells. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. INF-γ was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

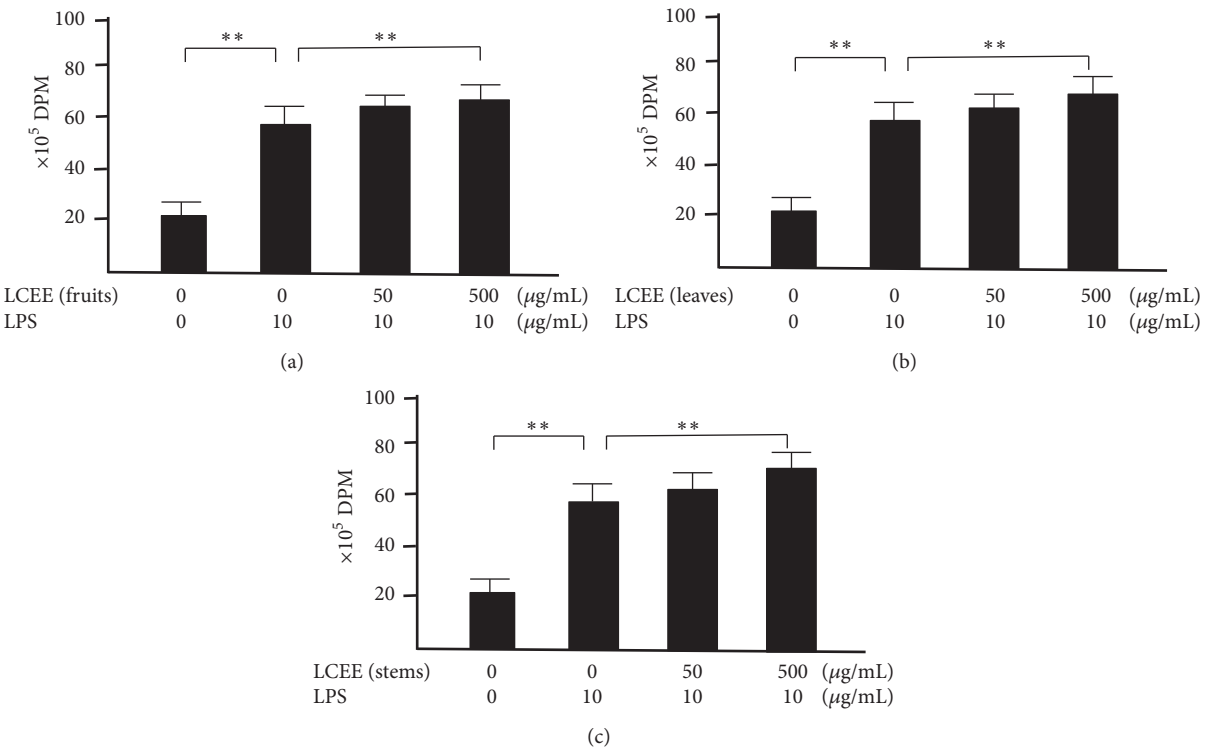

Figure 7: ^{3}H-thymidine-uptake assay in splenocytes with LPS stimulation. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

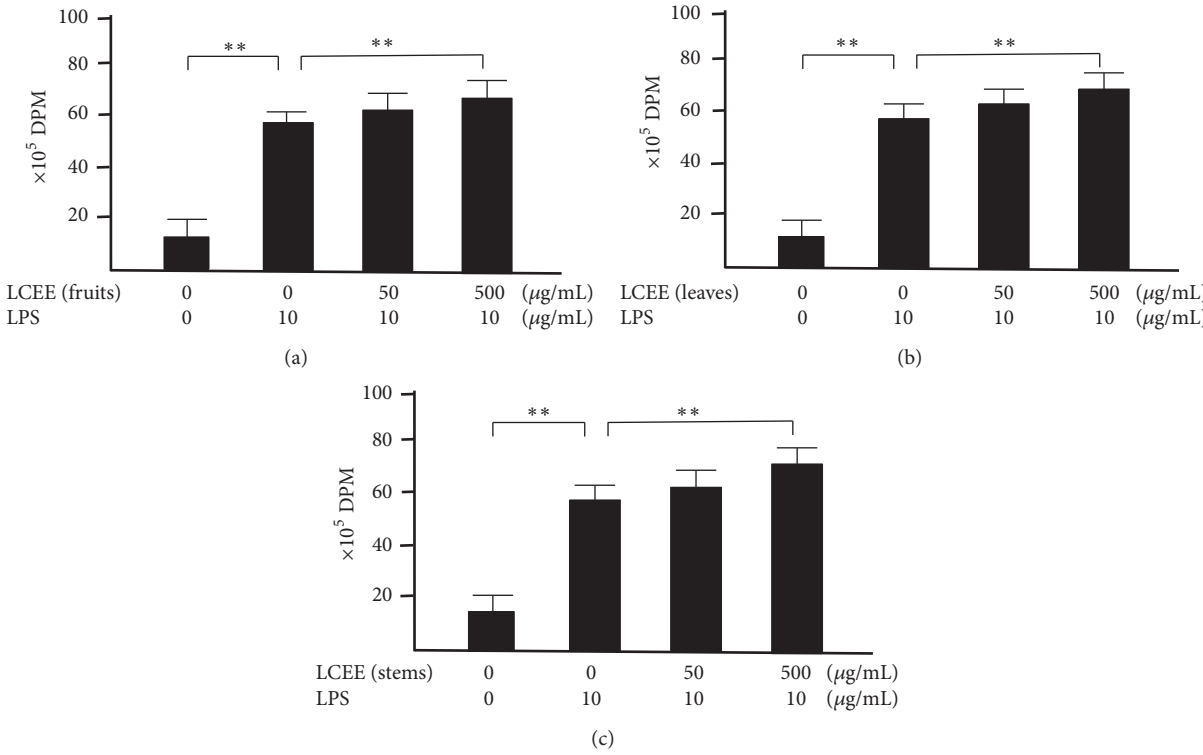

FIGURE 8: ^{3}H-thymidine-uptake assay in mesenteric lymph nodal cells with LPS stimulation. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

3.5. Evaluation of Proliferative Response of Splenocytes and Mesenteric Lymph Nodal Cells without LPS. We also tried to clarify whether splenocytes treated with LCEEs showed elevated activities without LPS, we performed ^{3}H-thymidine uptake analysis. As shown in Figure 11, the uptake of ^{3}H-thymidine into splenocytes treated with LCEEs (500 μg/mL) was significantly higher than that of control (0 μg/mL). We also confirmed that the uptakes of ^{3}H-thymidine into mesenteric lymph nodal cells treated with LCEEs (500 μg/mL) exhibited significantly higher activity than those without the treatments (Figure 12).

3.6. Lignin Contents. The concentrations of acid insoluble and soluble lignins in LCEE prepared from leaves were significantly higher than that in LCEEs from the fruits, and that from the stems was significantly higher than that in LCEEs from the leaves (Figure 13). Thus, the concentrations of total lignins in stem were the highest among three parts of LCE.

4. Discussion

To our knowledge, this is the first experimental study that leaves and stems of the LCE would be effective in *S. pyogenes*-caused murine model. Our result revealed that LCEE prepared from the leaves and stems showed a stronger immunostimulative action than that from fruits. As leaves and stems are unused resources, they will be potentially useful materials that can be expected for future medical applications.

It is not surprising that the leaves and stems of the LCE are used as a crude drug because an herbal medicine having a detoxifying action. Especially, some physiological activity is recognized on the stems of the *Lonicera japonica* [14]. We first evaluated the possibilities of some parts of LCE as anti-infective materials in animal study. From these results, we next investigated the immunostimulatory effects of LCEEs by the inducible effects on GM-CSF secretion from lymph nodes. The maximum concentration of LCEE *in vitro* study was set at 500 μg/ml because this concentration refers to the fact that 4% of the extract was absorbed and distributed in blood when human being takes 50 g of the extract. From our experimental results, the extraction efficiency from the fresh fruits of LCE was 12.6%. Let us assume that a human eats 100 g of fruits of LCE (whose amount of LCE extract is 12.6 g). If this is all absorbed and evenly distributed in the blood, the blood concentration of the LCE extract may be 2.7 mg/mL because the human blood volume is about 4.6 L. In fact, however, not all of the LCEE can be absorbed into the body. Now we do not know the exact absorption rate of the LCEEs, but if it is about 10% similar to the iron absorption rate [22], the blood concentration of the LCE extracts may become 270 μg/mL. Since this hypothesized blood concentration is included between 50 to 500μg/mL of this setting concentration, we think that our concentration setting of LCEEs is reasonable in this study. There is no report on the improvement of GALT-related immune

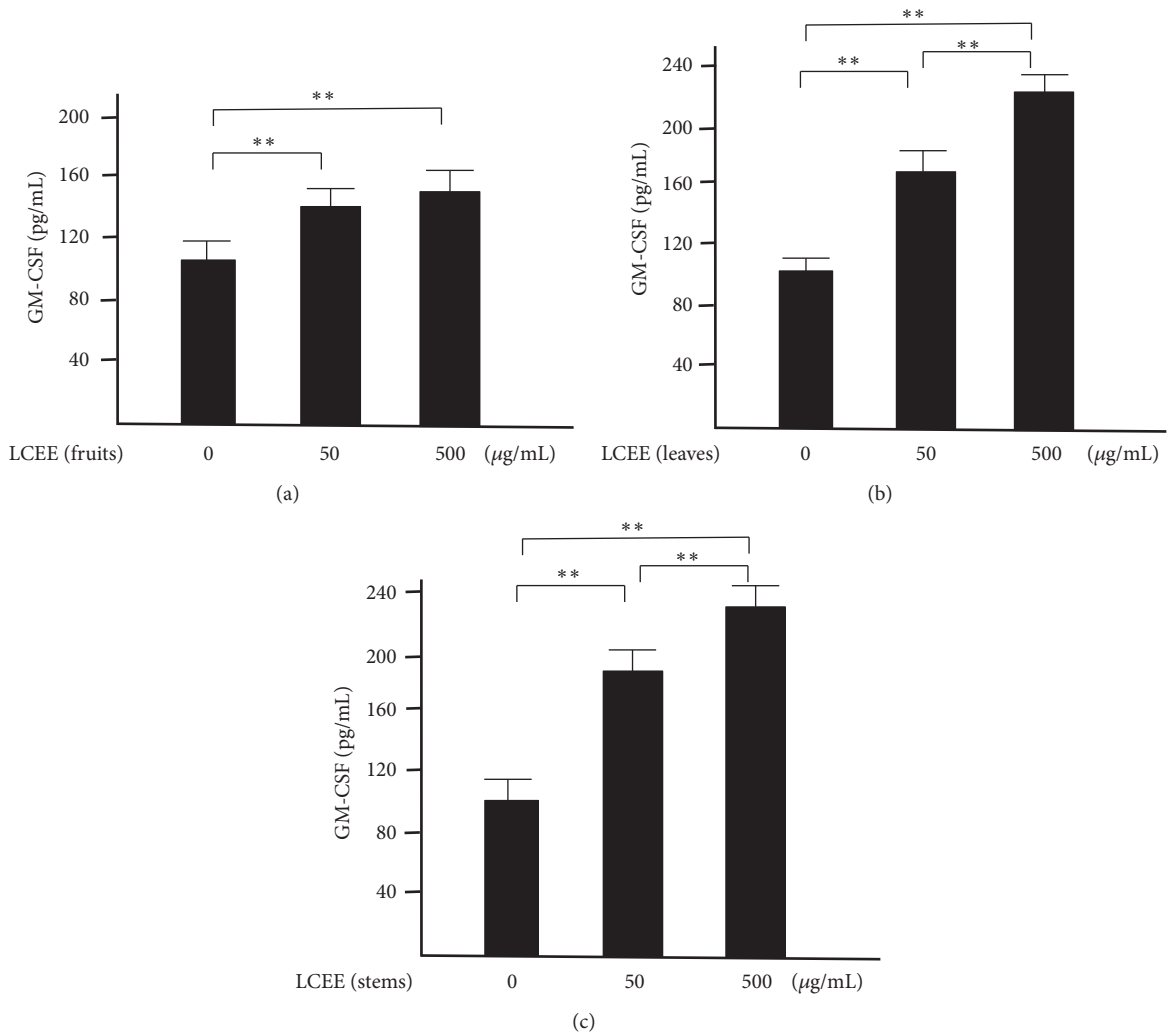

Figure 9: GM-CSF levels in culture medium of splenocytes lymph nodal cells. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. GM-CSF was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

mechanism by *Lonicera* genus including in LCE. However, in other plant investigation, murine GALT function was improved via improvement of Th2 cytokine IL-4 level when cranberry proanthocyanidin was administered to low GALT function-mouse [23]. Furthermore, in traditional Japanese Kampo medicine and traditional Chinese medicine, both of which are originated from ancient Chinese medicine, the stimulation of lymphocytes derived from spleen, mesenteric lymph node, and Peyer's patch of mice orally administered with Juzen-Taiho-To with concanavalin A causing the production of IFN-γ was enhanced in spleen, mesenteric lymph node, and Peyer's patch-derived lymphocytes [24]. As LCEEs may also have same mode of action for improving GALT function as cranberries and Juzen-Taiho-To, further research is necessary for this point. G-CSF and GM-CSF are cytokines that stimulate the production of granulocytes and are clinically used to treat neutropenia and to prevent immune deficiency induced by chemotherapy [25, 26]. In other cases, the extract of hochuekkito, one of the immunostimulatory agents in Japanese traditional Kampo formulation, stimulated G-CSF secretion from intestinal epithelial cells, and its active ingredients were polysaccharides [27]. Regarding LCEE, the same kind of mode of action may be suggested.

Although the various parts of *Lonicera* species have been utilized in folk medicine for many decades, several phenolic matrix constituents have been suggested as the main components responsible for the health benefits of the edible honeysuckle recently [4]. The phenolic fraction of LCE fruits may be beneficial for the adjunctive treatment of periodontitis as an agent for attenuation of the inflammatory process [28]. It also inhibited LPS-induced upregulation of interleukin-1β and interleukin-6 in gingival fibroblast and it suppressed expression of cyclooxygenase-2 [29]. This immunological result was almost coincided with our result. An LCEEs also showed endotoxin-induced uveitis. The possible mechanism for these effects may depend especially on the ability to inhibit activation of NF-κB and the subsequent production of proinflammatory mediators such as TNF-α. As murine macrophage cell lines were stimulated with LPS in the presence of blue honeysuckle extract, the treatment with this

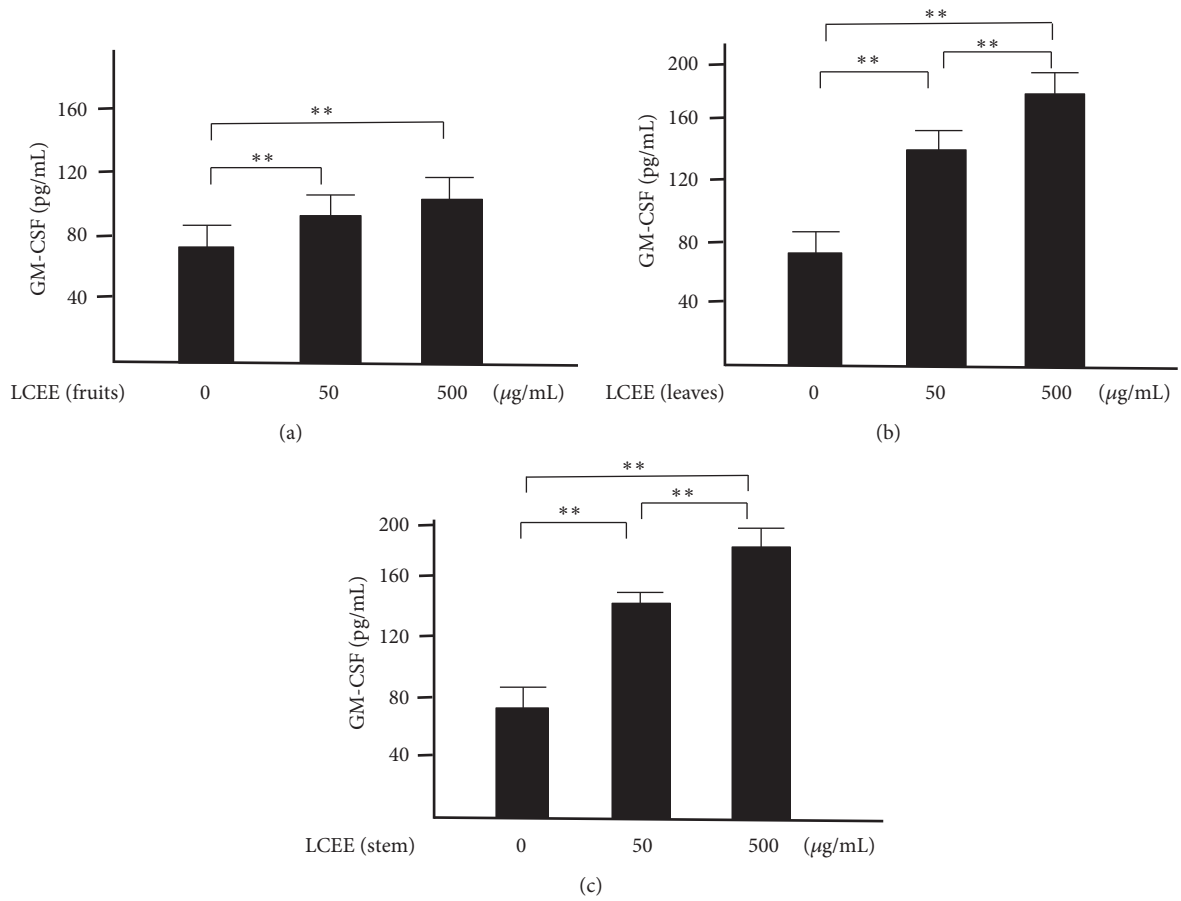

Figure 10: GM-CSF levels in culture medium of mesenteric lymph nodal cells. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. GM-CSF was measured by ELISA. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

extract significantly reduced the inflammatory cell migration and the levels of TNF-α.

Our results demonstrated that the stems and leaves of LCE had more immunological activity than the fruit. We focused on lignins since lignin-carbohydrate complexes (LCCs) are major cell wall components formed by the dehydrogenation of three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols. LCCs stimulated the iodination of myeloperoxidase-positive human monocytes, neutrophils, and promyelocytic leukemia that may be involved in the bacterial killing mechanism. LCCs stimulated splenocyte proliferation and showed both pro- and anti-inflammatory activity in activated macrophage. Preliminary DNA array analysis demonstrated the activation of the signal pathway of chemokine expression via Toll-like receptor 2. Broad and potent antiviral activity and synergism with vitamin C suggested functionality of LCCs as alternative medicine [30]. Various lignified materials, including pine cone extract, stimulated the morphological change of mouse peritoneal macrophages. The results strongly suggest the importance of lignin-structure in macrophage activation [31].

The water-soluble lignin in the extract of the solid culture medium of *Lentinus edodes mycelia* has been known to have immunopotentiating activities *in vivo* and *in vitro*. It activated the cytotoxicity of natural killer cells and macrophages and activated T cells in vitro. It also had antiviral and immunopotentiating activities [32]. Since there has been no report on the analysis of the immunological effect on lignins in LCE, future research is strongly desired from the viewpoint of searching for new drugs.

In summary, LCEEs prepared from the fruits, leaves, and stems may improve the immunological effect on immunocompromised condition. Furthermore, LCEEs from the leaves and stems had more effective than that from the fruit. We suggest LCEE as the therapeutic candidate for novel effective therapy on bacterial infectious disease caused by *S. pyogenes*.

Data Availability

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

Conflicts of Interest

All authors have no conflicts of interest.

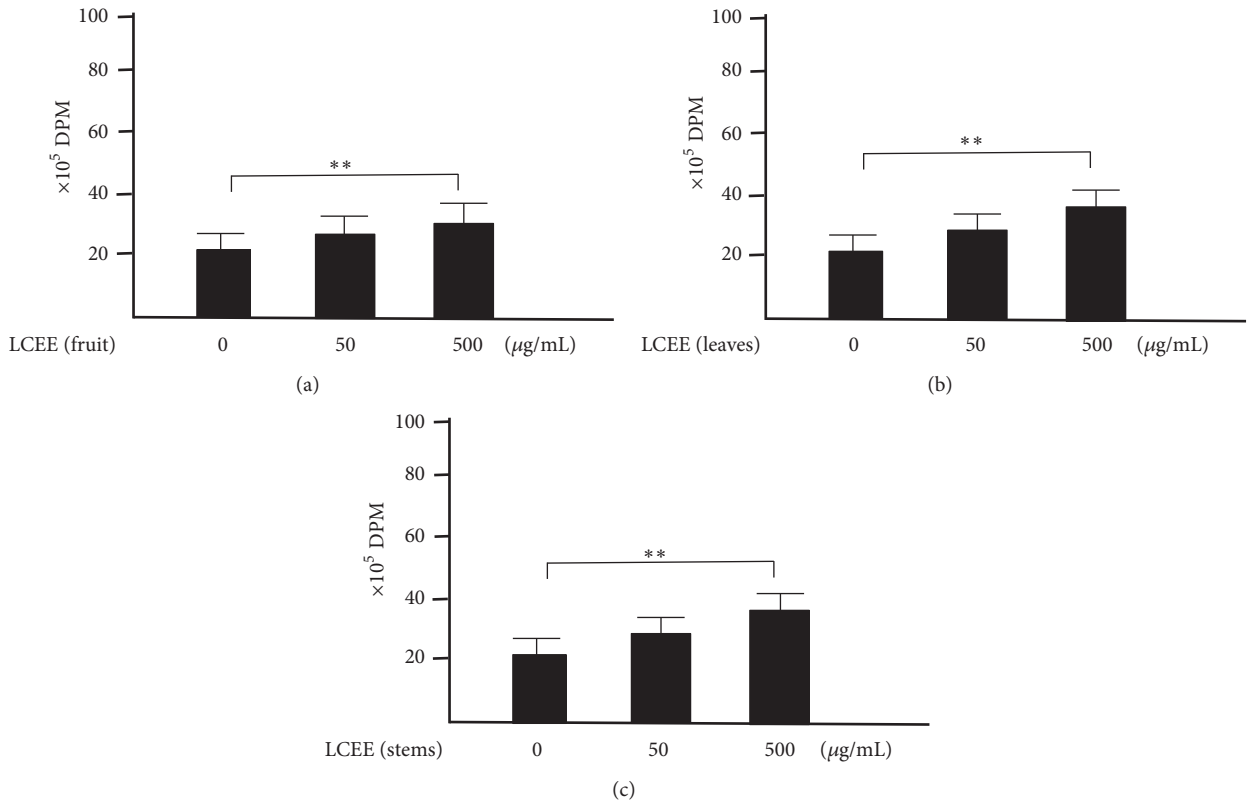

Figure 11: ^{3}H-thymidine-uptake assay in splenocytes without LPS stimulation. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

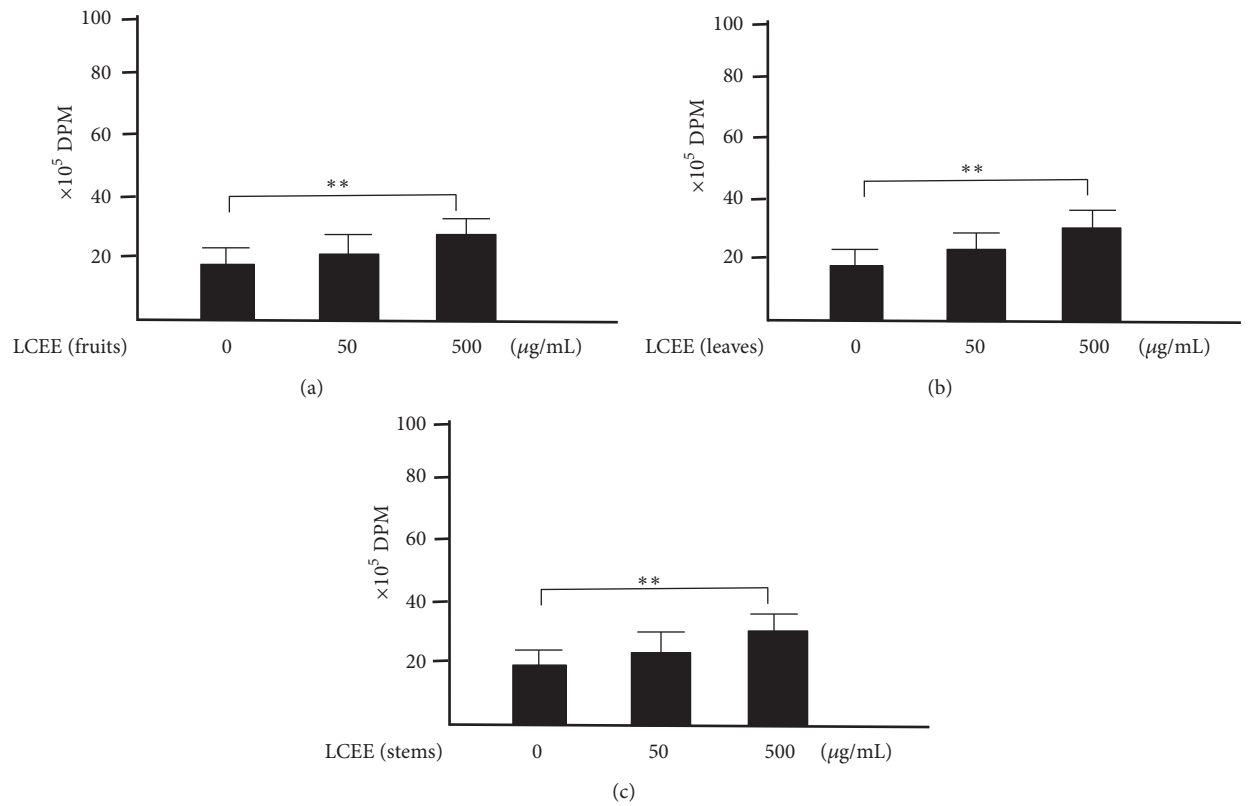

Figure 12: ^{3}H-thymidine-uptake assay in mesenteric lymph nodal cells without LPS stimulation. LCEEs ((a) fruits, (b) stems, and (c) leaves) were added to cells. Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

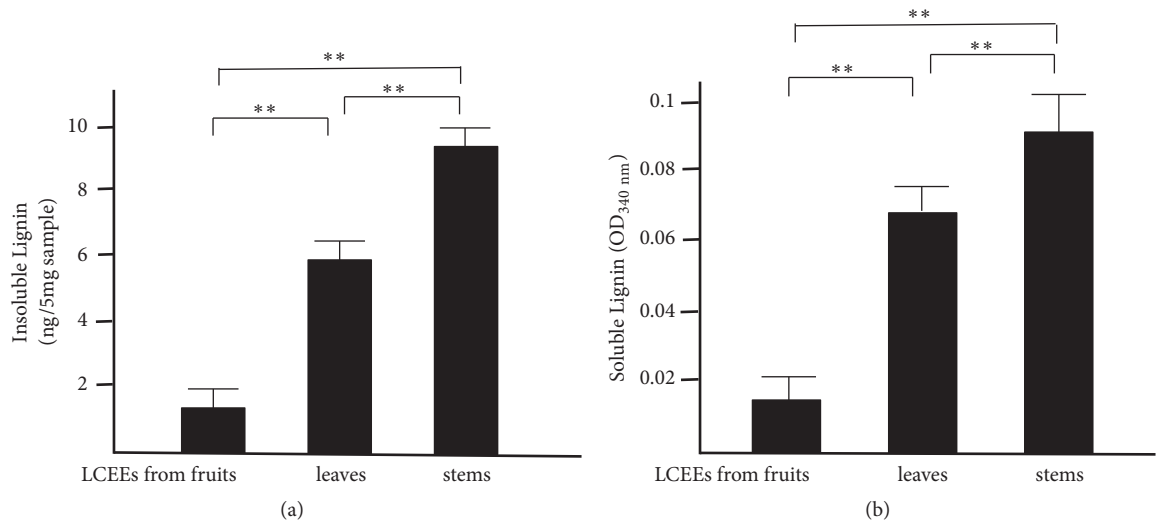

Figure 13: Lignin concentration in some parts of LCEEs. Insoluble lignins were measured as weight. Soluble lignins were measured as optical density ($OD_{340\,nm}$). Data represent the mean ± SD ($n = 6$). $**p < 0.01$ by Bonferroni-Dunnett's t-test.

Acknowledgments

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Research Article

Antimicrobial and Antibiofilm Activity against *Streptococcus mutans* of Individual and Mixtures of the Main Polyphenolic Compounds Found in Chilean Propolis

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Dental caries is multifactorial disease and an important health problem worldwide. *Streptococcus mutans* is considered as a major cariogenic agent in oral cavity. This bacteria can synthetize soluble and insoluble glucans from sucrose by glucosyltransferases enzymes and generate stable biofilm on the tooth surface. Biological properties of Chilean propolis have been described and it includes antimicrobial, antifungal, and antibiofilm activities. The main goal of this study was to quantify the concentrations of main flavonoids presents in Chilean propolis and compare some biological properties such as antimicrobial and antibiofilm activity of individual compounds and the mixture of this compounds, against *S. mutans* cultures. Chilean propolis was studied and some polyphenols present in this extract were quantified by HPLC-DAD using commercial standards of apigenin, quercetin, pinocembrin, and caffeic acid phenethyl ester (CAPE). MIC for antimicrobial activity was determined by serial dilution method and biofilm thickness on *S. mutans* was quantified by confocal microscopy. Pinocembrin, apigenin, quercetin, and caffeic acid phenethyl ester (CAPE) are the most abundant compounds in Chilean propolis. These polyphenols have strong antimicrobial and antibiofilm potential at low concentrations. However, pinocembrin and apigenin have a greater contribution to this action. The effect of polyphenols on *S. mutans* is produced by a combination of mechanisms to decrease bacterial growth and affect biofilm proliferation due to changes in their architecture.

1. Introduction

Dental caries is an infectious disease that affects people from developed and underdeveloped countries. In Chile, it is an important health problem that affects adults and children from a low socioeconomic status [1]. This multifactorial disease leads to tooth destruction and removing the enamel by means of degradation of mineral material.

Streptococcus mutans (*S. mutans*) has been indicated as the major cariogenic agent in oral cavity. Such bacteria can synthetize soluble and insoluble glucans from diet sucrose mediated by glucosyltransferases enzymes (gtfs), which allows extracellular aggregation for stable biofilm formation on the tooth surface [2–4]. However, gtfs are encoded by GTFB, GTFC, and GTFD genes and previous studies have demonstrated that polyphenols-rich extract of Chilean propolis decreases GTFs gene expression levels and exerts a functional effect on its enzymatic capacity for synthetizing insoluble glucans at subinhibitory concentrations [5].

The biological properties of propolis have been established from some years and include antifungal, antiatherogenic, antioxidant, and antimicrobial activities [6–8]. High content of polyphenols in Chilean propolis can inhibit the growth of the *S. mutans* and reduce biofilm formation

Table 1

Standard	LOD	LOQ	LR	Regression equation	R^2
Quercetin	3.91	13.03	13.03 – 40.00	y = 16902x + 145755	0.9931
Apigenin	2.23	7.43	7.43 – 50.00	y = 59358x + 24261	0.9987
Pinocembrin	1.34	4.45	4.45 – 100.00	y = 151122x + 80657	0.9999
CAPE	0.29	0.98	0.98 – 20.00	y = 72216x - 84427	0.9998

LOD: limit of detection; LOQ: limit of quantification; LR: linear range; R^2: coefficient of correlation

without bactericidal effect [9, 10]. Moreover, polyphenols from Chilean propolis can affect the expression of genes involves in *S. mutans* virulence and the capacity for forming a biofilm [11, 12].

The composition of Chilean propolis extract from La Araucanía Region showed high concentrations of polyphenols and the presence of different families of flavonoids with pinocembrin being the predominant compound [13].

This study aimed to compare the antimicrobial activity of the individual polyphenols quantified in Chilean propolis and a mixture of this compounds applied to *S. mutans* cultures. Thus, we evaluated different concentrations in a mixture of these compounds to confirm its synergistic action in order to describe this effect as a main property of polyphenols to change a biofilm structure and affect its size as additional factor to decrease the virulence of this microorganism.

2. Materials and Methods

2.1. Preparation of Polyphenol-Rich Extract of Propolis (EP). To evaluate the effect of polyphenols from EP in *S. mutans* antimicrobial activity and biofilm formation, the propolis was collected during the spring of 2008 from La Araucanía Region (Chile). Propolis crude sample was kept frozen (-20°C) and later crushed in cold, and 30 grams was dissolved in 100 mL of ethanol (70%) and macerated for 7 days at room temperature. The ethanolic extract of propolis (EEP) was filtered with Whatman 2.0 paper and centrifuged at 327 g, during 20 minutes at 5°C. Finally, the solvent was evaporated at a temperature of 40°C, for 2 hours in a Rotavaporator (Buchi, R-210, Germany) and dissolved for 24 h with sterile DMSO (0.01%) to obtain Polyphenol-rich Extract of Propolis (EP).

2.2. Determination of Total Phenolic Content in EP. The content of total polyphenols in EP was quantified by Folin-Ciocalteau reaction by a modification of Popova and collaborator's methodology [14]. For this assay, 100 μL of EP was mixed with 100 μL of distilled water and 2 mL of Folin-Ciocalteau reagent (Merck, Germany). The resulting solution was incubated for 8 minutes, and finally 3 mL of sodium carbonate 20% (w/v) was added. The absorbance of this solution was measured at 760 nm after 2 hours of incubation at room temperature. The concentration of polyphenols was calculated from a calibration curve and was expressed in mg mL^{-1} equivalent to the pinocembrin-galangin standard mixture 1:1.

2.3. Identification and Quantification of Polyphenolic Compounds Present in EP. Four compounds were identified and their concentrations were calculated by the direct injection method in a liquid chromatograph of high resolution (Shimadzu, Japan), equipped with a LC-20AT pump connected to a UV-Visible detector SPD-M20A UV (HPLC-DAD). The separation was carried out in a LiChrospher RP-18 column, with particle size 5 μm x 250 mm and stove CTO-20AC at 25°C. The elution was realized at 40°C using a acetonitrile, methanol, water, and formic acid 5% mixture in a flow of 1.0 ml min^{-1}, with a gradient from 30 to 70% and 20 μL of the diluted EP sample (1:50) were injected. To calculate compounds concentrations, we used solutions at 5 ppm of apigenin, quercetin, pinocembrin, and caffeic acid phenethyl ester (CAPE) under commercial standards (Sigma-Aldrich, St Louis, MO), and the concentrations were expressed in mgL^{-1} by interpolation in a calibration curve (see Table 1).

2.4. Cellular Culture Conditions and Biofilm Generation. S. *mutans* strains were obtained from samples of fluids of the oral cavity from children with tooth decay. The cultures were made in Petri plates. Biofilm samples for studies were obtained after inoculation of 5 x 10^5 UFC mL^{-1} in 96-well microplates. For confocal microscopy studies, the biofilm was obtained in Fluorodish microplates (World Precision Instrument Inc., China). All cultures were incubated for 24 hours, at 37°C and 5% of CO_2 with Trypticase Soy (TSB) (Becton Dickinson and Co, NY, USA) and sucrose (1%) in a container (Anaerobic Generator GasPak EZ (Becton, Dickinson and Co., NY, USA). The presence of *S. mutans* was confirmed by polymerase chain reaction (PCR) as previously described [15].

2.5. Antimicrobial Activity of Individual and Mixtures of Polyphenols. The Minimum Inhibitory Concentration (MIC) was determined by serial dilution method following the CLSI guidelines [16]. The strains of *S. mutans* suspension 5 x 10^5 CFU mL^{-1} were inoculated in a 96–well microplates containing 100 μL of TSB and sucrose 1% supplied with 100 μg mL^{-1} of EP or 25 μg mL^{-1} of commercial polyphenols in DMSO (0.1%), respectively. We used different controls: a positive control 10 μL of chlorhexidine digluconate (0.2%) and a control without propolis, as a negative control (vehicle) was included. All tests were run in triplicate.

2.6. Evaluation of Biofilm Formation. Biofilm samples were prepared in FluoroDish microplates in TSB and sucrose

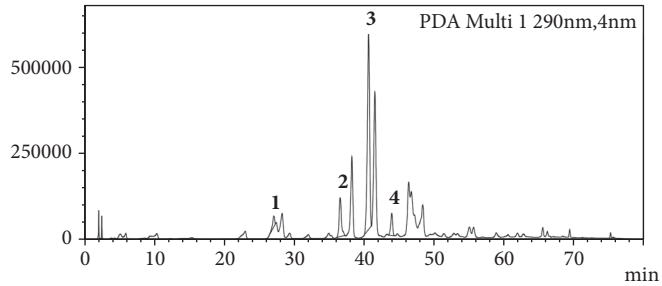

Figure 1: Compounds identified in EP2008 by HPLC-DAD. 1. Quercitin, 2. Apigenin, 3. Pinocembrin, and 4. CAPE.

Table 2: Polyphenolics compounds quantified by HPLC-DAD in EP.

Compounds	Tr. (minutes)	Area (mm^2)	Concentrations ± SD ($mg\,L^{-1}$)
Apigenin	36.59	2409968	40.2 ± 0.6
CAPE	43.96	1234518	19.2 ± 0.3
Pinocembrin	40.66	12714319	83.6 ± 0.9
Quercetin	27.13	657840	21.0 ± 0.3

1%, polyphenols at 25 $\mu g\,mL^{-1}$, and a mix of polyphenols (apigenin, pinocembrin, quercetin, and CAPE), in concentrations of 6.25; 12.5 and 25 $\mu g\,mL^{-1}$. The bacterial biofilm was incubated for 1 hour at room temperature with 100 μL of the probe Calcein Biofilm Tracer™ (Invitrogen, the USA), and later it was washed with sterile PBS. The structure of the biofilm was observed by a confocal microscope Olympus Fluoview 100, equipped with lens of watery immersion (To x 60, 0.21 NA). The images were captured by means of direct acquisition by excitement at 480 nm and the sign of fluorescence was detected by means of the green channel. The confocal planes for the three-dimensional image obtained in intervals of 15 seconds and 0.5 μm for plane and a scan of 512 x 512 pixels. The images were processed in the software ImageJ Mac Biophotonic.

2.7. Statistical Analysis. Statistical analyses were performed using the computational software package Prism 5 (Graph Pad Software Inc., San Diego, USA). Experimental values of MIC means obtained from antimicrobial test were estimated by statistical analysis of variance (ANOVA) and a Posttest of Tukey. To compare the values from individual's experiments of biofilm size, we applied unpaired two-sample t-tests statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Total Polyphenols Content in Chilean Propolis and Compounds Quantification. In EP the entire polyphenols contents in equivalence of pinocembrin-galangin mixture were quantified by Folin-Ciocalteau reaction and it was 137.7 ± 0.7 $mg\,g^{-1}$. This result is concordant with similar results from other studies [14, 17].

The main flavonoids were identified on the Chilean propolis by means of the HPLC-DAD as shown in Figure 1. In accordance with the times of retention (Tr) and the direct comparison with commercial standards, it was possible to verify the presence of quercetin, apigenin, pinocembrin, and caffeic acid phenethyl ester (CAPE). Concentrations of these polyphenols were quantified by HPLC-DAD (Table 2).

3.2. Antimicrobial Activity of Individual and Mixtures of Polyphenols Found in Propolis. An antimicrobial test was carried out in order to quantify MIC in bacteria cultures submitted to treatment with solutions of some polyphenols identified in the EP at the same concentrations (25 $\mu g\,mL^{-1}$). The values of MIC for *S. mutans* when the plates supplied with Polyphenols Mixture exhibit a similar potential as traditional chlorhexidine (1.6 $\mu g\,mL^{-1}$). Table 3 shows that flavonoids, as apigenin and pinocembrin, had lower values of MIC when compared with a mixture. All treatments showed significant statistical differences in comparison of quercetin and CAPE ($p < 0.5$ and $p < 0.001$, respectively).

3.3. Effect of the Polyphenols in the Reduction of Bacterial Biofilm Thickness. Figure 2 represents results in the reduction on biofilm's architecture generated from cultures of *S. mutans*. As shown in Figure 3, the cariogenic bacteria produced a thinner biofilm in plates supplied with individual compounds and polyphenols mixtures. The biofilm generated by cells in the control group without antimicrobial treatment reached values higher than 20 μm, as observed in confocal microscopy image of Figure 3(a). Samples treated with polyphenols mixture at low concentration (6.25 $\mu g\,mL^{-1}$) showed significant statistical differences ($p< 0.01$) when compared with untreated biofilm control. Again, some individual compounds in the solution, as pinocembrin (25 $\mu g\,mL^{-1}$) and apigenin (25 $\mu g\,mL^{-1}$) applied on *S. mutans* cultures, showed better potential in the reduction of the biofilm in comparison with a traditional synthetics products used for dental caries

Table 3: Antimicrobial activity of individual compounds and polyphenols mixture on *S. mutans* cultures.

Compounds	MIC	p-value *vs* Quercetin	p-value *vs* CAPE
Polyphenols Mixture	1.6 ± 0.4	p<0.5	p<0.001
Apigenin	1.3 ± 0.4	p<0.01	p<0.001
Pinocembrin	1.4 ± 0.4	p<0.01	p<0.001
Chlorhexidine	1.6 ± 0.2	p<0.5	p<0.001
Quercetin	4.1 ± 0.8	-	NSD
CAPE	5.2 ± 0.8	NSD	-

MIC: minimum inhibitory Concentration. MIC values were expressed in $\mu g\,mL^{-1}$ as Mean ± Standard Deviation. P-value was calculated as significant differences after ANOVA Multiple Comparison and test Tukey's Posttest. NSD: Nonstatistical Differences.

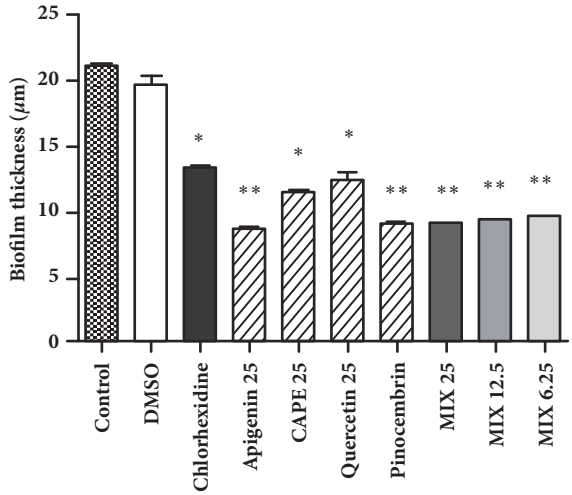

Figure 2: Evaluation of bacterial biofilm thickness in *S. mutans* cultures treated with individual and mixtures of polyphenols. Each values of individual experiments were expressed as a mean ± standard deviation. P-value was determined by ANOVA and Tukey Post Test. $*p < 0.5$; $**p<0.01$, when compared with the control without treatment.

treatment as chlorhexidine (Figure 3(b)). The aspect and thickness of the biofilm that result from the bacterial cellular aggregation are detailed in Figures 3(c)–3(f).

4. Discussion

Since many years ago, the *S. mutans* has been identified as the main microorganism responsible for initiating the colonization of the oral cavity, and it is capable to generate an acidic ambience favorable for the degradation of tooth mineral and protein material [18]. The diversity in its biological activity has been investigated in numerous studies with high concentrations of entire polyphenols present in EP, especially certain types of polyphenols as flavonoids.

Previously, a seasonal effect has been demonstrated (collection time) on the Chilean propolis composition with changes in polyphenols families, identified in the samples where they were observed. Pinocembrin and apigenin were quantified in the EP extract obtained from La Araucanía. These polyphenols have been related to antimicrobial activities against *S. mutans* [19, 20].

The employment of chemical compounds as chlorhexidine in the treatment of oral diseases had not had entirely successful outcomes due to several local side effects as the bacterial tolerance, changes in teeth coloration, taste disorders, and alterations in oral cavity microorganism that provokes a resistance to the treatments in patients [21, 22]. These aforementioned circumstances make it necessary to conduct searches toward new substances with anticariogenic activity with higher antimicrobial potential and at the same time they do not cause toxicity for the human organism.

Certainly, the antimicrobial actions demonstrated in this study for some flavonoids as apigenin and pinocembrin have allowed us to obtain MIC values similar to the administration of chlorhexidine. Nevertheless, when only quercetin or CAPE was added, the values of MIC were very high (4.1 and $5.2\,\mu g\,mL^{-1}$, respectively). However, when four compounds were applied to the antibacterial treatments they then increased its antimicrobial polyphenol's effect. Although quercetin and CAPE were present in the mixture, the additive actions of polyphenols confirm these results, and also they can act at low concentrations. Prior to this study it was not possible to define which group of compounds had a greater contribution to this action.

Some studies showed that adherence of *S. mutans* in tooth surface depended on hydrophobicity and therefore affect cell-surface proteins as Antigen I/II involved in initials steps for adherence and biofilm formation [23]. In some of these investigations, the antimicrobial activity of the apigenin found in EP has been established and operates on the gtf C enzyme, and they prevent the synthesis of insoluble glucans for the formation of the extracellular matrix [24]. Actually the potential of that compound for disruption in biofilm accumulation and reduction of gtf D activity can explain its action in mind and late-exponential phase of bacteria growth [25, 26], but the mechanism of action of these polyphenolics compounds is not completely known yet.

Quercetin and its derivatives, isorhamnetin and quercetin-3-glucuronide, may reduce the expression of some inflammatory genes. Its effects have been described in cellular cultures on the oxigenase-1 protein and the transduction of nuclear factor NFkB also and the decrease in the expression of the gene Nrfk2 and the inactivation of miR-155 with proinflammatory activity [27]. In the case of the phenylates flavonoids identified in natives propolis of the Pacific Ocean, the strong antimicrobial potential is due to their direct

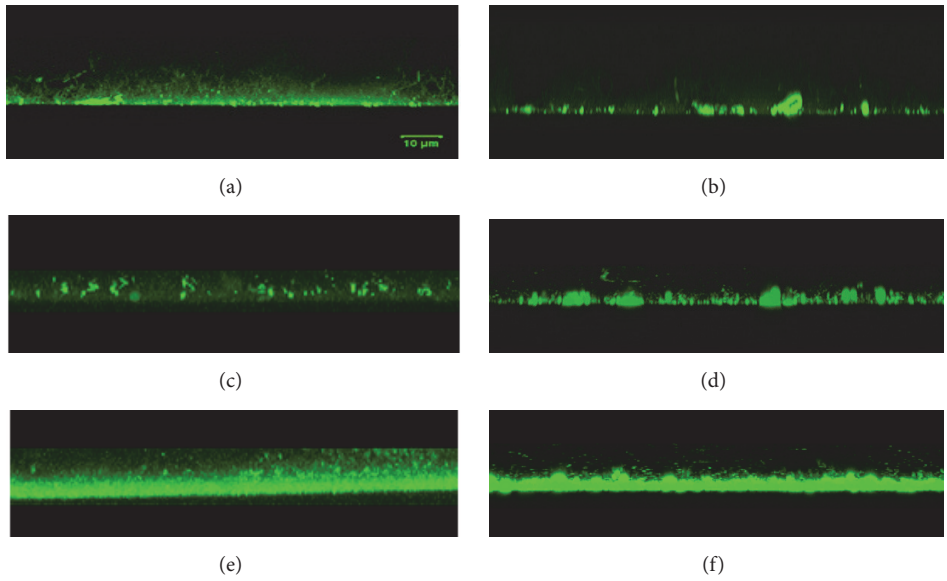

Figure 3: Images of bacterial biofilms from *S. mutans* cultures obtained by confocal microscopy. (a) Control group. (b) Chlorhexidine. (c) Pinocembrin. (d) Apigenin. (e) CAPE. (f) Quercitin.

effects on the bacterial cell membrane [28]. Quercetin and kaempferol, besides, have been related to inhibition of a glycolytic enzyme F-ATPase and subsequently increased the intracellular pH in *S. mutans* [29, 30].

One of the main virulence factors is the capacity for generating extracellular polymers (glucans) by means of glucosyltransferases activity in *S. mutans*. In this study, we also evaluate the potential of the polyphenols as agents that affect the formation of a stable bacterial biofilm. The growth process of the biofilm needs some steps that involve the cellular adherence to solid surfaces and the interactions of the cell-to-cell in microcolonies structures [31, 32].

The antimicrobials also produce changes in the structures of the biofilm and in the form of cellular aggregation due to changes in the levels of protein expression and enzymatic actions, but some phenolic acids, for example, caffeic acid and its phenethyl ester (CAPE), have a significant role in cancer cells apoptosis and cellular cycle, and it might affect bacterial multiplication [33, 34].

Confocal planes allowed visualizing changes in the architecture of the bacterial biofilm. A clear organization of the *S. mutans* biofilm architecture that grows upwards can be observed in the image of the control group, without treatment (Figure 3(a)). Flavonoids effects, such as pinocembrin and apigenin, are modifying the structures in the biofilm architecture of *S. mutans* (Figures 3(c) and 3(d), resp.). Figure 3(b) shows the reduction in thickness of the biofilm, while Figure 2 shows the significant statistical differences between polyphenols mixtures with respect to the control without treatment. Previous results demonstrate the additive action of polyphenols at small doses, achieving an inhibitory effect on biofilm formation [35]. The effect of pinocembrin and apigenin in relation to the control also showed a reduction in the biofilm thickness associated with enzymatic gtf C inhibition. Both compounds also have the highest antimicrobial activity, which are the flavonoids that contribute most to this action. So these mechanisms are very important for preventing tooth decay and consequently decrease *S. mutans* virulence and limiting extra oral colonization [36, 37]

These results demonstrate that the effect of polyphenols found in the Chilean propolis on *S. mutans* is produced by a combination of mechanisms, not only because their antimicrobial potential, since a significant reduction of the cellular adhesion and biofilm structure is achieved as an additional mechanism for tooth colonization.

5. Conclusion

These results suggest that polyphenols found in Chilean propolis exhibit antimicrobial activity against *S. mutans* at low concentrations. In addition, they decrease biofilm proliferation due to changes in their architecture. Moreover, pinocembrin and apigenin have strong antimicrobial and antibiofilm activity. This allows us to explain the contribution of these flavonoids to antimicrobial activity of polyphenols.

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 paper.

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Research Article

Antimicrobial Characteristics of Lactic Acid Bacteria Isolated from Homemade Fermented Foods

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Objective. Lactic acid bacteria (LAB) were isolated from fermented foods, such as glutinous rice dough, corn noodle, chili sauce, potherb mustard pickles, and stinky tofu, in northeast China. LAB strains with antimicrobial activities were screened, and seven of these *Lactobacillus* strains were identified as *L. plantarum*, *L. pentosus*, and *L. paracasei* through 16S rRNA gene analysis. After the supernatant of LAB was treated with proteinase K, pepsin, and papain, their antibacterial effect almost disappeared. Most strains with antibacterial activities were highly resistant to heat (65°C–121°C), acidity (pH 2–6), and alcohol. The antimicrobial effect of most strains treated with the Tween-80 surfactant was significantly reduced, and the antibacterial property of T4 was even lost. Ammonium sulfate precipitation, PCR, and nanoLC-ESI-MS/MS results confirmed that T8 produced antibacterial substances belonging to a protein family, and its zone of inhibition against pathogens significantly increased (>13 mm). In bacterial growth inhibition experiments, the colony count of *Staphylococcus aureus* was up to 10^{15} CFU/mL in the 3∗de Man, Rogosa, and Sharpe (MRS) group, and this value was more than that in the 3∗S6 supernatant group (10^{12} CFU/mL) and the control group (10^{10} CFU/mL) at 12 h. This study provided a basis for the selection of antimicrobial peptides and the development and utilization of LAB.

1. Introduction

Food-borne diseases associated with the consumption of fresh and minimally processed agricultural products have resulted in remarkable outbreaks and caused health problems. The side effects of the improper use of artificial preservatives and antibiotics have become serious. In the food industry, traditional sterilization methods involve the use of chemical sanitizers or high-temperature heating sterilization. However, in this way, harmful bacteria are incompletely eradicated, and organoleptic qualities decline [1]. As such, current studies aim to extend shelf life and antibacterial property by using antibacterial substance from microorganisms via [2].

Lactobacillus is widely used as probiotics in fermented foods. In vitro analysis found that lactic acid bacteria (LAB) have antioxidant effects and can chelate ferrous ions and degrade nitrite and cholesterol [3, 4]. LAB are natural microbes, and their metabolites are generally regarded as safe [5]. For example, nisin, which is an antimicrobial preservative, is the only allowed food preservative from the *lactococcus lactis* to prevent the growth of specific pathogens and spoilage caused by organisms and bacteria. LAB metabolic products, such as acid, hydrogen peroxide, and bacteriocin, can inhibit some bacteria and fungi [6]. Some strains do not elicit antimicrobial effects because their metabolic production is insufficient or minimal [7]. Therefore, LAB with high antibacterial activities should be screened, and their antibacterial components should be analyzed.

LAB isolated from yogurt have been screened and functionally analyzed, and studies have revealed that their antibacterial effects are not evident. However, LAB isolated from homemade fermented foods should be examined. In this experiment, five kinds of homemade fermented foods in Northeast China were selected as raw materials. Considering that LAB have these effects in vitro and that their metabolites may target and play a role in the competitive exclusion of pathogens, we should screen bacteria that produce numerous

antimicrobial peptides and acids that are good biopreservatives for pickled products [8]. Therefore, our study aimed to extend the screening of LAB and to obtain novel and good strains.

2. Materials and Methods

2.1. Bacterial Isolation and Identification. *Salmonella enterica* (ATCC14028), *Staphylococcus aureus* (ATCC 6538p), *Escherichia coli* (ATCC 8739), and *Bacillus cereus* Frankland (CICC 20551) used in the experiment were purchased from the China Center of Industrial Culture Collection (CICC).

A total of 231 strains were randomly isolated from fermented foods, namely, nianmianzi (glutinous rice dough), tangzimian (corn noodle), chili sauce, potherb mustard pickles, and stinky tofu, through serial dilution in MRS agar (Qingdao Hopebio Co.) and LAB purification twice [9]. H, T, L, and S were the abbreviations used for the fermented foods and the number of isolates corresponding to the strain name. Plate count agar was utilized to monitor viable bacteria for the reserve concentration and stored at −80°C in MRS with glycerol before use. LAB were identified using a 16s rRNA gene with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [10]. Thereafter, sequencing was performed in Basic Local Alignment Search Tool in the EzTaxon-e database with a sequence-matching program.

2.2. Culture Conditions. All of the isolated strains were incubated in MRS broth (37°C, 48 h). The second culture was incubated (1%, v/v) for 1 day, and the viable cell count was adjusted to 10^8 colony forming units (CFU)/mL. The strains were stored at −20°C in all of the experiments.

Salmonella, *S. aureus*, *E. coli*, and *Bacillus cereus Frankland* were grown for 24 h and incubated in 1% inoculation at 37°C with shaking at 200 rpm in CM0002 broth (CICC). The second culture was incubated overnight at 37°C and 200 rpm.

2.3. Screening for the Antimicrobial Activity. The antimicrobial activity of the LAB supernatant was analyzed. All of the supernatants were precipitated via centrifugation at 4000 × *g* for 20 min at 4°C when 1% inoculum was incubated for 24, 48, and 72 h at 37°C. pH was adjusted to 6.0 to rule out acid inhibition. All of the treated supernatants were stored at 4°C. Testing was subsequently performed against all of the indicator strains by using an Oxford cup (internal diameter of 6.0 mm) diffusion method. The spread plate method was prepared by adding the indication inocula of 100 μL of 1.2 OD_{600} into the CM0002 agar plate[11, 12]. Ampicillin (25–100 μg/mL, Beijing Solarbio Science & Technology) and nisin (500 μg/mL, dissolved in 0.05% acetic acid/0.1 M EDTA, Shanghai Seebio Biotech, Inc.) were used as a positive control in the plate. Furthermore, 100 μL of the treated supernatant was applied to an Oxford cup in the plate at 37°C for 24 h.

2.4. Effect of Enzymes on the Antimicrobial Activity. Enzymes were added to the supernatant of the selected strains to evaluate their effect on bacteriocin-like inhibitory substances. The supernatants were treated with catalase (5220 U/mg, Beijing Solarbio Science & Technology Co.) in a water bath at 25°C for 1 h, filtered, and stored at 4°C for the succeeding experiments. $CaCl_2$ buffer (0.05 mol/L Tris, 5 mmol/L $CaCl_2$, and pH 7.0) was added at 1 mg/mL enzymes, such as proteinase K (>30 U/mg, Beijing Solarbio Science & Technology Co.), α-amylase (100,000 KSB, Aobox Biotechnology), lysozyme (20,000 U/mg, Aobox Biotechnology), and papain (400 U/mg, Beijing Dingguo Changsheng Biotechnology Co.). For another experiment, citrate buffer solution was prepared (pH 3), and 1 mg/mL pepsin (250 U/mg, Beijing Solarbio Science & Technology Co.) was dispensed. All of the solutions were filter sterilized. The treated supernatants were set at 37°C for 3 h, and the mixture was boiled for 3 min to inactivate the enzymes. Bacteriostatic effects were analyzed using the Oxford cup diffusion method.

2.5. Antimicrobial Activity after Treatment under Varying Conditions. The treated supernatants were selected and incubated for 48 h. The influence of temperature on the antimicrobial activity of the supernatants heated at 65°C, 85°C, 100°C, and 121°C was estimated [13]. The effect of pH was determined by adjusting the pH of the supernatant to 2, 3, 4, 5, 6, 8, and 14. The supernatant was exposed to 30°C for 1 h. Finally, pH was modified to 6.0. *n*-Butanol, methanol, and ethanol were added to the supernatant at 1:9 (vol/vol) and placed at 30°C for 30 min (organic solvent from Beijing Chemical Works). Afterward, 1% (w/t) sodium citrate, potassium chloride, and Tween-80 were added to the supernatant and mixed. All of the treated solutions of the antimicrobial experiments were incubated for 24 h at 37°C by using the Oxford cup diffusion method. The residual activity of the strains was determined by observing their zone of inhibition. Sterile water and untreated samples were used as the control.

2.6. Ammonium Sulfate Precipitation of the Concentrated Antibacterial Components. $(NH_4)_2SO_4$ was added to 100 mL of the supernatant of the cultured strain reaching 80% concentration (4°C, 24 h) for further purification to evaluate whether the antimicrobial components of LAB belong to a protein family[14]. The treated supernatants were then centrifuged similar to those in the preceding experiments. The supernatants were subsequently dialyzed, and the antimicrobial activities of the concentrates were determined using the Oxford cup method against *S. aureus* and *Salmonella*.

2.7. Inhibition of Bacterial Growth. The inhibitory effects of the supernatants were identified by adding them to the indication broth of *S. aureus* and *Salmonella*. Afterward, 1 and 3 mL of the supernatants at pH 6.0 (48 h) were added at approximately 10^7 CFU/mL at the initial stage to 100 mL of CM0002 broth to determine the growth curves of indicators at 37°C and 200 rpm. At an interval of 2 h, the bacterial suspensions were measured at an optical density of 600 nm (OD_{600}) until they were incubated for 12 h. The initial MRS, nisin–MRS (500 μg/mL), ampicillin–MRS (100 μg/mL), and kanamycin–MRS (100 μg/mL) were prepared as described above at 37°C for 48 h as the control. Flat colony counting

method was applied to determine the total number of colonies at 37°C (12–24 h).

2.8. Identification of Antimicrobial Peptides. The target gene of seven strains was amplified using primers (forward 5′-ATGAAAAAATTTCTAGTTTTGCGTGAC-3′andreverse 5′-CTATCCGTGGATGAATCCTCGGACAGC-3′) via PCR. PCR was performed in accordance with the ExTaq (Takara) reaction protocol as follows: 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s. The T8 sample was further analyzed using nanoLC-ESI-MS/MS (ProtTech, Suzhou, China).

2.9. Data Analysis. All of the experiments involved three randomly selected replicates per treatment. SPSS was used to analyze the data through one-way ANOVA with a significance level of 0.5%.

3. Results and Discussion

3.1. Antimicrobial Activity Evaluation. The preliminary effect of the antimicrobial activity of the strains was determined via the diffusion method with 1 M HCl/NaOH to remove the acid that could inhibit the production of pathogenic bacteria in the supernatant. Thirty-five strains presented antimicrobial activity against *S. aureus* (Table 1). Eleven strains also elicited a strong antibacterial effect with an inhibition zone of more than 8.9 m. The inhibition zones of S6, L2, T8, and H9 were greater than 9.2 mm. Similar conditions were observed in the *Lactobacillus* inhibition of *Salmonella*. Eight strains covered an inhibition zone of greater than 9 mm. In comparison with the control group and the treatment group against *Bacillus cereus*, pathogenic bacteria slightly grew in the middle of the inhibition zone in the treatment group. Some spores might grow in the pathogenic *B. cereus* congenicstrain, and LAB were not well inhibited. As such, *B. cereus* was excluded from further investigation. Most strains did not inhibit *E. coli* except L2, L11, L19, T4, T8, T30, H9, H12, and S8 strains with an inhibition zone of 8 mm. Although most strains were limited against one pathogen in our study, seven strains displayed a broad-spectrum bacteriostatic effect. In general, the inhibitory effect of the isolated antibacterial strains on *S. aureus* and *Salmonella* was greater than that on *E. coli* and *Bacillus*. The results suggested that the concentration and type of the produced antibacterial substance differed from those of LAB. Thus, different LAB showed various degrees of inhibitory activity against pathogenic bacteria. Lanhua Yi [15] revealed that *Lactobacillus* can be enhanced to inhibit pathogenic bacteria selectively and confirmed that *L. coryniformis* XN8 exhibits a broad-spectrum antimicrobial activity and induces a strong antibacterial effect against *S. aureus*. This finding confirmed the characteristics of the selective inhibition of LAB [16].

pH was roughly used to determine the acidity of the produced *Lactobacillus.* Table 1 shows that the amount of acid (pH < 3.7) secreted by two strains, namely, T30 and S6, was higher than that produced by other strains. This finding indicated the varying transport regulation and metabolism of the lactose system of LAB and the acid production ability of the different strains. Therefore, S6 shows potential as a biopreservative and fermentation agent in fermented food production[17].

Seven strains, namely, L2, L16, L19, T4, T8, H9, and S6, and the indicator bacteria, namely, *S. aureus* and *Salmonella*, were examined for further relevant experimentation.

3.2. Strain Identification. The seven isolates with antimicrobial activities were identified using a 16s rRNA gene. The results were compared with the data in the EzTaxon-e database. Table 2 shows three *Lactobacillus* species, namely, *L. plantarum* (L2, L16, T4, and T8), *L. pentosus* (L19 and S6), and *L. paracasei* (H9). The strains reached more than 99% similarity.

3.3. Identification of Antimicrobial Components. Most strains lost their antimicrobial activity after acid was removed. L19 slightly decreased the antibacterial effect of the inhibited bacteria after catalase treatment was administered. This finding suggested that the main antimicrobial effect of some strains is dependent on acid and confirmed that hydrogen peroxide elicits a bacteriostatic effect [18]. Table 3 shows the bacteriostatic effect of the seven strains after enzyme treatment was administered. These supernatants of the strains were partially inactivated by using α-amylase and lysozyme and consequently induced to decrease their antimicrobial activities. The antimicrobial activities of the strains treated with proteinase K, pepsin, and papain were completely inactivated, but the antimicrobial activity of S6 was slightly retained after papain was administered. NatarajanDevi [19] demonstrated that bacteriocin produced by *L. sakei* GM3 isolated from goat milk is unstable after pepsin, trypsin, papain, and proteinase Kare administered. This finding suggested that the primary antimicrobial activity of the seven strains was also dependent on peptides after acid and catalase were removed. Moreover, the results showed that antimicrobial substances may contain a carbohydrate that promotes inhibition to a certain extent.

3.4. Effects of Temperature, pH, Additives, and Organic Compounds on Antimicrobial Components. After the treatments were administered at different temperatures, their effects on the antimicrobial activity of LAB were stable at a low temperature for 30 min (Table 4). Similarly, bacteriocins produced by the strains isolated from salpico are thermostable at 100°C for 20 min[20]. The bacteriocins produced by *L. bulgaricus* BB18 and *L. lactis* BCM5 were highly stable at high temperatures, and their antimicrobial activities were retained after 60 min at 100°C. However, the antimicrobial activity of L16 and pathogenic growth in the zone against the indicator bacteria was reduced to some extent at 121°C for 20 min. Most antimicrobial components remained stable at high temperatures. The molecular weight of antimicrobial peptides, which are secondary protein structures (α-helix, β-folding, β-rotation angle, and random crimp), is between 3 and 10 kDa. Their low molecular weight and secondary structure may lead to the high-temperature resistance of most antimicrobial peptides [21]. This finding indicated that the antibacterial components of LAB could be applied as

Table 1: Antimicrobial activity of the supernatant of the strains against pathogenic bacteria.

Strain	pH (48 h)	Inhibition zone (mm) of S. aureus			Inhibition zone (mm) of Salmonella		
		24 h	48 h	72 h	24 h	48 h	72 h
T4	3.88±0.01	9.13±0.12	9.17±0.15	9.07±0.06	9.09±0.07	9.04±0.04	9.03±0.10
T5	3.82±0.02	8.03±0.15	8.13±0.06	8.17±0.12	8.67±0.08	8.71±0.08	8.69±0.07
T6	3.76±0.01	8.00±0.20	8.03±0.06	8.33±0.35	8.18±0.12	8.14±0.04	8.19±0.11
T8	3.72±0.04	9.27±0.25	9.23±0.05	9.20±0.20	9.03±0.13	9.05±0.13	9.03±0.06
T12	3.76±0.03	-	8.46±0.12	8.26±0.21	7.85±0.18	8.16±0.10	8.09±0.04
T13	4.06±0.04	-	7.83±0.21	7.97±0.12	9.07±0.11	8.98±0.12	9.09±0.09
T18	3.88±0.01	8.89±0.15	8.87±0.08	8.73±0.31	8.48±0.06	8.59±0.11	8.44±0.14
T19	3.96±0.06	7.70±0.26	7.93±0.12	7.90±0.10	-	-	-
T20	4.02±0.16	7.83±0.02	7.85±0.01	8.13±0.05	8.33±0.05	8.24±0.08	8.34±0.15
T24	3.81±0.04	9.00±0.17	8.87±0.13	8.93±0.06	-	-	-
T25	3.95±0.02	-	7.86±0.02	7.94±0.05	8.70±0.11	8.64±0.05	8.61±0.03
T26	3.92±0.08	8.66±0.03	8.74±0.05	8.61±0.03	-	-	-
T27	3.97±0.01	7.50±0.26	7.37±0.23	7.40±0.27	7.92±0.09	8.01±0.15	7.87±0.03
T28	3.87±0.03	8.06±0.11	8.70±0.10	8.80±0.26	8.66±0.17	8.72±0.07	8.68±0.18
T30	3.69±0.03	8.63±0.06	8.70±0.17	8.64±0.07	8.51±0.16	8.45±0.08	8.52±0.19
T52	3.87±0.02	8.13±0.07	8.17±0.08	8.08±0.08	-	-	-
L1	3.90±0.01	8.20±0.02	8.16±0.03	8.12±0.08	7.94±0.21	7.85±0.09	7.83±0.05
L2	3.86±0.04	9.33±0.08	9.28±0.03	9.25±0.15	9.29±0.06	9.40±0.26	9.23±0.10
L8	3.88±0.02	9.22±0.15	9.28±0.12	9.21±0.16	8.38±0.14	8.39±0.13	8.31±0.14
L11	3.82±0.01	-	7.79±0.02	7.83±0.01	-	-	-
L12	3.95±0.03	-	-	-	7.89±0.02	7.96±0.04	7.91±0.08
L13	3.97±0.06	8.23±0.03	8.22±0.03	8.17±0.07	8.88±0.08	8.85±0.06	8.83±0.08
L14	3.88±0.02	8.29±0.06	8.18±0.02	8.15±0.04	8.35±0.18	8.42±0.13	8.36±0.04
L15	3.90±0.05	8.42±0.03	8.41±0.08	8.37±0.08	7.03±0.26	7.72±0.37	7.65±0.19
L16	3.84±0.02	9.18±0.09	9.17±0.05	9.17±0.10	9.06±0.07	9.16±0.14	9.22±0.25
L18	3.99±0.03	-	-	-	8.29±0.05	8.30±0.05	8.21±0.13
L19	3.79±0.01	9.23±0.06	9.26±0.06	9.14±0.04	9.17±0.08	9.19±0.02	9.11±0.06
L20	3.99±0.04	-	8.27±0.08	8.21±0.04	8.23±0.07	8.34±0.06	8.31±0.02
L21	3.85±0.05	8.31±0.15	8.27±0.05	8.39±0.03	7.89±0.14	7.81±0.05	7.82±0.09
L25	3.91±0.02	8.65±0.05	8.57±0.11	8.54±0.02	8.22±0.04	8.16±0.08	8.13±0.04
L27	3.87±0.07	-	7.32±0.09	7.32±0.03	8.23±0.07	8.77±0.07	8.50±0.00
L31	4.12±0.10	8.15±0.09	8.09±0.04	8.15±0.06	7.75±0.39	8.29±0.08	8.43±0.09
S6	3.67±0.04	9.42±0.18	9.39±0.09	9.42±0.10	9.03±0.06	9.07±0.03	9.06±0.02
S8	3.90±0.11	9.12±0.03	9.20±0.13	9.09±0.09	8.18±0.09	8.27±0.10	8.23±0.03
S13	3.92±0.05	8.07±0.06	8.05±0.05	8.08±0.11	-	-	-
H9	3.92±0.04	9.38±0.09	9.32±0.09	9.27±0.08	9.08±0.07	9.17±0.09	9.03±0.06
H12	3.88±0.03	8.50±0.05	8.48±0.03	8.56±0.07	8.23±0.03	8.23±0.05	8.26±0.17
H27	3.97±0.02	8.06±0.04	8.10±0.08	8.04±0.01	7.90±0.03	8.08±0.10	7,97±0.05
nisin		14.53±0.15	14.43±0.28	14.48±0.07	12.73±0.16	12.65±0.05	12.73±0.14
ampicillin		19.21±0.07	19.18±0.13	19.20±0.10	14.96±0.11	15.05±0.10	15.05±0.05

biological preservatives for high-temperature treatments of food.

Most strains were inhibited on two indications when three types of organic solvents were added to the supernatants (Table 4), and this observation was consistent with that reported by Natarajan Devi [19], who demonstrated that bacitracin can be soluble in organic solvents. However, the antibacterial effect was reduced to some extent when methanol was added to T4 and L19 supernatants possibly because the surface structure of various antimicrobial agents caused intolerance to methanol.

The antimicrobial activity of the supernatant against the indicator was stable within a wide pH range (2.0–6.0). The antimicrobial activity of LAB was also retained at pH 8 for 30 min, but the inhibitory effect of S6 was evidently reduced. The antimicrobial activities at pH 14 were completely lost, and this finding was consistent with *Pediococcus pentosaceus* bacteriocin ALP57 that loses its antimicrobial activity at

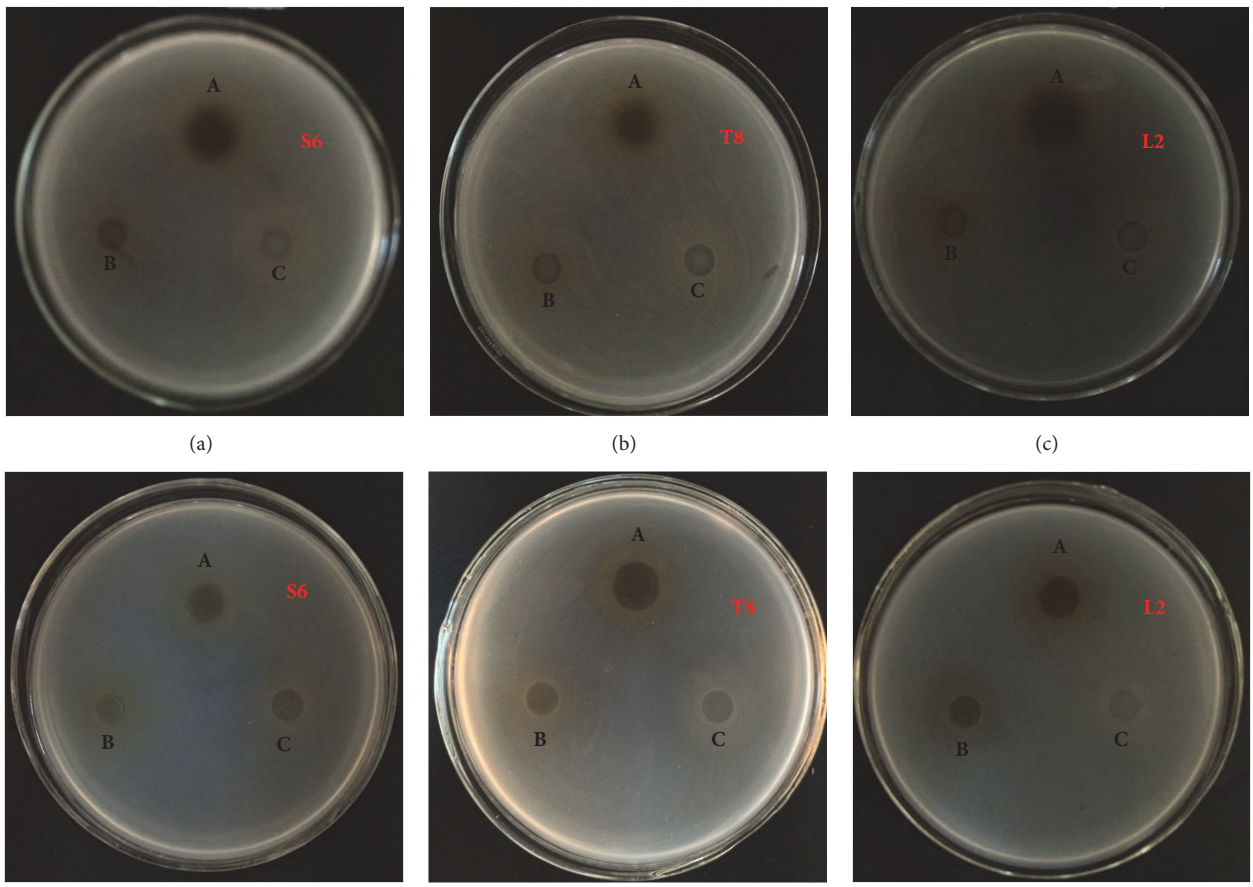

Figure 1: Crude extraction test after preliminary purification against *S. aureus* (a, b, and c) and *Salmonella* (d, e, and f). A: sample concentration. B: MRS concentration. C: water in each figure.

Table 2: Identification of strains.

Strain	Isolation source	Identified	%Similarity
L2	chili sauce	*Lactobacillus plantarum*	100%
L16	chili sauce	*Lactobacillus plantarum*	100%
L19	chili sauce	*Lactobacillus pentosus*	99.79%
T4	corn noodle	*Lactobacillus plantarum*	100%
T8	corn noodle	*Lactobacillus plantarum*	100%
H9	glutinous rice dough	*Lactobacillus paracasei*	100%
S6	stinky tofu	*Lactobacillus pentosus*	100%

pH 12[22]. In our study, high antimicrobial activities were detected at low pH.

All of the supernatants were treated with additives, such as sodium citrate, potassium, and surfactants, to verify the effect of food additives and other chemicals on the antibacterial components of LAB (Table 4). LAB showed a stable antimicrobial activity against the indicator bacteria treated with sodium citrate and potassium. After Tween-80 was added, the antimicrobial activity remarkably differed, and T4 lost its antimicrobial activity. Priscilia Y [23] reported that the antimicrobial activity of *Lactobacillus* spp. isolated from Mexican Cocido cheese against *S. aureus*, *Listeria innocua*, *E. coli*, and *S. typhimurium* decreases when anionic compounds are added. However, the bacteriostatic effect of bacteriocin CM3 is stable when different surfactants are added. These behaviors could be explained by LAB from different sources producing bacteriocin-like substances (BLS), whose surface structure varies and consequently results in different sensitivities to Tween-80. Overall, the different surface structures of the BLS affect their antimicrobial activity, and their substance tolerance varies.

L2, S6, and T8 strains were selected for further experiments based on our results.

3.5. Analysis of the Antibacterial Components after Purification. In the experiment, the concentrate that inhibited the indicator was enhanced after ammonium sulfate precipitated, revealing that the antimicrobial components of these strains were proteins (Figure 1). The comparisons were drawn between the inhibition zones of the untreated and concentrated samples. All of the inhibition zones of the concentrate against *S. aureus* were enlarged by >12 mm (Figures 1(a), 1(b), and 1(c)). The inhibition zone of S6 reached 15.22±0.13 mm. The concentrations of T8 and L2 remarkably inhibited *Salmonella* (Figure 1), and the inhibition zone of

Table 3: Effects of antimicrobial after treatment with enzymes.

Enzyme	Strains						
	L2	L16	L19	T4	T8	H9	S6
Proteinase K	-	-	-	-	-	-	-
Pepsin	-	-	-	-	-	-	-
Papain	-	-	-	-	-	-	+
α-Amylase	+++	++	+	++	++	+++	+++
Lysozyme	++	++	++	+	++	++	++

ps: "-":inhibition zone < 6 mm; "+":inhibition zone: 6–8 mm; "++":inhibition zone: 8–9 mm; "+++":inhibition zone > 9 mm.

Table 4: Residual antimicrobial activity of *Lactobacillus* treated under different conditions.

Treatment concentration	Strains						
	L2	L16	L19	T4	T8	H9	S6
Temperature/time							
65°C/30 min	+++	+++	+++	+++	+++	+++	+++
85°C/30 min	+++	+++	+++	+++	+++	+++	+++
100°C/30 min	+++	+++	+++	+++	+++	+++	+++
121°C/20 min	+++	+	++	++	++	+++	++
pH							
2	+++	+++	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++	+++	+++
8	++	++	++	++	+++	++	+
14	-	-	-	-	-	-	-
Organic solvent							
Methanol 10% (vol/vol)	++	++	+	++	++	+++	++
Ethanol 10% (vol/vol)	+++	+++	+++	+++	+++	+++	+++
n-Butanol 10% (vol/vol)	+++	+++	+++	+++	+++	+++	+++
Additive							
Potassium chloride 1% (wt/vol)	+++	++	+++	++	++	++	+++
Sodium citrate 1% (wt/vol)	+++	++	++	++	+++	+++	+++
Tween-80 1% (wt/vol)	+	+	++	-	+	+	++

ps: "-":inhibition zone < 6 mm; "+":inhibition zone: 6–8 mm; "++":inhibition zone: 8–9 mm; "+++":inhibition zone > 9 mm.

T8 was more than 13 mm. Overall, the concentrate could enhance the antimicrobial activity, and the observation was the same as that in previous studies, which showed that the ability of pure plantaricin NC8 to inhibit food-borne pathogens is evidently higher than that of the untreated group [24]. Ammonium sulfate precipitate produced from different strains elicited varying inhibitory effects against bacteria, suggesting that LAB selectively inhibit pathogens, and their antimicrobial components belong to a protein family [15].

S6 and T8 were selected for the subsequent experiments because the concentrated T8 significantly enhanced the bacteriostatic effect of the two pathogenic bacteria, and S6 inhibited *S. aureus* to a greater extent than other strains.

3.6. Effect of Various Factors on the Growth Curve of the Indicator Bacteria. Figure 2 shows the influence of various factors on the growth of pathogenic bacteria. Lanhua Yi [15] reported that LAB can inhibit bacterial growth to some extent. The antibiotic–MRS completely inhibited the non-drug-resistance genes of *S. aureus*. However, OD_{600} and the population of the indicator in the MRS group were higher than those of the experimental group with the same volume until 12 h. Meanwhile, the population of *S. aureus* in 3∗supernatant (S6) ranged from 12 $\log^{10}$ CFU/mL to 15 $\log^{10}$ CFU/mL compared with that in 3∗MRS, which was significantly higher than that in the 1∗ group as revealed by the results of the colony counting method at 12 h. The positive control group of antibiotic–MRS also favors the growth of *Salmonella* to some extent [25, 26]. This finding is consistent with that described in a recent work, which showed the resistance of *Salmonella* to gentamicin, ciprofloxacin, aminoglycosides, and tetracycline [27, 28] (Figures 2(c) and 2(d)). This phenomenon remarkably altered the OD_{600} and the colony number of *Salmonella* in the antibiotic–MRS group. In this study, the OD and clump count revealed that the supernatant or the antibiotic could decrease the growth

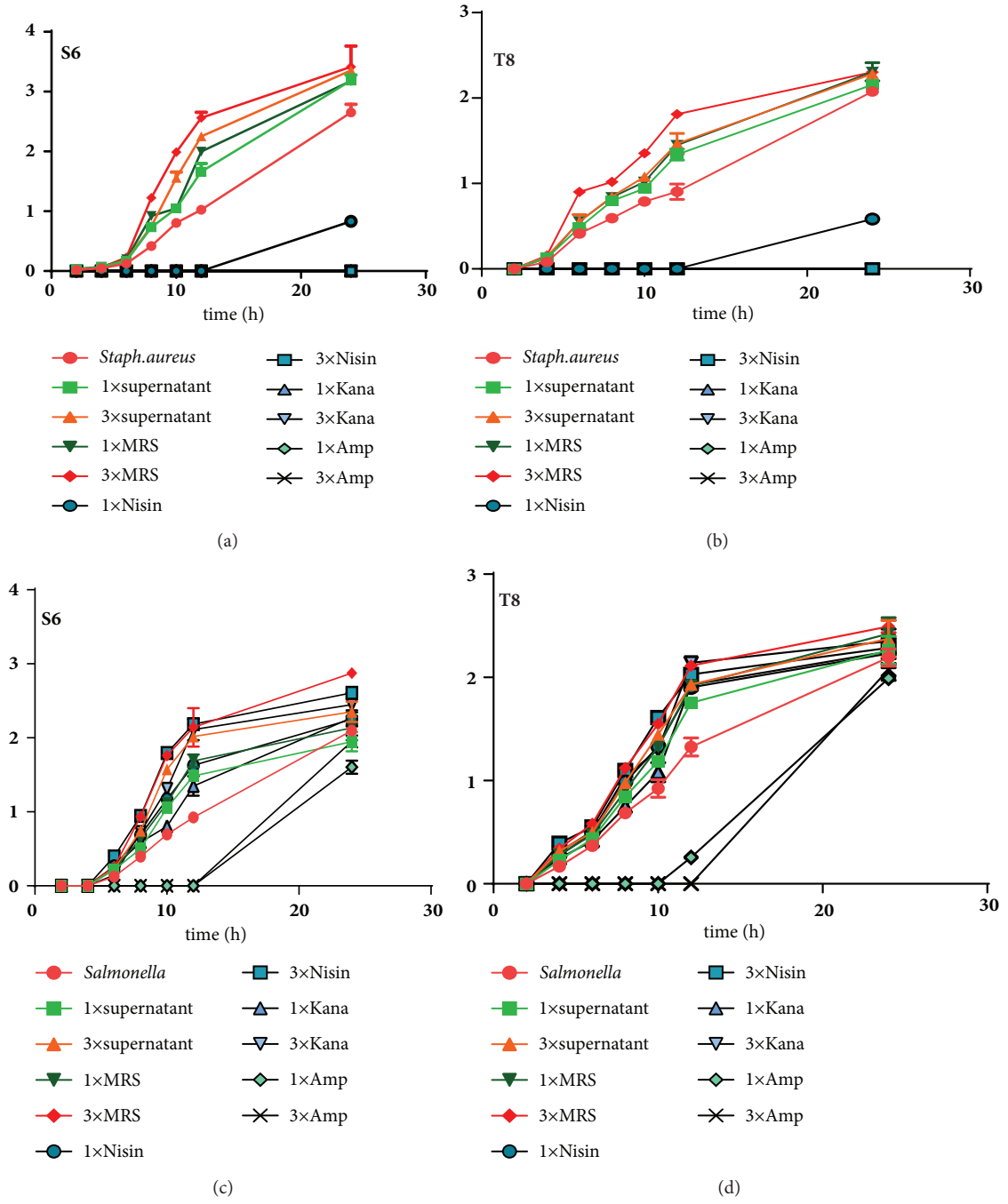

Figure 2: Effects of various factors on the growth of strains. *S. aureus* (a, b) and *Salmonella* (c, d) are the indicator bacteria.

of the indicator bacteria compared with that of the control group with MRS.

3.7. Identification of Bacteriocin. PCR showed a 150–200 bp DNA fragment in Figure 3 (L19, L16, L2, T4, and T8). Sequence analysis revealed that the target DNA fragment reached 100% similarity compared with that of PlnF in the National Center for Biotechnology Information. nanoLC-ESI-MS/MS indicated that the peptide had a molecular weight of 5729.03 Da (Table 5). In comparison with UniProt, the submitted peptide was bacteriocin peptide plnF, whose relative abundance reached 97.8%. Albert [29] reported the presence of plantaricin-encoding genes, such as plnA, plnB, plnE/F, and plnF, in LAB.

4. Conclusions

In vitro studies showed that the antibacterial components of seven LAB isolated from nianmianzi, tangzimian, chili sauce, potherb mustard pickles, and stinky tofu were resistant to certain temperature ranges (65°C–121°C), acidity (pH 2–6), alcohol, and some additives. After preliminary

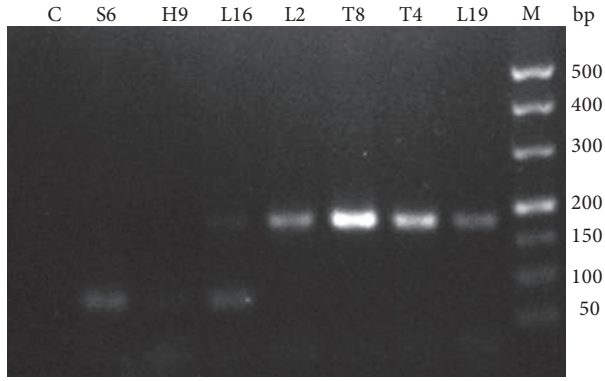

Figure 3: Agarose gel electrophoresis of the PCR product. Line L19–S6: PCR result of the seven strains. Line C: negative control.

Table 5: nanolc-ESI-MS/MS.

Protein Mass	No. of Peptides	Sequence Header	Link	Relative Abundance	Probability
5729.03	87	Bacteriocin peptide PlnF OS = Lactobacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) GN=plnF	F9UU07	97.8%	99.0%

enrichment was performed using ammonium sulfate, the bacteriostatic effect of *L. pentosus* S6 against *S. aureus* was up to 15.22±0.13 mm, the bacteriostatic effect of *L. plantarum* T8 increased to 13.08±0.15 mm, and the inhibition zone of T8 against *Salmonella* increased to 13.37±0.19 mm. These findings demonstrated that the crude extract of T8 elicited good broad-spectrum antibacterial effects. The results of the growth inhibition experiment of pathogenic bacteria confirmed that LAB had certain supernatant fluid that inhibited bacterial growth. nanoLC-ESI-MS/MS and PCR results indicated that the bacteriocin peptide plnF existed in T8. Our results provided a basis for performing future studies on the heterogeneous expression of antimicrobial peptides and screening other suitable culture media for LAB growth of LAB.

Abbreviations

MRS: de Man, Rogosa, and Sharpe
BLS: Bacteriocin-like substances
EDTA: Ethylene diamine tetraacetic acid
CFU/mL: Colony forming unit per milliliter
S. aureus: *Staphylococcus aureus*
E. coli: *Escherichia coli*.

Data Availability

Most of the data about antimicrobial activity have been showed in Table 1; and according to the partial data, we draw Figure 2. Please feel free to contact Dayong Ren when you need the whole database.

Conflicts of Interest

The authors declare no conflicts of interest in the publication of this paper.

Authors' Contributions

Dayong Ren and Jianwei Zhu contributed equally to this paper. Dayong Ren, Jianwei Zhu, and Hansong Yu conceived and designed the experiments. Jianwei Zhu and Shengjie Gong performed the experiments. Dayong Ren, Jianwei Zhu, and Hongyan Liu analyzed the data and wrote the manuscript.

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Research Article

Peptide Extract from *Olivancillaria hiatula* Exhibits Broad-Spectrum Antibacterial Activity

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Increasing reports of infectious diseases worldwide have become a global concern in recent times. Depleted antibiotic pipelines, rapid and complex cases of antimicrobial resistance, and emergence and re-emergence of infectious disease have necessitated an urgent need for the development of new antimicrobial therapeutics, preferably with novel modes of action. Due to their distinct mode of action, antimicrobial peptides offer an interesting alternative to conventional antibiotics to deal with the problems enumerated. In this study, the antimicrobial potential of the peptide extract from the marine mollusc, *Olivancillaria hiatula,* was evaluated *in vitro.* Agar diffusion and broth dilution techniques were used to evaluate microbial susceptibility to the peptide extract. Microplate-based assays were also used to investigate time-dependent growth inhibition profiles of microbes in the presence of peptide and evaluate the peptide's ability to modulate the activities of standard antibiotics. Both Gram-positive and Gram-negative bacteria were inhibited by the peptide extract in the agar diffusion assay. The minimum inhibitory concentration (MIC) of peptide against test microorganisms was between 0.039 and 2.5 mg/mL. At the MIC, the peptide extract was bacteriostatic towards all tested microorganisms but bactericidal to *Staphylococcus aureus.* In the presence of the peptide extract, a prolonged lag phase was observed for all microbes, similar to standard ciprofloxacin. When administered together, peptide extracts enhanced the activities of ciprofloxacin and cefotaxime and were antagonistic towards erythromycin but indifferent towards metronidazole. Taken together, these results show the broad-spectrum antibacterial activity of peptide extract from *Olivancillaria hiatula* and demonstrate that antimicrobial peptides can be employed in combination with some conventional antibiotics for improved effects.

1. Introduction

At the beginning of the 20th century, infectious diseases were reported to be the leading cause of global morbidity and mortality. The discovery of the penicillins and other antibiotics improved this grim outlook a bit, with increased optimism that the war against infectious diseases was under control [1, 2]. Between 1930 and 2000, there was a tremendous supply of antibiotics and arsenals of other antimicrobials for clinical and veterinary use. Antimicrobials such as penicillins, tetracyclines, macrolides, cephalosporins, quinolones, aminoglycosides, oxazolidines, and glycopeptides revolutionized the field of medicine and increased life expectancy remarkably [3, 4]. Infectious diseases, however, still remain a concern. Globally, they are the second leading cause of deaths and the third leading cause of death in developing countries [5, 6].

In the last few decades, the world has undeniably faced a postantibiotic era characterized by multidrug resistance, where most microbes are escaping the effect of existing antibiotics [7, 8]. To further compound the situation, there is a marked decline in research and development of antimicrobials [7] and this is a major threat to global health. This decline in the antibiotic pipeline, the inevitable development of resistance that follow the introduction of new antibiotics [9] coupled with emergence, and reemergence of infectious diseases have led to a pressing need for new antimicrobial agents to be unearthed to salvage this dire situation.

To overcome the menace of antimicrobial resistance (AMR), various strategies have been proposed. These include

combination therapy [10], supplementing antibiotics with adjuvants [11], modifying old antibiotics to improve antimicrobial activity [12], and searching nature for new antimicrobial agents [7]. While combination therapy seems to be at the risk of toxicity and antagonism, modifying old antibiotics could expand the spectrum of resistance acquisition strategies employed by microorganisms and lead to even further complications. The search for novel antimicrobial agents, antibiotics, or lead compounds with unconventional modes of action from nature is potentially a promising route to tackle the problem.

Most antibiotics owe their source to the terrestrial ecosystem: fungi, soil-borne bacteria, and some plants are examples. The aquatic (marine) ecosystem has languished behind the terrestrial ecosystem in the search for remedies with novel mechanisms of action [13]. However, exploring sources such as the marine environment could lead to the discovery of chemical and biological novelties as well [14]. A number of works on several extracts of marine organisms have shown interesting antimicrobial, antioxidant, antimalarial, anti-inflammatory, and anticancer activities. In fact, some metabolites possessing these properties have been isolated and characterized [15]. Antimicrobial peptides from marine invertebrates provide a novel class of compounds possessing remarkable antimicrobial activities as well as slower rates of resistance acquisition by bacteria [16, 17] that could be explored in the quest for new antimicrobial therapeutics.

Antimicrobial peptides (AMPs) are abundant in nature among plants and various animal families. They are mostly cationic and amphipathic. Due to their amphipathicity, they are able to achieve high concentrations in both aqueous environments and within membranes of organisms. AMPs exhibit a broad-spectrum antimicrobial activity since they constitute the first line of defense of both animals and plants against the attack of microbes. Microbial killing is usually as a result of rapid interaction of the AMP with the microbial outer membrane which leads to membrane disruption, release of cytoplasmic constituents, and a halt to cellular activities [18–21]. Little work is ongoing concerning peptides from Ghanaian marine invertebrates, but crude peptides of *Galatea paradoxa* and *Patella rustica* have been reported to possess some antimicrobial activity [22].

Olivancillaria hiatula (*O. hiatula*), a marine gastropod belonging to the family Olividae, is ubiquitous on the shores of Eikwe in the Western Region of Ghana. *O. hiatula* is benthic, and its sessile life form makes it prone to harsh environmental conditions and varying microbial attacks. We have recently shown that solvent extracts from the body tissue of *O. hiatula* possess impressive ability to reduce inflammation *in vivo* [23]. We hypothesized its whole-body tissue as a potential source of antimicrobial peptides. The antibacterial activities and antibiotic-modulating effect of peptides extracted from the whole-body tissue of *O. hiatula* were therefore investigated in this study.

The peptide extract from *O. hiatula* was observed to possess broad-spectrum antimicrobial activity against selected human pathogens. Bacterial growth kinetic studies demonstrated a prolonged lag time with a high reduction in bacterial growth within that period in the presence of peptides at subminimum inhibitory concentrations. Generally, bacteriostatic activity was observed for most of the organisms. Modulation studies revealed that the peptides enhanced the activity of ciprofloxacin and cefotaxime, antagonistic towards erythromycin but indifferent towards metronidazole activity.

2. Methods

2.1. Sample Collection and Identification. Samples were collected by convenience sampling from Eikwe (4° 58 '00" N 2° 28 '47" W), a town in the Nzema East Municipality of the Western Region of Ghana. They were transported on ice to laboratories in the Department of Chemistry, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, and stored at 4°C. Organism was identified with help from the Fisheries and Marine Sciences Department of the University of Ghana, Legon. The Global Biodiversity Information Facility (GBIF) database [24] was used to confirm the taxonomy and sample was identified as *O. hiatula*.

2.2. Peptide Extraction. The shells of the molluscs were removed and the whole-body tissues washed and blended. Hundred grams of the blended body tissue was homogenized with 60 mL of 10% (v/v) acetic acid and kept for 12 hours at 4°C. The extract obtained was centrifuged at 5000 rpm (SciSpin ONE, UK) for 10 minutes and the supernatant decanted. Ice-cold acetone (25 mL) was then added to the supernatant and kept at 4°C for 24 hours to precipitate peptides. The precipitates were collected by centrifuging at 5000 rpm for 15 minutes and discarding the supernatant. Precipitates were then frozen at -80°C. Nitrogen gas was used to blow out traces of solvents after freezing at -80°C. The peptides were reconstituted in 25% acetonitrile (ACN) prepared in 0.1% trifluoro acetic acid (TFA) to give 20 mg/mL stock solution [28] and stored at 4°C prior to use.

2.3. Characterization by Infrared Spectroscopy. The infrared spectrum of the peptide was determined using a Fourier Transform infrared (FTIR) equipment (UATR Two, PerkinElmer). The regions between 4000 cm^{-1} and 400 cm^{-1} were scanned. This was then followed by baseline correction. Dried extract obtained from lyophilization was used.

2.4. Antimicrobial Assays

2.4.1. Microbial Cultures. In this study, nine test bacterial strains (2 Gram-positives and 7 Gram-negatives) were used to assess the antimicrobial properties of the extracts. The Gram-positive bacteria used were *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Enterococcus faecalis* ATCC 29212 (*E. faecalis*). Gram-negative bacteria included *Escherichia coli* ATCC 25922 (*E. coli*), *Proteus mirabilis* ATCC 4175 (*P. mirabilis*), *Pseudomonas aeruginosa* ATCC 4853 (*P. aeruginosa*), and clinical strains of *Klebsiella pneumonia* (*K. pneumonia)*, *Salmonella paratyphi* (*S. paratyphi)*, *Neisseria gonorrhea* (*N. gonorrhea),* and *Vibrio cholera* (*V. cholera)*. All microbial strains were obtained from the Department

of Pharmaceutical Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences of the College of Health Science, KNUST.

2.4.2. Inoculum Preparation. Bacterial isolates were streaked onto nutrient agar (Oxoid, United Kingdom) plates and incubated for 18–24 hours at 37°C. Using the direct colony suspension method, suspensions of the organisms were made in nutrient broth and incubated overnight at 37°C. These overnight cultures were used for the determination of antimicrobial activity using the well diffusion assay. For the remaining tests, colony suspensions in sterile saline was adjusted to 0.5 McFarland standard and further diluted in sterile double strength nutrient broth (~2×10^5 CFU/mL) [29].

2.5. Agar Well Diffusion Assay. Twenty-five milliliters of freshly prepared sterile nutrient agar (cooled to 40–50°C) was poured into sterile Petri dishes containing 10 μL of overnight cultures and swirled to ensure a homogenous spread of the organisms. This was allowed to solidify. Three equidistant wells of 6 mm in both diameter and depth were made on the plates using sterile cork borers. 100 μL of prepared peptide solution was then dispensed into the wells, allowed to equilibrate at room temperature for 30 minutes, and then incubated overnight at 37°C. Zones of growth inhibition (in mm) were measured as the diameter of the clear zone around each well. The assay was performed in independent triplicates and the averages of the three experiments taken. Ciprofloxacin (Sigma Aldrich, Michigan, USA) was used as reference antimicrobial agent (positive control) for bacteria strains while 25 % ACN in 0.1 % TFA was used as negative control [28].

2.6. Minimum Inhibitory Concentration. Minimum Inhibitory Concentration (MIC) of the peptide extract was determined by the broth microdilution method described by Wiegand [29]. Ten to twenty-four serial twofold dilutions of peptide or standard antibiotic (Ciprofloxacin) were prepared to obtain a final concentration range of 2.5 to 4.88×10^{-3} mg/mL and 500 to 5.96×10^{-5} μg/mL for peptide and ciprofloxacin, respectively, in a microtiter plate. Fifty microliters of double strength nutrient broth containing an inoculum size of ~2.0×10^5 CFU/mL was added to each well. The total volume of each well was 100μL. The plates were covered and incubated at 37°C for 24 hours. Twenty microliters of 1.25 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 30 minutes at 37°C. The MIC was determined as the lowest concentration of peptide extract or drug that inhibited growth of test organism. This was indicated by the absence of purple coloration upon the addition of the MTT dye and incubation. All tests were performed in triplicate.

2.7. Minimum Bactericidal Concentration (MBC). Minimum bactericidal concentrations (MBC) of the peptide extracts were determined by the same procedure as the MIC assay. After the 24-hour incubation period, 50 μL aliquots from wells with peptide concentrations greater than the MIC were plated on sterile agar plates. Agar plates were incubated at 37°C for 24 hours. MBC was recorded as the lowest extract concentration killing 99.9 % of the bacterial, i.e., least peptide concentration that showed no visible growth of the microorganisms on the surface of the nutrient agar. Each experiment was repeated three times.

2.8. Evaluation of Bactericidal and Bacteriostatic Capacity of Peptide Extract. The ratio of MBC/MIC was used to characterize the antimicrobial activity of peptide extracts. When the ratio of MBC/MIC ≤ 2, the effect was considered as bactericidal and a ratio ≥ 4 defined as bacteriostatic [25].

2.9. Microplate-Based Turbidimetric Growth Inhibition Assay. Growth inhibition of test organisms in the presence of peptide was studied using the microplate inhibition assay [30, 31] with slight modifications. In this assay, peptide extract was serially diluted from 4× MIC concentration through to 0.25× MIC peptide concentration for each organism after which 50 μL of nutrient broth containing a microbial inoculum size of ~2.0×10^5 CFU/mL was added. Microplates were incubated at 37°C and optical density at 600 nm (OD_{600}) determined at 2 hourly intervals with a microplate reader (Synergy H1 multimode plate reader, Germany). The OD_{600} values obtained were plotted against time and were used to illustrate the inhibitory activity of the peptide of *O. hiatula* against the various test organisms.

2.10. Modulation Studies. The ability of peptide extracts at sub-MIC concentrations to modulate the activity of standard antibiotics was evaluated. In this experiment, the MIC of standard antibiotics against the microbes and the MIC of the antibiotics in the presence of sub-MIC concentration of the peptide were determined. The microbial resistance modulation tests were performed according to a modified procedure described by Wiegand and coworkers [29]. Twenty-four serial twofold dilutions of standard antibiotics; Ciprofloxacin (Sigma Aldrich), Metronidazole (Sigma Aldrich), Erythromycin (Alfa Aesar), and cefotaxime (Alfa Aesar) were prepared to obtain final concentration ranges of 500 to 5.96×10^{-5} μg/mL. Fifty microliters of nutrient broth containing a microbial inoculum size of ~2.0×10^5 CFU/mL was added to each well. The reference antibiotics were tested against all microorganisms. MICs were determined after incubation of plates for 24 hours and upon the addition MTT to the medium in the wells.

Subinhibitory concentrations of 20 μg/mL of the peptide solution and various dilutions of standard antibiotics plus the same inoculum size were mixed and then incubated overnight at 37°C. MICs of antibiotics in the presence of the peptides were determined as described earlier. All tests were performed in triplicate.

Modulation factor (MF) was calculated and used to evaluate the antimicrobial effects of the peptide extract on the MIC of various antibiotic used.

$$\mathrm{MF} = \frac{\mathrm{MIC\ (antibiotic)}}{\mathrm{MIC\ (antibiotic + modulator)}}. \quad (1)$$

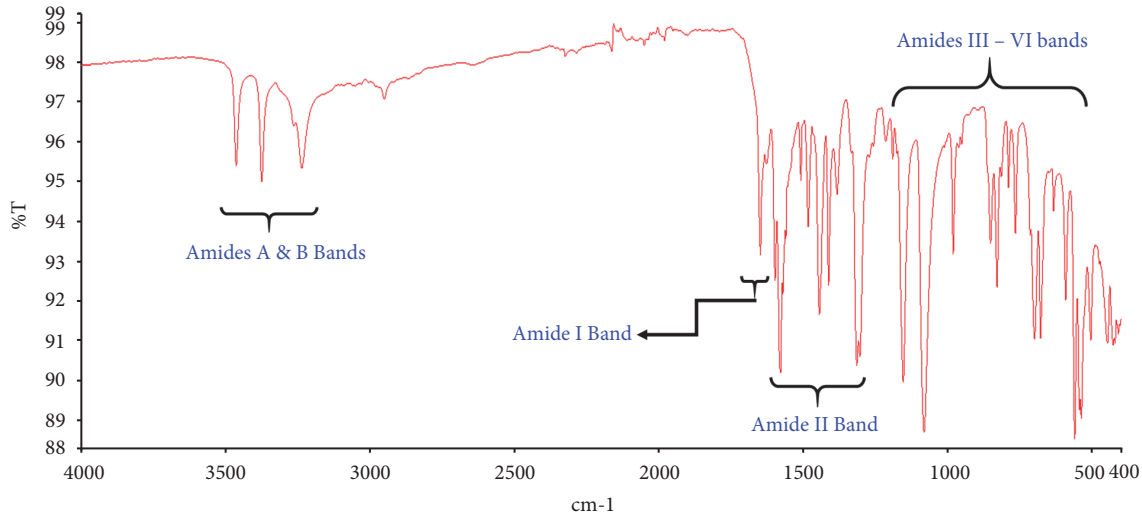

Figure 1: Fourier Transform infrared (FTIR) spectrum of peptide extract of *Olivancillaria hiatula*. Amides A&B bands spans 3100–3500 cm^{-1}, Amide I band is from 1600 to 1700cm^{-1}, Amide II band is from 1480 to 1600 cm^{-1}, and the region from 500 to 1300 cm^{-1} represents Amides III–VI bands [26, 27].

Table 1: Zones of inhibition (mm) of peptide extract against test microorganisms.

		Zone of inhibition (mm)	
Microorganism (Gram status)	Peptide Extract (5 mg/ml)	Positive control Cipro (1mg/ml)	Negative control
E. coli (-)	30.9 ± 0.2	51.5 ± 1.3	-
K. pneumonia (-)	28.9 ± 0.8	48.8 ± 1.2	-
S. paratyphi (-)	27.4 ± 0.5#	25.0 ± 6.1	-
P. mirabilis (-)	12.1 ± 0.6	41.0 ± 0.2	-
N. gonorrhea (-)	31.0 ± 0.4	40.0 ± 0.8	-
V. cholera (-)	30.0 ± 0.7∗	40.3 ± 0.6	-
P. aeruginosa (-)	27.0 ± 0.7	53.3 ± 0.9	-
S. aureus (+)	28.5 ± 2.1	39.2 ± 0.9	-
E. faecalis (+)	33.2 ± 3.0#	46.5 ± 1.9	-

Values reported as mean ± standard deviation of three replicate experiments; ∗ and # activity at 10 mg/mL and 50 mg/mL, respectively (ZI not observed at 5mg/mL); negative control (25 % ACN in 0.1 % TFA).

A modulation factor >2 was set as the cut-off for biologically significant modulation [32].

The change in MIC was computed using [33]

$$\text{Change in MIC} = \frac{(MIC\ (Antibiotic) - MIC\ (Antibiotic + Peptide))}{MIC\ (Antibiotic)} \times 100. \quad (2)$$

2.11. Data Analyses. GraphPad Prism Version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2007 were used for all data analyses and graphs.

3. Results

3.1. Infrared Characterization. The spectrum obtained from the FTIR showed prominent peaks of a typical peptide. Prominent peaks consistent with stretching and bending vibrations of N-H, C=O and C-H were observed (Figure 1).

3.2. Antimicrobial Assay. The peptide extract showed a broad-spectrum antimicrobial activity with impressive activities against all microorganisms. All extracts were tested at a concentration of 5 mg/mL for the agar diffusion assay. The highest zone of inhibition (ZI) was recorded against *N. gonorrhea* while no zone of clearance was observed against *V. cholera, S. paratyphi,* and *E. faecalis* at this concentration. When the concentrations were increased between 10 and 50 mg/mL, however, clear zones of inhibition were observed for those 3 microorganisms (Table 1).

3.3. Minimum Inhibitory Concentration (MIC) of Extracts. Peptide extract from *O. hiatula* demonstrated really good antimicrobial activity with very low MICs recorded. MICs ranged from 2.5 to 0.039 mg/mL against all test organisms. Gram-positive organisms recorded a relatively high MIC

Table 2: MIC, MBC, bacteriostatic and bactericidal effects of peptide extract.

Microorganism	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
E. coli (-)	0.625	>2.5	>4	static
K. pneumonia (-)	1.25	>2.5	>2	static
S. paratyphi (-)	0.625	2.5	4	static
P. mirabilis (-)	0.039	>2.5	>4	static
N. gonorrhea (-)	0.156	2.5	>4	static
V. cholera (-)	0.315	2.5	>4	static
P. aeruginosa (-)	0.039	1.25	>4	static
S. aureus (+)	2.5	2.5	1	cidal
E. faecalis (+)	2.5	>2.5	>1	Cidal / static

MIC and MBC experiments were replicated thrice; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBC/MIC ≤ 2 implies bactericidal; MBC/MIC ≥ 4 implies bacteriostatic [25].

Table 3: Co-modulation studies: MICs of ciprofloxacin plus 20 μg/mL of peptide extract.

Organism	MIC (μg/mL)		MF	Change in MIC (%)
	Cip	Cip + P		
E. coli (-)	1.95	0.00095	2053	99.95 ^{R}
K. pneumonia (-)	0.00095	0.00012	8	87.50^R
S. paratyphi (-)	125.00	0.00763	16383	99.99^R
P. mirabilis (-)	0.00048	0.00003	16	50.00 ^{R}
N. gonorrhea (-)	125.00	62.5	2	50.00^R
V. cholera (-)	3.91	0.00095	4116	99.98^R
P. aeruginosa (-)	0.24	0.00191	126	99.99^R
S. aureus (+)	15.63	1.95	8	87.50^R
E. faecalis (+)	0.24	0.0038	64	98.44 ^{R}

MIC experiments were replicated thrice; change in MIC computed using equation (2).
MF: modulation factor, Cip: ciprofloxacin, P: peptide extract, and *R*: reduction in MIC

of 2.5 mg/mL while the Gram-negative bacteria, especially, recorded much lower MICs (Table 2). *P. mirabilis* and *P. aeruginosa* in particular had very low MICs (39 μg/mL) as can be seen in Table 2.

3.4. Minimum Bactericidal Concentration (MBC). The MBC and the ratio of MBC to MIC were determined for the peptide extract against all test organisms. This ratio indicated the microbiostatic or microbicidal nature of the peptide extract against the test organisms. The lowest MBC (1.25 mg/mL) was recorded for *P. aeruginosa* while relatively higher MBC (≥ 2.5 mg/mL) of peptide were recorded for the remaining test organisms. From the ratio of MBC to MIC, the peptide was seen to have a microbicidal effect against *S. aureus* and a microbiostatic action against the remaining test organisms (Table 2).

3.5. Microplate Turbidimetric Growth Inhibition Assay. In the growth inhibition assay of the peptide extract against the test organisms, the growth curves of the test organisms in the presence of 4× MIC, 2× MIC, MIC, 0.5× MIC, and 0.25× MIC of the peptide extract were reduced comparative to the growth curves of the control (test organism in the absence of peptide). The lag phases of the test organisms were prolonged for an average of 16 hours while the log phases were also reduced in the presence of the peptide. Growth curves of most test organisms flattened during the 24-hour incubation period in the presence of 2× MIC and 4× MIC of peptide concentration while this effect was observed at even the MIC of ciprofloxacin (Figures 2 and 3). The effects of the peptide extract in inhibiting the growth of the test organism were observed to be concentration dependent (Figure 2).

3.6. Antibiotic Modulation. Peptide extract of *O. hiatula* at sub-MIC concentration of 20 μg/mL had noticeable effects on the response of test organism to antibiotics with modulation factor ranging from <0.25 to 524288 (Tables 3–6). When 20 μg/mL of peptide extract was added to varying concentrations of ciprofloxacin and test organisms, the MIC of ciprofloxacin reduced markedly for all test organisms by a factor as high as about 16, 000 (Table 3). Sub-MIC concentration of peptide extract also modulated the action of cefotaxime positively against test organisms (Table 4). There was a reduction in the MIC of cefotaxime in the presence of the peptide extract for all test organisms except *S. aureus and N. gonorrhea* where an increase in MIC was observed. The MIC of *N. gonorrhea* actually doubled under the experimental conditions (Table 4).

The peptide extract did not have any noticeable effect on metronidazole (Table 6) but was antagonistic to erythromycin (Table 5).

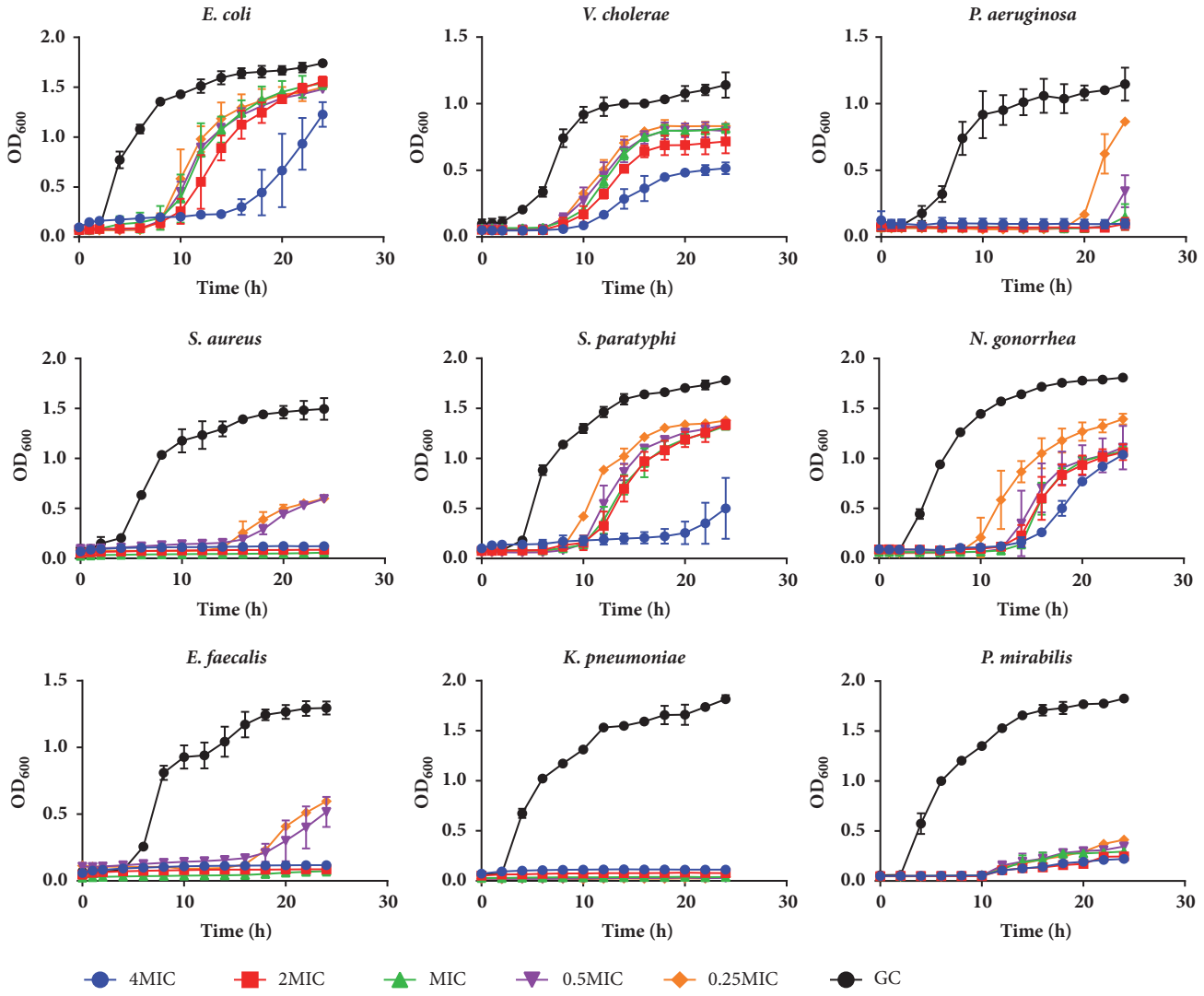

FIGURE 2: Growth curves of test microorganisms in the presence of varying concentrations of peptide extract. Each data point is the average of 3 replicate experiments (MIC, minimum inhibitory concentration; GC, growth control).

TABLE 4: Co-modulation studies: MICs of Cefotaxime plus 20 μg/mL of peptide extract.

Organism	MIC (μg/mL)			
	Cef	Cef + P	MF	Change in MIC (%)
E. coli (-)	31.25	1.95	16	93.75^{R}
K. pneumonia (-)	31.25	3.91	8	87.50^{R}
S. paratyphi (-)	62.5	3.91	16	93.75^{R}
P. mirabilis (-)	31.25	0.24	130	99.22^{R}
N. gonorrhea (-)	31.25	62.50	0.5	100.00^{I}
V. cholera (-)	62.5	1.19×10^{-4}	525210	99.99^{R}
P. aeruginosa (-)	31.25	1.19×10^{-4}	262605	100.00^{R}
S. aureus (+)	250.00	>250.00	<1	100.00^{I}
E. faecalis (+)	31.25	1.95	16	93.75^{R}

MIC experiments were replicated thrice; change in MIC computed using equation (2).
MF: modulation factor, Cef: cefotaxime, P: peptide extract, and R: reduction in MIC, *I*: increase in MIC.

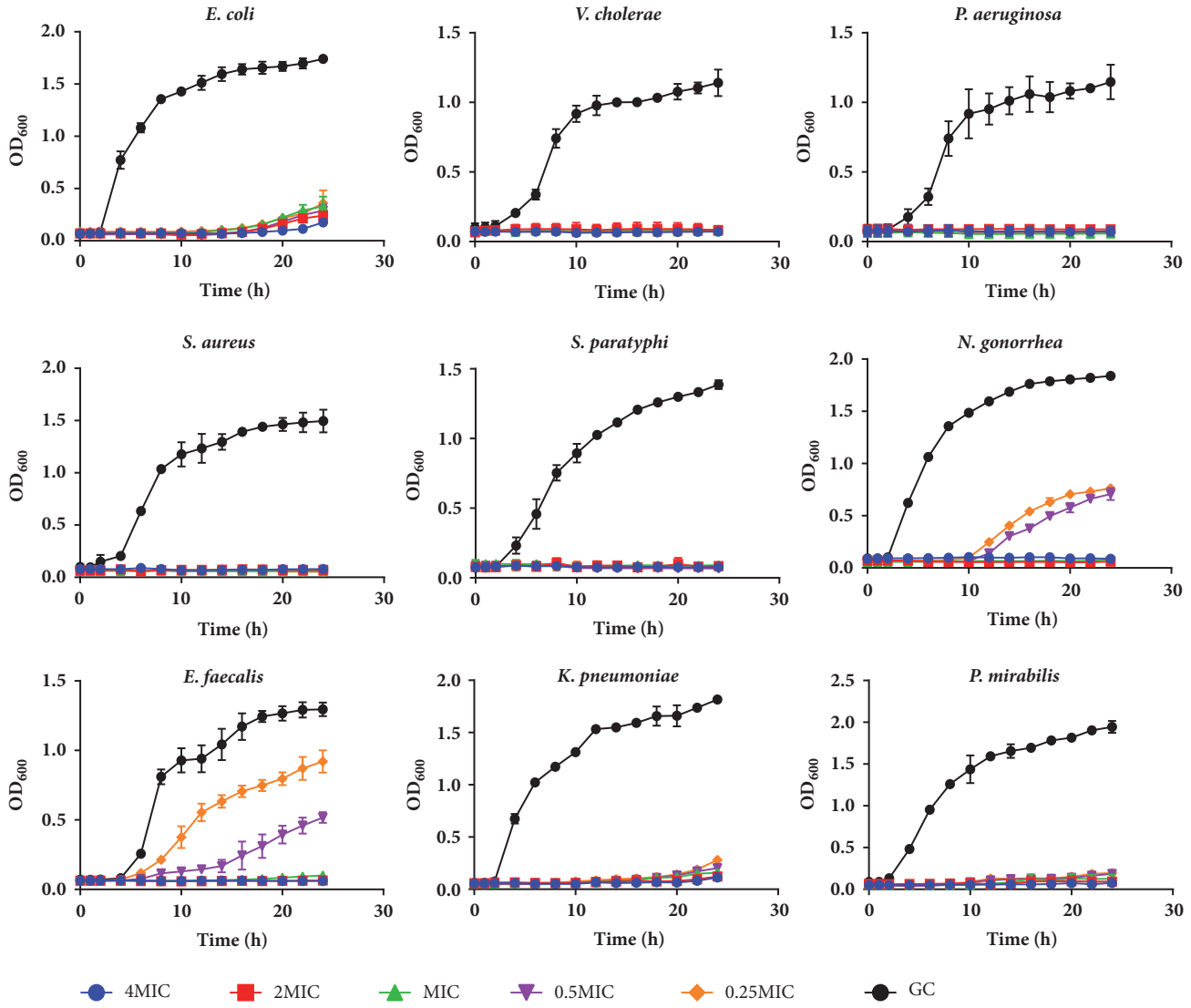

Figure 3: Growth curves of test microorganisms in the presence of varying concentrations of standard ciprofloxacin drug. Each data point is the average of 3 replicate experiments (MIC, minimum inhibitory concentration; GC, growth control).

Table 5: Co-modulation studies: MICs of Erythromycin plus 20 μg/mL of peptide extract.

Organism	MIC (μg/mL)		MF	Change in MIC (%)
	Eryt	Eryt + CP		
E. coli	125	125	1	*N*
K. pneumonia	7.8	>7.8	<1	> 100^I
S. paratyphi	250	>500	<0.50	> 100^I
P. mirabilis	125	250	0.50	100^I
N. gonorrhea	500	>500	<1	> 100^I
V. cholera	125	>500	<0.25	>100^I
P. aeruginosa	125	>500	<0.25	>100^I
S. aureus	>500	>500	<1.00	> 100^I
E. faecalis	0.24	>7.8	<0.03	>100^I

MIC experiments were replicated thrice; change in MIC computed using equation (2).
MF: modulation factor, Eryt: erythromycin, P: peptide extract, *N*: no change, and *I*: increase in MIC

Table 6: Co-modulation studies: MIC values of mtronidazole plus 20 μg/mL of peptide extract.

Organism	MIC (μg/mL)			
	Met	Met + CP	MF	Change in MIC (%)
E. coli	>500	>500	<1	$>100^{I}$
K. pneumonia	>500	>500	<1	$>100^{I}$
S. paratyphi	>500	>500	<1	$>100^{I}$
P. mirabilis	>500	>500	<1	$>100^{I}$
N. gonorrhea	>500	>500	<1	$>100^{I}$
V. cholera	>500	>500	<1	$>100^{I}$
P. aeruginosa	>500	>500	<1	$>100^{I}$
S. aureus	>500	>500	<1	$>100^{I}$
E. faecalis	>500	250	>1	$<50^{R}$

MIC experiments were replicated thrice; change in MIC computed using equation (2).
MF: modulation factor, Met: Metronidazole, P: peptide extract, *R*: reduction in mic, and *I*: increase in MIC

4. Discussions

Various methods exist for the isolation of peptides from marine invertebrates. In this work, we have utilized the whole-body tissue of *O. hiatula* as our source of antimicrobial peptides. Ice-cold acetone precipitation of peptides from whole-body tissue homogenate afforded crude peptides in appreciable quantities.

The FTIR spectrum of the extract obtained was consistent with reported vibrational spectra of peptides [26, 27, 34]. Amide I band, which is a direct consequence of the carbonyl (C=O) stretching vibrations, was observed at about 1650 cm^{-1}. N-H bending and C-N stretching vibrations are the major contributors to Amide II bands and are usually observed from 1480 to 1575 cm^{-1}. In this spectrum, Amide II bands turned out to be more prominent with strong absorptions recorded in this region. Amides A and B bands can be observed between 3200 and 3500 cm^{-1}. These are usually due to N-H stretching vibrations. Peaks corresponding to Amides III–VI regions (500–1300 cm^{-1}) can also be seen in the spectrum. Together, these peaks are indicative of a sample predominantly made up of peptides. IR spectra can be used to estimate secondary structural elements. It is difficult to make any such deductions from the spectrum of this extract since it is presumably a mixture and could contain a number of different peptides. However, the absence of strong Amide I absorptions is conspicuous. Based on this observation, it could be speculated that the extract is rich in α-helical peptides [26, 34].

Antimicrobial peptides (AMPs) usually exhibit broad-spectrum antimicrobial activity and have been suggested as an alternative to counter the menace of antimicrobial resistance. Because AMPs are usually membrane targeting, microbial resistance would probably involve the architectural redesigning and/or compositional variation of the entire cell lipid membrane of the microorganism [21]. Such a venture would most likely be very costly and difficult to achieve for microorganisms. AMPs therefore represent a viable therapeutic option.

Peptide extract from *O. hiatula* was active against both Gram-positive and Gram-negative bacteria. Microbial susceptibility was evaluated using the agar well diffusion and broth microdilution methods. Even though some microbes (*S. paratyphi, V. cholera,* and *E. faecalis*) required much higher peptide concentration for activity to be observed in the agar diffusion assay, they showed really good activities in the broth microdilution test. The broth microdilution assay is regarded as being more sensitive relative to the agar diffusion assay for screening antimicrobial natural products [35]. Properties of the natural product such as pH, solubility, volatility and diffusion in agar all influence results of the agar diffusion assay but not broth microdilution assay [36, 37]. The low MICs recorded against *N. gonorrhea* and *P. aeruginosa* is impressive and hence extract is considered to be very active [37]. In general, the MIC values recorded are much lower than those recorded for peptide extracts from *Patella rustica* and *Galatea paradoxa*[22] as well as methanol and ethyl acetate extracts of *Littorina littorea* and *Galatea paradoxa* [38]. These MICs, however, are in the range of those recorded for the antimicrobial peptide pexiganan, an antimicrobial peptide that has advanced furthest in clinical trials for the treatment of diabetic foot ulcers. MICs for pexiganan ranged from 16 to 32 μg/mL [39, 40]. There is a strong positive correlation between α-helical content and antimicrobial activity [41, 42]. The impressive activities recorded against both Gram-positive and Gram-negative bacteria supports the notion that the major secondary structural elements in *O. hiatula* peptide extract are α-helices, which was speculated from the IR data.

To investigate the kind of inhibitory effects that the peptide extract had on the various bacteria studied, the minimum bactericidal concentration (MBC), defined as the lowest extract concentration killing 99.9 % of the bacterial inocula after 24-hour incubation at 37°C, was recorded. At the MIC, a bacteriostatic effect was observed for all bacteria, except *S. aureus* where a bactericidal effect was observed. Above the MIC, peptide extract was found to possess a bactericidal effect. The activity of most AMPs is concentration dependent. An increase in peptide: lipid ratio across the membrane of microorganism greatly enhances the peptide's ability to penetrate and disrupt membrane integrity. Ion channel formation, transmembrane pore formation, and membrane rupture which all result in microbial death are more prevalent at higher peptide concentrations [43]. This effect can be observed clearly in the growth curves of the

various bacteria in the presence of varying peptide concentrations where a prolonged lag phase is recorded at 2× - and 4× MIC. The growth curves of *S. aureus, S. paratyphi, P. mirabilis,* and *P. aeruginosa* and to a lesser extent *E. faecalis* in the presence of peptides (Figure 2) were similar in shape to that of the standard drug, ciprofloxacin (Figure 3).

While therapeutic agents can be used in isolation to elicit specific effect(s), combination therapy is fast becoming the norm due to several advantages associated with it. Combination therapy could possibly reduce emergence of drug resistant microbes as the microorganism has to adapt to two or more drugs with different *modus operandi*. Toxicity associated with high doses could also be eliminated in combination therapy since lower doses of the drugs will be required to achieve comparable levels of efficacy in single drug therapy. Finally, the range of pathogens that could be targeted may be expanded depending on the individual drugs present in that particular combination [44]. Identification of AMPs that can be combined with orthodox antibiotics to be used for the treatment of infections has a good potential to expand available therapeutic options.

To evaluate the possible effect of the peptide extract of *O. hiatula* on some standard antibiotics, modulation experiments were set up. Subinhibitory concentration of peptide extract remarkably decreased the MICs of ciprofloxacin against all test microorganisms. When peptide was combined with cefotaxime, the MICs against almost all test microorganisms were also reduced. For erythromycin and metronidazole, a different trend was observed, with higher MICs being recorded for erythromycin and no appreciable change observed in the case of metronidazole. Both sets of antagonistic and synergistic effects of antimicrobial peptides in combination with antibiotics have been reported in literature [45, 46]. The synergistic interaction between peptides and antibiotics could be a result of the membrane permeability action of peptides or pore formation in the bacterial membrane. This leads to disruption of membrane integrity and easy penetration of antibiotics into bacterial cells where they cause greater damage [39, 40, 46]. The antimicrobial peptides, WR12 and D-IK8, have been shown to possess potent synergism with most topical antibiotics (fusidic acid and mupirocin) and systemic antibiotics (daptomycin, teicoplanin, vancomycin, linezolid, ciprofloxacin, meropenem, and oxacillin) [46]. Short peptide chains are known to confer bacterial resistance towards some macrolide antibiotics, especially erythromycin. Macrolide resistance occurs via modification of the drug binding site (either via allosteric mutations of direct mutations of amino acid residues in the vicinity of the binding pocket) [45, 47], action of specialized antibiotic efflux pumps [48], and the action of short peptides [45, 49]. Short peptides bind to the macrolide and form an inactive complex or act directly on the ribosome to inhibit or terminate translation [45].

5. Conclusions

The broad-spectrum antibacterial activity of the peptide extract of *O. hiatula* has demonstrated this study. Peptide extract was shown to be bacteriostatic at the MIC but bactericidal at twice and quadruple MICs. In the presence of the peptide extract, a prolonged lag phase was observed in the growth patterns of all test microorganisms. The peptide extract was also found to be synergistic when used with ciprofloxacin and cefotaxime but antagonistic towards erythromycin and indifferent to metronidazole. Together, these results demonstrate the utility of peptide extracts from *O. hiatula* as potential source of potent antimicrobial agents. Efforts to isolate and characterize the antimicrobial peptides in the extract mix are currently underway in our laboratories.

Data Availability

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

Disclosure

Part of this work was presented as a poster at the "7th Ghana Science Association. Research Seminar and Poster Presentations" held at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, in April 2018.

Conflicts of Interest

All authors declare no competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Authors' Contributions

Lawrence Sheringham Borquaye conceived the study. All experiments were designed by Lawrence Sheringham Borquaye, Edward Ntim Gasu, and Hubert Senanu Ahor. Samples were collected by Edward Ntim Gasu. Hubert Senanu Ahor and Edward Ntim Gasu carried out all the experiments. Data analysis was by Lawrence Sheringham Borquaye, Edward Ntim Gasu and Hubert Senanu Ahor. Manuscript was prepared by Lawrence Sheringham Borquaye, Hubert Senanu Ahor, and Edward Ntim Gasu. All authors read and approved the final manuscript.

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Research Article

Functional Probiotic Assessment and *In Vivo* Cholesterol-Lowering Efficacy of *Weissella* sp. Associated with Arid Lands Living-Hosts

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The research and the selection of novel probiotic strains from novel niches are receiving increased attention on their proclaimed health benefits to both humans and animals. This study aimed to evaluate the functional properties of *Weissella* strains from arid land living-hosts and to select strains with cholesterol-lowering property *in vitro* and *in vivo*, for use as probiotics. They were assessed for acid and bile tolerance, antibiotic susceptibility, membrane properties, antibacterial activity, antiadhesive effect against pathogens to host cell lines, and cholesterol assimilation *in vitro*. Our results showed that the majority of strains revealed resistance to gastrointestinal conditions. All the strains were nonhemolytic and sensitive to most of the tested antibiotics. They also exhibited high rates of autoaggregation and some of them showed high coaggregation with selected pathogens and high adhesion ability to two different cell lines (Caco-2 and MIM/PPk). Particularly, *W. halotolerans* F99, from camel feces, presented a broad antibacterial spectrum against pathogens, reduced *Enterococcus faecalis* and *Escherichia coli* adhesion to Caco-2 cells, and was found to reduce, *in vitro*, the cholesterol level by 49 %. Moreover, *W. halotolerans* F99 was evaluated for the carbohydrate utilization as well as the serum lipid metabolism effect in Wistar rats fed a high-cholesterol diet. *W. halotolerans* F99 showed an interesting growth on different plant-derivative oligosaccharides as sole carbon sources. Compared with rats fed a high-fat (HF) diet without *Weissella* administration, total serum cholesterol, low-density lipoprotein cholesterol, and triglycerides levels were significantly ($p<0.001$) reduced in *W. halotolerans* F99-treated HF rats, with no significant change in high-density lipoprotein cholesterol HDL-C levels. On the basis of these results, this is the first study to report that *W. halotolerans* F99, from camel feces, can be developed as cholesterol-reducing probiotic strain. Further studies may reveal their potential and possible biotechnological and probiotic applications.

1. Introduction

Probiotics are defined as "live microorganisms, which, when administrated in adequate numbers, confer a health benefit to the host" [1]. Lactic acid bacteria (LAB), especially Lactobacilli, are widely used in food production and represent the most common microorganisms employed as probiotics in functional foods [2–4]. The probiotic concept is gaining much worldwide attention, due to the perceived beneficial effects of these bacteria on human and animal health [2, 5, 6]. As well, the use of probiotics has been rising in order to ovoid negative effect induced by the abusive use of antibiotics in human and veterinary medicine [7, 8]. Particularly, the use of antibiotic as growth promoters in animal feed has been suspected to be responsible for the emergence of multidrug-resistant pathogens [9–11]. Therefore, the possibility of using probiotics as preventive/curative treatment or human and animal health promoters constitutes an important subject in applied microbiology [12, 13]. Probiotic strain selection must satisfy many criteria related to their safety, persistence,

and the required functional characteristics [14]. The tolerance to gastric acid and bile toxicity, the adhesion ability to intestinal cells, and inhibition of pathogenic bacteria are among the most important probiotic properties to consider in candidates selection for gastrointestinal tract colonization [2, 14]. Probiotic bacteria have their origins mainly from fermented foods or the gut microflora of humans and animals. However, based on many clinical studies the efficacy of some probiotics is highly questionable, such as *Lactobacillus rhamnosus* GG strain causing sepsis in children and adult patients linked to their ingestion as probiotic supplements [15–18]. Thus, the rigorous characterization and evaluation of the probiotic abilities are the most important factors for probiotic candidates. Particularly, bacterial species have not been before reported as probiotics. Moreover, the isolation and the selection of novel probiotic strains from other ecological niches could have the advantage to obtain strains with new beneficial functional properties, potentially useful for technological and/or probiotic applications. Organisms living in arid lands represent a valuable source to prospect for the selection of potential probiotic bacteria. The difference of origin should lead to specific bacterial characteristics, which might provide a new or prominent probiotic effect to the patients. Such organisms may select specific microorganisms having particular metabolic traits in response to their adaptation to hard conditions [19]. This concept of the possible implication of gut microbiome of many living organisms, especially for insects, in the survival/ adaptation of their hosts, becomes noticeable and well-argued [19–21]. In this work, LAB from desert plants and gastrointestinal microflora of camels and *Cataglyphis* ants were studied and evaluated for their probiotic potential. In fact, camels and *Cataglyphis* ants have a high capacity of adaptation to survive in semiarid, arid, and desert areas, which are characterized by poor nutrients, high temperatures, salt stress, desiccation, and UV radiations [20]. In addition, *Cataglyphis* ants are one of the most distinctive groups of insects that live in arid regions. They are commonly considered a model organism for studying many advanced adaptation traits [20]. Moreover, most ant species and their larvae are edible in different parts of the world, in order to satisfy the growing demand for sustainable feed and food sources [22]. Therefore, these distinctive physiological characteristics gained by such desert host-organisms may emphasize the presence of a peculiar gut microbiota, endowed with interesting metabolic properties contributing to the adaptation of their hosts. *Weissella* was proposed as a relatively new genus among the members of LAB, based on 16s rRNA gene sequences phylogeny data [23]. It is among the most widespread lactic species in different ecological niches [24]. Bacteria of the genus *Weissella* encompassing 19 species are reported to be isolated from a variety of fermented plant-based foods, soil, plants, animal products, human feces, and the gastrointestinal tract of human or animal [24–26]. They are facultative anaerobes and commonly grow at temperatures between 15 and 42°C. Only few studies have reported the evaluation of *W. kimchii, W. confusa*, and *W. cibaria* strains as potential probiotics [26, 27]. The aim of this study was to evaluate the probiotic prospective of some selected *Weissella* strains, isolated from unexploited source (bacterial communities associated with camel feces, gut of *Cataglyphis* ants, and desert plants), and to assess, *in vivo* the cholesterol-lowering effect of selected probiotic potential *Weissella* strains.

2. Materials and Methods

2.1. Sampling Methods. Samples of desert plants (*Euphorbia guyoniana*) (13), camel feces (49), and ants (15) were collected from arid land of southern Tunisia in March 2010 and 2011. The different samples were collected in sterile bags autoclaved or falcon tubes (Thermo Scientific Nunc, 50 ml), kept below 10°C, and treated within 7 days. LAB from plant samples were isolated by the enrichment method as described by Fhoula et al. [28]. LAB isolation from feces was performed as described by Foo et al. [29]. The *Cataglyphis* ants were transferred in sterile containers for organ dissection before microbial isolation. The ants were surface-disinfected with 70% ethanol and rinsed twice with sterilized water prior to dissection. Each adult ant was aseptically dissected using sterilized fine-tip forceps and the entire gut was removed from the body. Guts were placed in 1.5 ml tubes with 500 μl of physiological saline (0.85% NaCl). After that, they were macerated with a plastic pestle and used for bacteria culturing.

2.2. Bacterial Strains, Culture Conditions, and Cell Lines. From a total of 69 environmental LAB isolates of the *Weissella* genus, nine strains were retained for this study based on a preliminary selection of resistance to low pH (see below), one of the more important selection criteria for probiotics (Table 1). *Enterococcus faecium* MMRA [30] was associated with this study. LAB were cultured in De Man, Rogosa, and Sharpe MRS broth (Biolife) at 37°C. Other bacterial strains used for antibacterial activity, including *Escherichia coli* DH5α, *Listeria monocytogenes* L15, *Salmonella* Typhimurium IPT13, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 6538, were grown in BHI broth (Biolife) at 37°C. Identification of the isolated strains was performed as described by Fhoula et al. [28] using 16S rRNA gene sequence analysis. The obtained DNA sequences were deposited in the GenBank database and the corresponding accession numbers are indicated in Table 1. For long-term storage, the strains were stored at −80°C in 15% glycerol. The human Caucasian colon adenocarcinoma Caco-2 cell line was purchased from *Sigma*-Aldrich. The Murine enteric glial MIM/PPk cell line [31] was provided as a gift by Prof. Anne Ruehl (University of Munich). The opportunistic pathogens (*Escherichia coli* N176 and *Enterococcus faecalis* P592) used for the inhibition adhesion to Caco-2 cells experiment were obtained from the collection of Biomedical Sciences Department, Section Experimental and Clinical Microbiology, University of Sassari, Italy. All chemicals required for cell culturing and adhesion studies were purchased from Sigma-Aldrich, USA. The Caco-2 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich). The MIM/PPk cells were cultured in DMEM-F12 medium (Dulbecco Minimal Essential Medium, GIBCO) supplemented with 10% FBS and

Table 1: Origin and survival rate (%) of selected *Weissella* strains to low pH and different bile concentrations.

Strains	Source	Survival rate (%) at pH2.2	Survival rate (%) in bile after 3 h	
			0.3%	0.5%
W. cibaria V28 (KM100709)	Plant (*Euphorbia guyoniana*)	61.63±0.20	64.46±0.01	82.9±0.03
W. confusa F81 (KM100707)	Camel feces	53.28±0.13	76.26±0.10	50.96±0.01
W. confusa F80 (KM100708)	Camel feces	68.89±0.17	77.80±0.03	85.16±0.01
W. halotolerans F99 (KM100706)	Camel feces	74.24±0.12	78.78±0.03	96.28±0.05
W. halotolerans FAS23 (KM100711)	Ant gut	89.29±0.03	72.00±0.25	54.65±0.02
W. halotolerans FAS22 KM100710)	Ant gut	60.20±0.09	29.86±0.34	-
W. halotolerans FAS3 (KM100705)	Ant gut	69.97±0.18	73.96±0.05	56.33±0.02
W. halotolerans FAS65 (KF198087)	Ant gut	44.45±0.14	75.33±0.05	50.23±0.09
W. halotolerans FAS24 (KF198085)	Ant gut	72.10±0.11	68.00±0.80	42.60±1.10
En. faecium MMRA	Dairy product	85.42±0.01	89.14±0.03	75.60±0.02

W, *Weissella*; *En*., *Enterococcus*. Each value represents the mean value ±standard deviation (SD) from three trials. (-), No growth; (∗), Survival rate of bacterial cells successively treated in a low pH and high bile.

100 U/ml penicillin-streptomycin. Incubation was made at 37°C in the presence of 5% CO_2. Cells were seeded at a concentration of 1x10^5 cells per well on coverslips inside 24-well tissue culture plates.

2.3. Tolerance to Low pH and Bile. The tolerance of *Weissella* strains to low pH was tested as described by Klayraung et al. [32]. Acid resistance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 min at 37°C was used as preliminary screening for probiotic strain evaluation. The ability of *Weissella* strains to resist this pH was determined by single streaking on MRS agar plates after 48 h of anaerobic incubation at 37°C. Tolerance of the selected strains to pH 2.2 was conducted as follows: cell pellets were washed twice in 0.01M phosphate buffered saline (PBS, 0.14 M NaCl, 1.5 mM K_2HPO_4, 6.0 mM Na_2HPO_4, 3.0 mM KCl; pH 7.4) and resuspended in 10 ml of (0.05M) phosphate buffer pH 2.2 (adjusted using 1N HCl) to achieve 10^7-10^8 CFU/ml and were held at 37°C for 2 h. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH 6.2). To test the resistance to bile salts, *Weissella* strains were grown in MRS broth containing 0.3 and 0.5% (w/v) of bile salts for 3 h incubation at 37°C. The viable bacterial cells under acidic and bile conditions were determined by plating in triplicate on MRS agar after an incubation of 48 h at 37°C. The survival rate was calculated as the percentage of colonies growing on MRS agar, compared with the initial bacterial concentration.

2.4. Adhesion Assays to Caco-2 and MIM/PPk Line Cells. Adhesion ability of LAB strains to intestinal epithelial cells of the enterocyte-like Caco-2 cell line and to MIM/ PPk murine intestinal glial cells was investigated. Briefly, LAB strains were grown in BHI-GY medium for 18 h at 35°C. The cells were harvested (10000 x g, 10 min, RT), washed twice with sterile PBS, diluted in DMEM, and adjusted to 0.5 McFarland. Cell monolayers were washed with antibiotic-free DMEM and 1 ml of bacterial suspension (approximately 1x10^6 CFU/ml) was added to each well. Plates were centrifuged at 1500 x g for 10 min. After incubation for 3 h at 37°C with 5% CO_2, the plates were washed two times with PBS and fixed with methanol for 30 min. After staining with May-Gruenwald/ Giemsa solution (Riedel-de-Haën, Germany), bacterial adherence to the cells was visualized by light microscope (Zeiss optical microscope), under oil immersion, at a magnification of 100x [33]. Two independent experiments were performed for each strain and uninfected cells were included as a negative control. Adherent LAB in 20 random microscopic fields (40 in total for each strain) were counted.

2.5. Autoaggregation and Coaggregation. The autoaggregation and coaggregation assays for *Weissella* strains were determined according to Malik et al. [34]. The coaggregation capacity of *Weissella* isolates was examined with respect to the tested bacterial partner strains of *E. coli* DH5*α*, *S.* Typhimurium IPT13, and *St. aureus* ATCC 6538. The autoaggregation and coaggregation percentages were determined as the percent decrease of optical density (OD_{660}) of the nonaggregated cells in the supernatant after 60 min using the following equation: Aggregation % = $[(OD_{t0} - OD_{t60})/OD_{t0}]$ x 100.

2.6. Hemolytic Activity and Antibiotic Resistance. Fresh bacterial cultures were streaked in triplicate on base blood agar plates with 5% (v/v) horse blood and incubated at 30°C for 48 h. Blood agar plates were checked for β-haemolysis, α-haemolysis, or γ-haemolysis [35]. Susceptibility to antibiotics was determined by using the disk diffusion method on Muller Hinton agar (MHA) plates supplemented with 0.2% glucose and 0.4% yeast extract. The antibiotics used were ampicillin (AM; 10 μg), chloramphenicol (C; 30 μg), erythromycin (E; 15 μg), tetracycline (TE; 30 μg), clindamycin (CL; 2 μg), rifampicin (RA; 5 μg), and vancomycin (VAN; 30 μg) (Bio-Rad Laboratories, Hercules, CA, USA). MH plates were overlaid with soft MHA (containing 0.7% agar) inoculated at 0.5 McFarland with fresh bacterial culture. After 24 h of incubation at 37°C, the inhibition zone diameters around discs were measured, and the LAB isolates were categorized, according to the standard criteria [36], as resistant (R), intermediate resistant (I), or sensitive (S).

2.7. Inhibition of Pathogenic Bacteria. The inhibitory activity of *Weissella* strains against the indicator used strains was assayed by the agar spot test described by Schillinger and Lücke [37] with some modifications. Spots of 3 μl of each LAB culture were deposited onto the surface of LBP agar plates [tryptone (20 g), yeast extract (5 g), lactose (10 g), gelatin (2.5 g), agar (11 g), NaCl (0.4 g), sodium acetate (1.5 g), and distilled water (1L)]. Then, they were incubated at 35°C for 24 h to allow the colonies to develop. The indicator strains (*St. aureus*, *L. monocytogenes*, *En. faecalis*, *S.* Typhimurium, *P. aeruginosa*, and *E. coli*) were inoculated into 5 ml of soft agar (0.7% agar) at the concentration of 10^5-10^6 CFU/ml and poured over the plate on which the LAB isolates were grown. After incubation at 35°C for 24 h, the plates were examined for the presence of clear inhibition zones. Inhibition was considered positive when the diameter of the clear zone around the spot of the LAB isolates was more than 5 mm. All antibacterial tests were performed in triplicate.

2.8. Inhibition of Pathogen Adhesion to Epithelial Cells Caco-2. For exclusion assays of pathogen bacteria from adhering to Caco-2 cells, 100 μl of *Weissella* bacterial suspension (ca. 1× 10^8 CFU) was added to Caco-2 cells in each well, as described above, and incubated for 90 min at 37°C. Monolayers of Caco-2 cells were washed twice with 1 ml of sterile PBS to release unbound bacteria and then inoculated with 100 μl (10^8 CFU/ml) of one of the following opportunistic gastrointestinal and urogenital pathogens: *E. coli* N176 and *En. faecalis* P592 (resistant to Beta-lactamin, glycopeptides, penicillin, and vancomycin). After incubation, unbound bacterial cells were removed from wells and the Caco-2 cells were washed twice with 1 ml of sterile PBS, followed by 1 ml of 0.5% (v/v) Triton X-100 to release adhering bacterial cells. Serial dilutions were plated on MRS agar (Biolife), MacConkey agar (MCA, Biolife), and Bile Esculin agar (BEA, Biolife) media to enumerate *Weissella* species, *E. coli*, and *En. faecalis,* respectively. For competition assays, the competitive inhibition of the pathogens by the tested *Weissella* strains was determined as described previously, except that LAB strain and one of the pathogens (*E. coli* and *En. faecalis*) were added simultaneously to the Caco-2 cultures and incubated for 3 h at 37°C. Wells containing pathogenic bacteria alone served as controls. The capacity of selected *Weissella* strains to exclude or to inhibit the adhesion to Caco-2 cells from potential gastrointestinal pathogens was expressed as a percentage between the adhesion of pathogens in the presence and in the absence of the tested *Weissella* strain.

2.9. Phenotype Microarrays. The growth on different carbon sources (93) of two *Weissella* strains was assessed using Phenotype Microarray (PM) Technology (Biolog, Hayward, CA). Bacterial cells from a single colony, grown on BHI agar for 48 h, were suspended in the specific Biolog medium (adjusted to 65% of transmittance) and used to inoculate the phenotype microarray 96-well plates (PM1 and PM2), according to the manufacturer's instructions. PM plates were incubated for 72 h at 37°C. Data from a single experiment were analyzed with Omnilog-PM software. For each carbon source, the metabolic activity was measured quantitatively based on the area under curve. The two independent replicates of each PM plate showed the same results.

2.10. Cholesterol Assimilation. *Weissella* cells were inoculated into sterile MRS broth containing 0.3% (w/v) oxgall (Sigma) and 100 μg/ml filter sterilized water-soluble cholesterol (Sigma) and incubated anaerobically at 37°C for 24 h. Cells were harvested and the residual cholesterol concentration in the supernatant was determined using the o-phthalaldehyde colorimetric method of Rudel and Morris [38]. The percentage of cholesterol removed by the strain compared to the control was calculated as follows: [1- (residual cholesterol in cell-free broth)/cholesterol of control broth)] x 100.

2.11. In Vivo Cholesterol Assays

2.11.1. Animals and Experimental Design. Adult male Wistar rats, weighing 165.1 ± 5.2 g, were purchased from Pasteur Institute of Tunisia and housed two per clean plastic cages and allowed to acclimatize in the laboratory environment. The animal room was ventilated and maintained with 12 h light/dark at 24°C and a relative humidity of 50%. The rats were provided standard diet and water ad libitum. Animal experiments were carried out under strict compliance with the Guidelines for Ethical Control and Supervision in the Care and Use of Animals. After acclimatization, a total of 16 animals were randomly selected and divided into two groups (n=8 for each one). Groups I and II were fed with high-fat HF containing diet for 2 weeks and then treated as follows: group I received HF diet with PBS (control group), and group II received HF diet and *W. halotolerans* in PBS, 9×10^9 CFU/Kg body weight of suspension *W. halotolerans* F99 in PBS. A sterile gastric feeding tube was used for orally inoculating one of the two groups daily with 1 ml of *W. halotolerans* F99 suspension during 8 weeks at 9×10^9 CFU/Kg body weight. At the end of the experimental period, rats were sacrificed by decapitation in order to minimize the handling stress, and the trunk blood was collected. The serum was prepared by centrifugation (2500 × *g*, 10 min, 4°C), frozen, and stored at −20°C until it was analyzed for the plasma lipid profile. The HF diet contained 1% wt/wt cholesterol, 10 % oil fat, and a normal diet mix.

2.11.2. Serum Lipids. The concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) in serum were determined by enzymatic colorimetric methods using commercial kits (Elitech, France), while the low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald's formula [39].

2.12. Statistical Analysis. Statistical analysis was done through SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). Data obtained were analysed using one-way analysis of variance (ANOVA) and Tukey's test. Data were considered significantly different at p-value less than 0.05. All data are expressed as the mean ± standard deviation.

Table 2: Adherence of cells of *Weissella* strains to Caco-2 and MIM/PPk cell lines.

Strains	Adhesion[‡]	
	Caco-2	MIM/PPk
W. confusa F80	NA	+++
W. halotolerans F99	+++	++
W. *halotolerans* FAS23	++	++
W. *halotolerans* FAS3	+++	+++
W. *halotolerans* FAS24	+++	+++

(‡), NA: No significant adhesion (< 40); (+), weak adhesion, 40 ≤ Nb < 200); (++), Good adhesion (200 ≤ Nb <1000); (+++), strong adhesion (≥1000). Each value represents the mean value ±standard deviation (SD) from three trials. Adherence was evaluated in 20 random microscopic fields.

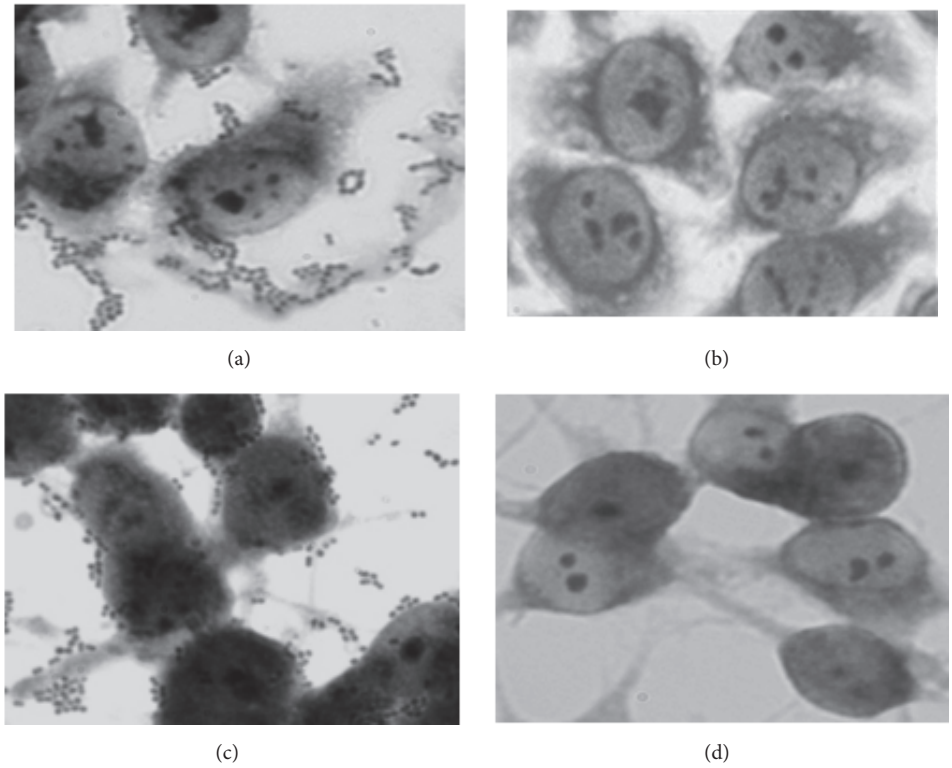
3. Results and Discussion

3.1. Tolerance to Low pH. Acid tolerance constitutes one of the first criteria used to select probiotic microorganisms for their ability to survive transit through the stomach [40]. The potential probiotic LAB strains isolated from different sources were first evaluated to survive to low pH (pH 2.5) condition. Tolerance to pH (2.2) was also checked for the selected strains. The results revealed that most LAB strains could survive approximately less than 68% up to 89.3% under low pH (Table 1) for 2 hours, which is the average time required for a classic passage of the food in the stomach [41]. The most tolerant strains were *W. halotolerans* FAS23, FAS24, and F99 with a survival rate ranging between 72.1 and 89.2 %. Particularly, *W. halotolerans* F99 survived better than probiotic *En. faecium* MMRA from dairy product (85.4%). Besides, *W. halotolerans* FAS65 was sensitive to this pH value at survival rate of 44.5% (Table 1). These results suggest that the resistance to low pH is a strain dependent property and the gut origin ecosystem could play an essential role for the bacteria to be able to adapt to the stress environments.

3.2. Bile Tolerance. Tolerance of LAB cells to different concentrations of bile salts (0.3% and 0.5%) in MRS was evaluated. The most strains, except *W. halotolerans* FAS22, showed a significant survival rate after 3 h of growth in physiological (0.3%) and high concentrations (0.5%) of bile (Table 1). But overall, survival rates were lower compared to positive control at 0.3% bile. Taking into consideration the acidity criterion, *W. halotolerans* FAS3, *W. halotolerans* F99, and *W. confusa* F80 were found among the resistant strains to 0.3% of bile, reaching a viability rate up to 73%. Besides, we noted an increase in the number of viable cells of some LAB in the high concentration 0.5% of bile (Table 1). These results highlight the potential of some *Weissella* isolates to survive under gastrointestinal conditions. Indeed, the high tolerance to bile salts represents an important factor that may considerably influence the viability of LAB in the host gastrointestinal tract and for the exploitation of these strains as probiotics. Hence, it is a prerequisite for the colonization and the contribution of metabolic activity of bacteria in balancing the intestinal microflora of their host [42]. Based on the gastrointestinal tolerance assays, five strains (F80, F99, FAS23, FAS3, and FAS24) were selected with survival rate of over 68% for further evaluation of other probiotic properties.

3.3. Adhesion to Caco-2 and MIM/PPk Cell Lines. The adhesion ability of *Weissella* strains was studied for two types of cell lines (Figure 1): the human colon carcinoma cell line (Caco-2), as an excellent *in vitro* enterocyte model, and the enteric glial cells (MIM/PPk), a major constituent of the enteric nervous system that appears to be essential for the maintenance of gut homeostasis and mucosal integrity [43–45]. Indeed, the enteric glial cells are known to play a complex and fundamental role in regulating many neuronal activities and seem to be involved in immunological and inflammatory processes in the gut [44, 45]. The adherence ability of *Weissella* toward the cell lines was different. They were able to adhere well or strongly to at least one of the two tested cell lines (Table 2). Particularly *W. confusa* F80 has presented strong specific adhesion only for MIM/PPk. The variable adhesion ability to different cell lines may reflect the mode of action of these bacteria [46]. Therefore, the strong adhesion of bacteria to Caco-2 line cells may facilitate the host colonization and the competitive exclusion of pathogenic bacteria from the epithelium surface. This is the case of strains *W. halotolerans* F99, FAS24, and FAS3, which could be selected as potential probiotic candidates. Considering this data, we could establish a correlation between biofilm formation on abiotic surface and the adherence to Caco-2 cells, except for the *W. halotolerans* FAS23. While the high adhesion to the enteric glial cells (EGC) may indicate that these strains could be involved in immune system modulation [45]. The mechanism of interaction of bacteria to EGCs is currently unknown [44]. In accordance with the suggestion of Ortua et al. [46], the use of combinations of potentially probiotic strains revealing different adhesion abilities may result in complementary effects, which could be exploited for different applications.

3.4. Autoaggregation and Coaggregation. Compared to the probiotic *En. faecium* MMRA strain, almost all strains showed a good autoaggregation percentage with an average of 64% (Table 3). The highest value of autoaggregation was observed for *W. confusa* F80 with an aggregation percentage up to 72% after incubation at room temperature for 1 h. Moreover, *W. halotolerans* FAS23, FAS3, and F99 exhibited a good autoaggregating phenotype with a percentage of 66.5%, 64.6%, and 64.1%, respectively. These results indicated that the majority of the tested strains possessed high potential ability to adhere to epithelial cells and mucosal surfaces. In fact, this ability of autoaggregation was related to cell



(a) (b) (c) (d)

Figure 1: **Adhesion of *Weissella* strains to Caco-2 epithelial (a) and MIM/PPK (b) enteric glial cells, as observed with Giemsa staining under a light microscope (magnification X 100).** (c) and (d), recognized as the cells without bacterial adhesion as a negative control.

Table 3: Percentage of autoaggregation and coaggregation of five selected *Weissella* strains.

Strains	Autoaggregation (%± SD)	Coaggregation (%± SD) with		
		Escherichia coli DH5α	*Salmonella* Typhimurium *IPT13*	*Staphylococcus aureus ATCC 6538*
W. confusa F80	72.27±0.55	68.26±0.35	69.24±0.27	59.65±1.18
W. halotolerans F99	64.12±1.81	68.33±1.36	71.70±0.98	50.90±0.91
W. halotolerans FAS23	66.51±1.12	74.00±0.63	81.13±0.11	75.38±0.33
W. halotolerans FAS3	64.61±0.39	80.61±0.49	79.25±0.17	67.08±1.35
W. halotolerans FAS24	52.10±0.64	10.40±1.06	78.81±0.07	15.64±0.23
En. faecium MMRA	54.20±0.42	21.10±1.81	68.35±0.67	46.12±1.53

Each value represents the mean value standard deviation (SD) from three trials. Values are significantly different ($P < 0.05$).

adherence properties [47], which is essential to be effective in the gut flora. On the other hand, the coaggregation of *Weissella* strains with three enteropathogens, *E. coli DH5α, S.* Typhimurium IPT13,and *St. aureus* ATCC 6538*,* was checked (Table 3). According to Kang et al. [48], the coaggregation percentage was significant when it reduced the level of enteropathogenic bacterial aggregation more than 30%. Excepting *W. halotolerans* FAS24 which demonstrated a weak coaggregation with *E. coli* (10.4%) and *St. aureus* (15.6%), most of *Weissella* strains showed an interesting coaggregation percentage with the tested enteropathogenic bacteria. This ability was particularly registered with *S.* Typhimurium, followed by *E. coli*, and *St. aureus.* The coaggregation abilities of probiotic strains with pathogens play an important role, enabling it to form a barrier that prevents colonization of harmful enteric pathogens usually involved in infectious disease [48, 49]. Likewise, it is also showed by Kang et al. [48] that the coaggregation abilities of some *W. cibaria*

isolates with the oral biofilm-forming pathogen *Fusobacterium nucleatum* play an important host defence mechanism against infection by their interference against the biofilm formation.

3.5. Safety Evaluation of Weissella Strains: Hemolysin and Antibiotic Susceptibility. Hemolytic test and resistance to some antibiotics were checked for the studied *Weissella* strains in order to evaluate their safety and to avoid their contribution to virulence. The results showed that the selected-*Weissella* strains were nonhemolytic and presented some resistance phenotypes (Table 4). Indeed, all strains were sensitive to chloramphenicol, clindamycin, and ampicillin, but also they had intrinsic resistance to vancomycin, which does not present potential risk for horizontal gene transfer [50]. *W. halotolerans* (FAS23 and FAS3) and *W. confusa* F80 were discarded from further studies on the basis of their acquired resistance to tetracycline (Table 4). In fact, the use of drug-resistant and/or virulent bacteria as probiotics represents a potential health hazard. For that reason, the safety evaluation of probiotics is required to avoid risks related to antibiotic and virulence gene transfer and dissemination, which contribute to the pathogenesis of virulent bacteria [51]. Moreover, additional tests of toxicity, pathogenicity, and infectivity should be performed in order to establish the "safety" status of the selected strains [52]. On the basis of these results, two potential probiotic strains of *W. halotolerans* (F99 and FAS24) were selected for further study.

3.6. Antibacterial Activity against Intestinal Pathogens. As shown in Table 5, the antagonistic effect of the two selected *Weissella* strains against six pathogenic bacteria was greatly variable. Contrary to *W. halotolerans* FAS24, *W. halotolerans* F99 presented a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative enteropathogenic bacteria. Particularly, it showed high inhibition activity against invasive *S.* Typhimurium and *P. aeruginosa* isolates, which were reported to penetrate epithelial cell monolayers and to cause intestinal infections [53, 54]. The isolate *W. halotolerans* FAS24 showed only an antibacterial inhibition of *St. aureus*. The different antibacterial responses observed in *Weissella* strains against various pathogenic bacteria indicated that these activities could not relate only to acidity. Indeed, the inhibitory activity of LAB is generally due to its ability to produce antibacterial molecules such as lactic acid, bacteriocins, H_2O_2, and other organic acids [55]. Besides, the antibacterial property detected in *W. halotolerans* F99 led to suggestion that it enables the bacteria to establish themselves and to dominate their environment.

3.7. Inhibition of the Adhesion of Pathogens to Caco-2 Cells by Two Weissella Strains. We examined the effect of two *Weissella* strains on the antiadhesion activity against *En. faecalis* P592 and *E. coli* N176 to Caco-2 cells in two conditions of competition and exclusion assays (Figure 2). Adhesion of the pathogens was inhibited by both *Weissella* strains. For competitive inhibition, the adhesion of *En. faecalis* was considerably reduced by *W. halotolerans* F99 (68%) and *W. halotolerans* FAS24 (58%) compared to *E. coli* (Figure 2). On the other hand, the two *Weissella* strains were tested for their ability to exclude pathogens. As shown in Figure 2, *W. halotolerans* FAS24 and *W. halotolerans* F99 significantly reduced the adhesion of *En. faecalis* with a high degree of exclusion of 94% and 81%, respectively, whereas they showed a moderate inhibition of enteropathogenic *E. coli* with an average of 50%. Surprisingly, the competition and exclusion inhibition profiles of *En. faecalis* by the two *Weissella* strains were nearly similar. This confirms that these two adhesion inhibition mechanisms of *En. faecalis* by these LAB strains are similar. Besides, our results suggest that the ability to reduce the pathogen adhesion was strain-dependent in both the LAB and the pathogen tested. This fact may be due to different factors such as the steric hindrance of available adhesion sites, adhesin receptors, and competition for attachment sites and to other factors such as coaggregation of both strains [56–58]. Similarly, the specific adhesion system to Caco-2 cell lines, which appears to be different between Gram-positive and Gram-negative bacteria, as well as the absence of antibacterial activity against *E. coli* could explain the moderate inhibitory activity of adhesion recorded with *E. coli* cells compared to *En. faecalis*. As revealed above, these findings suggest that the production of inhibitory substances can participate efficiently in the antiadhesive effect of the pathogen to epithelial cells [59].

3.8. Carbon Source Utilization by Phenotype Microarrays. The carbohydrate utilization profile of two selected strains (*W. halotolerans* F99 and FAS24) was investigated using Phenotype Microarray (Biolog) in order to determinate the metabolic functions of probiotic interest (Table S1 in file S1). Carbon source utilization was different between the tested strains (Table S1 in file S1). These strains were generally able to use glucosamine, gluconic acid, ribose, inosine, aminoethanol, dextrin, arabinose, and arbutin. *W. halotolerans* F99 was able to metabolize mannitol, xylose, arabinose, ribose, maltose, gentiobiose, glucoside, and tween. Particularly, *W. halotolerans* F99 displayed an important growth rate on plant-derivative complex carbohydrates such as xylose, cellobiose, trehalose, gentiobiose, and galactose [60]. Most of the metabolized oligosaccharides and particularly those containing arabinose and xylose substituents (commonly called arabinoxylan oligosaccharides or AXOS) cannot be degraded by human enzymes of the GIT. Probiotic fecal microbes are indispensable for the degradation of these molecules. This activity is responsible for the formation of partial carbohydrate breakdown products and short chain fatty acids, leading to the maintenance of a balanced gut homeostasis [61]. Moreover, such oligosaccharides are commonly used as prebiotic that stimulate the activity of specific probiotic bacteria of the colon and increase their abundance.

3.9. Cholesterol In Vitro and In Vivo Assays. The elevated serum cholesterol level is considered a risk factor of cardiovascular disease [62]. Therefore, cholesterol assimilation has become an important functional property for selection of probiotic strains to prevent disease. The cholesterol-reducing ability of LAB strains was evaluated *in vitro* in the presence of 0.3% bile. The results indicated that *W. halotolerans* F99 and FAS24 strains had the ability to remove cholesterol

Table 4: Antibiotic susceptibility, hemolytic activity of selected *Weissella* strains.

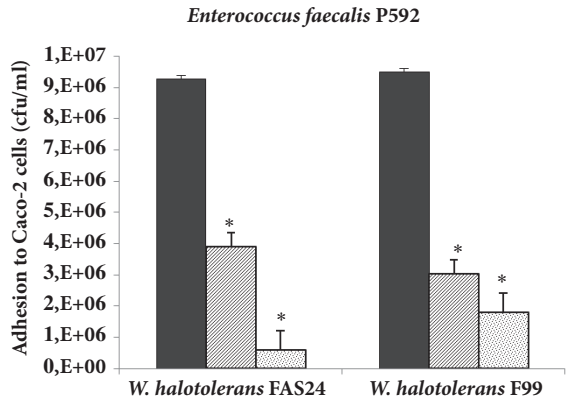
Strains	Vancomycin VA (30μg)	Erythromycin E (15μg)	Chloramphenicol CH (30μg)	Tetracycline TE (30μg)	Clindamycin CL (15μg)	Ampicillin AM (10μg)	Rifampicin RA (5μg)	Hemolytic Activity
W. confusa F80	6±0 (R)	36±2 (S)	24±1 (S)	14±0 (R)	34±2 (S)	27±2 (S)	27±1 (S)	γ-hemolytic
W. halotolerans F99	6±0 (R)	43±0 (S)	30±1 (S)	20±1 (S)	38±2 (S)	23±1 (S)	33±2 (S)	γ-hemolytic
W. halotolerans FAS23	6±0 (R)	25±0 (S)	22±1 (S)	14±0 (R)	29±1 (S)	25±1 (S)	27±1 (S)	γ-hemolytic
W. halotolerans FAS3	6±0 (R)	35±2 (S)	25±1 (S)	14±0 (R)	18±0 (S)	23±1 (S)	20±0 (S)	γ-hemolytic
W. halotolerans FAS24	6±0 (R)	37±1 (S)	31±1 (S)	22±0.5 (S)	30±0 (S)	23±0 (S)	31±0 (S)	γ-hemolytic

S, sensitive; R, resistant; The numbers represent the diameter of zone of inhibition (mm).

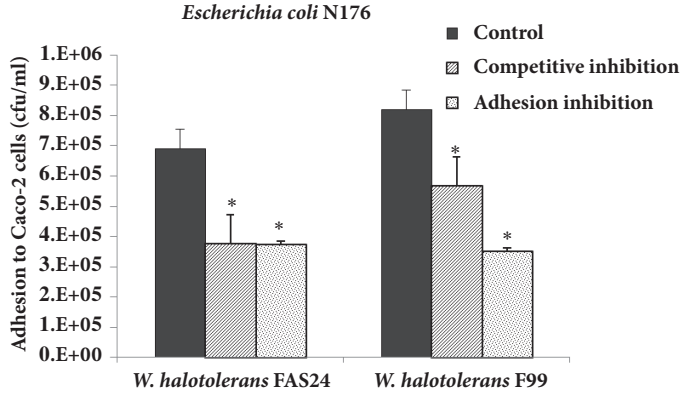
Table 5: The antibacterial activity of the selected *Weissella* strains against six pathogenic bacteria.

Strains	*Escherichia coli DH5α*	*Salmonella* Typhimurium *IPT13*	*Staphylococcus aureus ATCC 6538*	*Listeria monocytogenes LM15*	*Pseudomonas aeruginosa ATCC 27853*	*Enterococcus faecalis ATCC 29212*
W. halotolerans F99	-	21±0.4	12±1	12.3±1.5	24.1±0.6	12.4±0.3
W. halotolerans FAS24	-	-	12±1.6	-	-	-

Numbers indicated the diameter of the inhibition zone in mm; each value represents the mean value standard deviation (SD) from three trials.



(a)



(b)

Figure 2: **Competitive and exclusion inhibition of adhesion of *Enterococcus faecalis* (a) and *Escherichia coli* (b) to the Caco-2 cells by *Weissella halotolerans* (F99 and FAS24) strains.** Asterisks indicate significant differences ($* p < 0.001$).

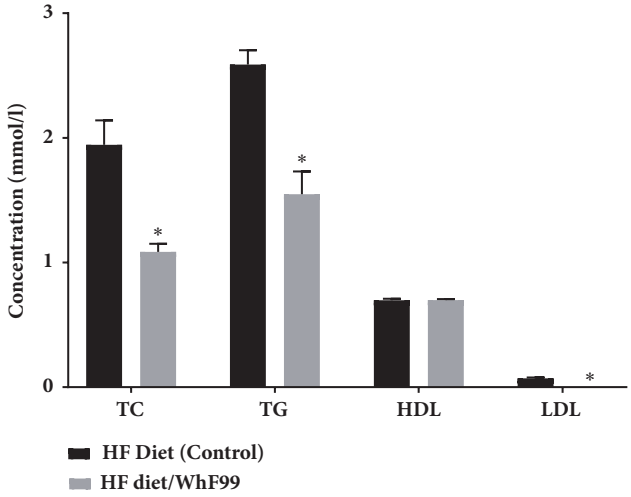


FIGURE 3: **Serum lipid levels of the control and treated groups after 8 weeks.** Control group: high-fat diet; treated group: high-fat diet+ *W. halotolerans* F99 (WhF99). Each concentration is the mean ± standard deviation (n = 8). Asterisks indicate significant differences (∗ $p < 0.001$ vs. control).

from the medium; however, they exhibited varying ratios of cholesterol-lowering ability of 49.04±0.04% and 19.63±0.10%, respectively. The assimilation rate *exhibited by W. halotolerans* F99 was comparable to the probiotics *Lb. rhamnosus* GG and *Lb. plantarum* NR74 showing an average of 48% [11] and higher than some probiotic reference *Lactobacillus* spp. reported in previous studies [63].

Owing to its high cholesterol assimilation ability, *W. halotolerans* F99 was assessed for the *in vivo* effect in Wistar rats. The administration of this *Weissella* strain to rats fed with high-fat diet was found to affect their serum lipid profile (Figure 3). Compared with the control group, the values for total cholesterol of rats serum (TC), triglyceride (TG), and LDL were reduced significantly ($p<0.001$) in group fed with *W. halotolerans* F99. However, for HDL this difference was not statistically significant (Figure 3). Similar results were also reported by Nocianitri [64] and Bendali [65] showing the effectiveness of probiotics to improve lipid profile *in vitro* and *in vivo*. These data provided for the first time the screening of *Weissella* strains for their cholesterol reduction ability. These results represent a preliminary basis for the promising role of *W. halotolerans* F99 as probiotic-based therapies, which may be used for the treatment and prevention of cholesterol metabolism and metabolic diseases, such as development of functional food with probiotic supplement.

4. Conclusions

In the view of our data, the *in vitro* assessment of probiotic properties of *Weissella* strains from arid land living-hosts was shown to be strain specific. The majority of the tested strains have showed to possess interesting probiotic features, including resistance to gastrointestinal conditions, cell surface properties, and adhesive ability to Caco-2 and MIM/PPk cells, as well as the inhibition and the competitive exclusion of harmful pathogens. The present study led to the first-line selection of *W. halotolerans* F99, from camel feces, as a putative strain for future studies as it was found to fit the almost required probiotic properties, including adhesion to epithelial cells, carbohydrate utilization, and cholesterol-lowering effect. It could be used as potential probiotic adjunct to improve the lipid profile in animal and human health. However, the mechanism(s) of regulating serum cholesterol needs further investigations by such a promising probiotic candidate. Based on the findings of this study the gut microflora of camel serves as a special source for model strains.

Data Availability

The nucleotide sequences data used to support the findings of this study are publicly available in the GenBank repository at National Center for Biotechnology Information NCBI (https://www.ncbi.nlm.nih.gov/genbank/). All data are provided in full in Results and Discussion in this paper. The results of Biolog phenotypic microarray analysis data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Supplementary Table S1: positive reaction for carbohydrate utilization by two *Weissella halotolerans* strains using Biolog phenotypic microarray. *(Supplementary Materials)*

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Research Article

Antimicrobial Fatty Acids from Green Alga *Ulva rigida* (Chlorophyta)

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This study deals with the antimicrobial potential assessment of *Ulva rigida,* in regard to collection period and sampling site. Besides, we assess the chemical composition of bioactive compounds. For this purpose, *Ulva rigida* was seasonally collected from two northern sites in Tunisia, Cap Zebib rocky shore (CZ) and Ghar El Melh lagoon (GEM). Crude organic extracts were prepared using dichloromethane and dichloromethane/methanol and tested against 19 indicator microorganisms using the disk diffusion method and microdilution technique to determine the minimum inhibitory concentration (MIC). Silica gel column and thin layer chromatography were used for purification of active compounds. Nuclear magnetic resonance (NMR) and gas chromatography were used for compounds identification. Samples of *Ulva rigida* collected from the two sites have uniform antimicrobial activity throughout the year. Algae collected from the lagoon showed the largest spectrum of activity and were used for subsequent analysis. Bioguided purification of extracts from *Ulva rigida,* collected at GEM, leads to 16 active fractions with antibacterial effect mainly against *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. These fractions were identified as fatty acids, mainly oleic (C18: 1 w9), linoleic (C18: 2 w6), palmitic (C16: 0), and stearic (C14: 0). MICs values ranged from 10 to 250 μg/ml.

1. Introduction

Seaweeds are a diverse group of marine organisms that have developed complex biochemical pathways to survive in a highly competitive environment, very different from the terrestrial one [1]. Such situations require the production of specific and potent bioactive substances that can lead to the development of new drugs and functional foods or nutraceuticals.

From an economic point of view, green algae (Chlorophyta) are sustainable biomass feedstock for the food and biotechnology industries, including possibilities for integrated multitrophic aquaculture (IMTA), bioremediation, and potential biofuel production [2, 3]. *Ulva* species are the most abundant representatives, being ubiquitous in coastal benthic communities around the world. Ulvacean are considered bioindicators species with increased importance in coastal ecosystem management, mainly related to green tides associated with eutrophication processes in shallow environments [3]. In addition *Ulva* species represent untapped resources for food, fuel, and high value-added compounds. Nevertheless, the genus *Ulva* remains considerably understudied [4].

In general, algal chemical composition and, therefore, its nutritional and biomedical value depend on many factors that include species and their development stages, geographical origin, collection period, growth and environmental conditions [1, 5] The green alga *Ulva rigida* is abundant on the coast of Cap Zebib as well as in the lagoon of Ghar El Melh, two environments with very different hydrobiological characteristics. The Ghar El Melh Lagoon is a shallow lagoon. The medium is hypereutrophic (low transparency, low dissolved oxygen concentration, high nitrogen, phosphorus and chlorophyll a); state generated by various land releases and amplified by water stagnation [6]. Cap Zebib is a region in beaten mode zone, with presence of marine vegetation that

enriches environment by the oxygen [7]. Therefore, in this work, *U. rigida* was selected with the aim of studying the effect of the collection period and the geographical site on the production of bioactive secondary metabolites and their characterization.

2. Materials and Methods

2.1. Alga Sampling and Identification. *U. rigida* C. Agardh samples were collected seasonally from July 2006 to June 2007 from the rocky shore of Cap Zebib (CZ) (37° 16.2′ N, 10° 3.6′ E) and from Ghar El Melh lagoon (GEM) (37° 10.8′ N, 10° 16.8′ E) in the region of Bizerte (Northern coast of Tunisia). Algae samples were collected by hand in shallow water (less than 2 m depth) at low tide and kept on ice till their transfer to the laboratory. Algae were taxonomically identified according to [8–10]. Specimen samples were conserved in 70% ethanol.

2.2. Physicochemical Parameters. Temperature, salinity, and pH were measured immediately after sampling using a multiparameter measuring device (HACH HQd field Case). The water quality of the two collection sites GEM and CZ was characterized seasonally through *in situ* measurement of temperature, salinity, dissolved oxygen, and pH. Analysis of nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), total nitrogen (TN), and total phosphorus (TP) is done using spectrophotometric methods [11]. Samples for chlorophyll *a* were filtered, extracted in 90% acetone, and quantified according to the method described by Strickland and Parsons [11].

2.3. Extraction Procedure. Fresh algae samples were rigorously washed three times with seawater and then with tapwater. Subsequently, they were dried in an oven at 40°C or for 15 days under ambient conditions in the shade. The dry biomass was crushed until a powder was obtained, which was kept at -20°C for later analysis. For algae crude extract preparation, 20 g of the dried algal biomass was extracted successively by 2 organic solvents of increasing polarity, dichloromethane (D) and dichloromethane/methanol (D/M) (1:1 v/v). These solvents are suitable to extract nonpolar and moderately polar compounds. Each extraction (24 h at room temperature) was repeated 3 times. The extracts were pooled and filtered. The filtrate was then concentrated in a rotary evaporator to obtain crud extract which was stored at -20°C until use.

2.4. Antimicrobial Test. *U. rigida* extracts and subsequent fractions (as described in purification, fractionation, and characterization analysis) were tested for antimicrobial activity against indicators microorganisms. The activity was evaluated by the discs diffusion method: 500 μg of algal crude extract was dissolved in dichloromethane (D) or dichloromethane/methanol (D/M) (10 μL) and placed on sterile filter paper discs (6 mm). After solvent evaporation, discs were placed on Tryptone Soy Agar (TSA) plates, already inoculated with a test culture (10^6 bacteria. mL^{-1}) in Tryptone Soy Broth (TSB). Simultaneously, a disc loaded with solvent only was used as a negative control. Plates were incubated overnight at 30°C. Inhibition diameters (mm) were measured after 24 h. Antimicrobial activity tests were conducted in triplicate.

2.5. Indicators Microorganisms. A set of pathogenic bacteria, Gram+ve (*Streptococcus agalactiae* (Pasteur Institute, Tunis), *Staphylococcus aureus* (Pasteur Institute, Tunis), *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, *Micrococcus* sp. (Pasteur Institute, Tunis) and Gram-ve (*Vibrio tapetis* CECT4600 (Department of Microbiology and Parasitology, University of Santiago de Compostela, Spain), *V. anguillarum* ATCC 12964T, *V. alginolyticus* ATCC 17749T, *Escherichia coli* O126-B16 (ATTC 14948), *E. coli* ATCC 25922, *E. coli* ATCC 8739, *Pseudomonas cepacia* (INSTM, Tunisia), *P. fluorescens* AH2 (Danish Institute for Fisheries Research, Denmark), *P. aeruginosa* ATCC 27853, *Aeromonas salmonicida* LMG3780, *A. hydrophila* B3 (RVAU-Denmark), *Salmonella typhimurium* C52 (Laboratoire Hydrobiologie Marine et Continentale, Université de Montpellier II, France), and the yeast *Candida albicans*ATCC10231, was used for testing antimicrobial activity of seaweed extracts and fractions.

2.6. Minimal Inhibition Concentration (MIC). The MIC was determined for selective active extracts and fractions on TSB media according to Khan *et al.* [12] and Ganière et al. [13]. Assays were performed in sterile culture plates of 96 round bottom wells. Suspensions of indicator bacterial inoculum were adjusted in the sterile broth medium TSB to the density of 0.5 Standard McFarland (Corresponding to 0.063 optical density at 600 nm, approximately 10^8 CFU mL^{-1}) and then diluted 10-fold twice to obtain a bacterial suspension density of about 10^6 CFU mL^{-1}. Microplates wells were inoculated with 180 μL of the culture containing the inoculum. 20 μL of each concentration of seaweed extract (diluted in dimethyl sulfoxide (DMSO)) was added to the wells containing bacterial culture suspension. The negative control contained 200 μL of culture medium only (without alga extract). Extracts (20 μL) were adjusted to give a concentration range of 1600 to 50 μg/mL (for alga crude extract) and 250 to 10 μg/mL (for alga fractions). Tests were performed in triplicate and plates were incubated for 18-24 h at 37°C. Subsequently, wells were examined by unaided eye for bacterial growth as indicated by turbidity [14]. The last concentrations in the dilution series that did not show visible growth (and showing only few colonies compared with other concentrations when spread on agar plates) correspond to the MIC of the antimicrobial agent. If difficulty is found to discern growth in some wells, MIC determination is then done with colony-forming units count.

2.7. Fractionation, Purification, and Characterization Analysis. In this study, Thin Layer Chromatography (TLC) analytic (TLCa) plates (Merck, Fluka) were used. The solvents system used for the fractions analysis is the n-Hex/EtOAc/DCM/MeOH with combinations and variable percentages according to the fractions. After their development, chromatograms were revealed by chemical reactives: the phosphomolybdic acid (PMA) and the liebermann. For

Table 1: Nutrients and chlorophyll *a* concentration values at the collection sites.

Locality	Season	$N\text{-}NO_2^-$ $\mu mol\ L^{-1}$	$N\text{-}NO_3^-$ $\mu mol\ L^{-1}$	$N\text{-}NH_4^+$ $\mu mol\ L^{-1}$	$P\text{-}PO_4^{3-}$ $\mu mol\ L^{-1}$	TP $\mu mol\ L^{-1}$	TN $\mu mol\ L^{-1}$	Chl *a* mgm^{-3}
Cap Zebib	Winter	0.24	0.98	2.55	0.41	2.04	16.25	3.26
	Spring	-	-	-	-	-	-	-
	Summer	0.14	0.47	1.11	0.34	2.35	10.23	3.13
	Autumn	0.22	0.76	4.70	0.56	3.77	15.22	2.52
Ghar El Melh	Winter	0.98	13.25	16.55	0.90	3.26	33.55	7.53
	Spring	-	-	-	-	-	-	-
	Summer	0.26	21.35	28.16	0.98	8.15	79.05	8.26
	Autumn	0.25	26.74	7.45	0.45	3.12	44.89	7.41

TP: total phosphorus and TN: total nitrogen.

Preparative Thin Layer Chromatography (TLCp) glass plates (20 x 20 cm) covered with silica gel (2 mm thickness) were used. This technique allows the purification of small product quantities (until ~100 mg). The band containing the cleansed product is scratched, and then the silica is extracted with a solvent. The solvent system used in this study is the n-hexane/EtOAc (1/3). The adsorption Column Chromatography on Silica gel (CCS) (pore Size 60 Å 0.063-0.200 mm (70-230 mesh) was used for *U. rigida* extracts and some fractions purification. The solvent system used is the n-hexane/EtOAc/DCM/MeOH. Sephadex LH-20 chromatography was used for separation of closely linked fractions (solvent system used was DCM/MeOH).

Fractions obtained were analyzed for their fatty acids composition by gas chromatography. Samples were homogenized with a chloroform/methanol (1:2 v/v) mixture and incubated during 12 hours in darkness. Residues were extracted 2-3 times with chloroform and methanol. The phase, containing the chloroform, was removed and vaporized. Samples were esterified in sulphuric acid (1%) in absolute methanol and extracted with hexane by phase separation. Samples were analyzed by means of a model leading chromatograph HP 19091N-133 equipped with a polar column INNOWAX (30 m of length; 25 μm of diameter; thickness of the film is of 0,25 μm) mark Agilent Technology. The oven temperature was from 150°C to 240°C with a gradient of 2°C/min. The injector temperature is 220°C, that of the detector is 275°C, flow 1ml/min, and injection volume is 1μl. The chromatogram peaks are identified compared with the retention time of standards peaks (SUPELCO), injected in the same conditions.

Nuclear magnetic resonance (NMR) was used for the active fractions chemical characterization. ^1H NMR and ^{13}C NMR spectra were recorded on an AVANCE 300 MHz instrument (Bruker). Extracts and fractions were solubilized in $CDCl_3$. Chemical shifts δ were expressed in parts per million (ppm), coupling constants J was expressed in Hertz (Hz). The identification of mixture constituents by NMR ^{13}C was realized by comparison of the chemical shifts of the mixture with those of the reference compounds contained in one or several spectra databases.

2.8. Statistical Analysis. Analytical determinations were realized in triplicate and the average values were registered. The data were analyzed by using the IBM SPSS Statistics (v. 20) and test Khi-2 used to determine significant variation of the activity ($P< 0.05$). Principal component analysis (PCA) was used to determine correlations between antimicrobial activity and chemical composition of positive fractions.

3. Results

3.1. Physicochemical Parameters. Registration of physicochemical parameters at GEM and CZ shows that the temperature varies from 13°C to 23°C in CZ and of 15°C to 24°C in GE. pH values, salinity, and dissolved oxygen are almost constant in both regions, whereas values of nitrate, ammonium, total phosphorus (TP), and *chl a* registered are clearly higher in the lagoon water (Table 1).

3.2. Antimicrobial Activity. D and D/M extracts of *U. rigida* collected from GEM showed significant antimicrobial activity during the four seasons with a variable activity spectrum (Table 2). No significant seasonal variability of the antimicrobial activity was detected. The P value (calculated according to the Khi-2 test) was > 0.05. The most sensitive bacteria were *A. salmonicida, S. typhimurium, Str. agalactiae, A. hydrophila, P. cepacia, S. aureus* and *E. faecalis*. MIC values were 0.8 mg/mL against both *P. cepacia* and *A. salmonicida*. The most resistant strains were *E. coli, Vibrio* spp, *Pseudomonas* spp., *Micrococcus* sp., and the yeast *C. albicans*.

Similarly, for *U. rigida* collected on CZ, no seasonal effect on the antimicrobial activity was observed (p> 0.05). Six of 19 tested indicator bacteria were sensitive to the extracts of *U. rigida* (CZ) (Table 3). D and D/M extracts show a strong activity against *S. aureus* ATCC 25923 and *Str. agalactiae*. No activity was detected against Gram-ve bacteria except on *A. salmonicida* for which the lowest MIC value (0.8 mg/mL) was recorded. Considering the activity spectrum, *U. rigida* collected from the lagoon presented a more pronounced antibacterial activity. This difference is especially observed with Gram-ve bacteria (58% of these Gram-ve bacteria were inhibited by *U. rigida* (GEM) while only 16% were inhibited by *U. rigida* (CZ)).

Considering that *U. rigida* from GEM showed the most relevant activity spectrum, it was chosen for subsequent fractionation, purification and chemical characterization.

Table 2: Antimicrobial activity of *U. rigida* collected seasonally from Ghar El Melh lagoon (data are in mm of inhibition diameter).

	Dichloromethane				Dichloromethane/methanol			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
E. coli O126 B16	-	-	-	-	8±0	11,3±1.1	7±0	8±0
V. tapetis CECT 4600	-	-	-	12±0	-	-	-	11,5±0.5
P. cepacia	-	-	11,8±0.2	12,6±0.5	11,6±0.5	9,6±0.5	14,3±0.5	12±0
P. aeruginosa ATCC 27853	-	11±0	7±0	-	-	-	-	-
A.eromonas salmonicida	14±0	14±0	14±0	14±0	12±0	10±0	11±0	11±0
*A. hydrophila*B3	-	11±0	-	15,6±0.5	9,3±1.1	10±0	14±0	15±1
S. typhymurium	11,3±1.1	-	-	12±0	12±0	15,6±0.5	11,6±0.5	14±0
Str. agalactiae	12,3±0.5	11,6±0.5	14±0	14,6±1.1	10,3±0.5	8,6±0.5	14,3±1.5	14,6±0.5
S. aureus	7±0	7±0	8,6±0.5	10±0	10±0	12,6±2.3	13,3±1.1	16±0
S. aureus ATCC 25923	13±1	17,3±1.1	17±0	16±0	10±0	16±0	18±0	16±0
S. aureus ATCC 6538	10,3±0.5	11±0	13±0	10,3±0.5	9±0	10±0	13,6±0.5	11±0
E. faecalis ATCC 29212	11,5±0.8	11,3±1.1	14,3±0.5	15,6±0.5	12±0	11,3±1.1	14±0	17±1

Extracts were tested at concentrations of 500 μg/disc; +/-: represents the standard deviation; the number of independent replicates was n=3.

Table 3: Antimicrobial activity of *U. rigida* collected seasonally from Cap Zebib shore (data are in mm of inhibition diameter).

	Dichloromethane				Dichloromethane/methanol			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
V. alginoliticus	-	10,3±0.5	8±0	7,6±0.5	-	-	-	-
A. salmonicida	**15±0**	12,8±0.2	9±0	-	11,6±0.5	-	8,3±0.5	8±1.7
Str. agalactiae	**15±0**	12,3±1.1	**15±1**	10,3±0.1	9,6±0.5	6,3±0.5	**16,3±0.5**	11±0
S. aureus	9,6±0.5	8,8±0.2	10,3±0.5	10,3±0.5	8,3±0.2	-	11,3±0.5	11,6±0.5
S. aureus ATCC 25923	12,8±0.2	12,3±0.5	12,6±0.5	10,8±0.2	9,1±0.2	6,6±1	**16,3±1.1**	10,6±0.5
S. aureus ATCC 6538	10±0	10,6±0.5	10±0.2	10,6±0.5	10,6±0.5	11±0	12,8±0.2	10±0

Extracts were tested at concentrations of 500 μg/disc; +/-: represents the standard deviation; the number of independent replicates was n=3.

3.3. U. rigida (Ghar El Melh) Crude Extract Purification. Given that all *U. rigida* (GEM) extracts, independently of the collection season, gave a significant antibacterial activity, they were grouped in a single extract for a better purification. The elution was realized in gradient mode by CCSs. Nine fractions (FG1-FG9) were obtained and tested for their antibacterial effect towards three indicator bacteria: *S. aureus*, *E. faecalis,* and *A. Salmonicida* which were the most sensitive bacteria to previously tested *U. rigida* (GEM) crude extracts (Table 4). The most active fraction FG1 was purified and a total of 27 sub-fractions (G1-G27) were obtained (Figure 1), which were also tested for their antibacterial potential.

Results showed that G4-G9, G11, G14-G16, and G26 fractions were active towards at least one of pathogenic tested bacteria with low values of MIC (Table 5). The TLC analysis and the PMA and LB revelation of G1 to G27 fractions show fatty acids (FA) characteristic spots, especially for the G1 to G10 fractions (Figure 2). The G4, G5, and G6 fractions contain FA in important quantity. These fractions were chosen for a final purification process (Figure 3) according to their higher antibacterial effect and lower MIC values. In addition, these fractions also showed sufficient weight for further purification. Table 6 shows antibacterial activity results for G4 G5 and G6 fractions, presenting lower MIC values that ranged between 10 and 40 μg/ml.

The successive purification of the *U. rigida* crude extract and the chemical revelation (Figure 3) showed that active fractions (16 fractions: G4-G9, G11, G13, G4 (4), G5 (5), B4, FX6, A, B, C, and D) had characteristic blue spots of FAs. Figure 4 shows antibacterial activity of *U. rigida* fractions against *S. aureus* ATCC 25923. Therefore, an NMR ^{1}H analysis was carried out to confirm the structure of active compounds. Subsequently, gas chromatography was applied to these fractions to determine their FAs composition. The NMR spectra of the G4, G5, FX6, B4, A, B, C, and D fractions possess typical NMR spectra of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) mixture. Figure 5 represents the NMR ^{1}H and ^{13}C spectra of G4 compound.

FA composition of G4-G9, G11, G13, G4 (4), G5 (5), and B4 fractions (having a sufficient weight) is shown in Table 7. Results showed that the fractions obtained contained saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), with variable quantities according to the fraction. Different fractions FA profiles showed that fractions (G4, G5, and G6) containing mainly SFA were the most active while those containing low amounts of PUFA were less active (G7, G8, G9, and G11).

In addition, fractions having a high amount in palmitic acid were the most active (G4, G5, G6, G4 (4), G5 (5), and B4). Furthermore, the increase in oleic acid amount in the fractions G4 and G5 is proportional to the increase of the

Table 4: Antibacterial activity of FG1-FG9 fractions obtained from *U. rigida* (Ghar El Melh).

Fraction	*S. aureus* ATCC 25923∗		*E. feacalis* ATCC 29212∗		*A. Salmonicida*∗	
	ID (mm)	MIC (μg/ml)	ID (mm)	MIC (μg/ml)	ID (mm)	MIC (μg/ml)
FG1	17±1	250±0	18.3±0.5	250±0	12.6±0.5	500±0
FG2	13.6±0.5	500±0	13.6±0.5	500±0	6.6±0.5	500±0
FG3	12.3±2	500±0	8.3±0.5	500±0	-	
FG4	8.6±0.5	500±0	-		-	
FG5	-		-		-	
FG6	-		-		14±0	250±0
FG7	6.3±0.5	500±0	-		10.6±0.5	500±0
FG8	9±1	500±0	-		-	
FG9	-		-		-	

∗: concentration 500 μg/disc, ID: inhibition diameter, MIC: minimal inhibition concentration, and -: not active

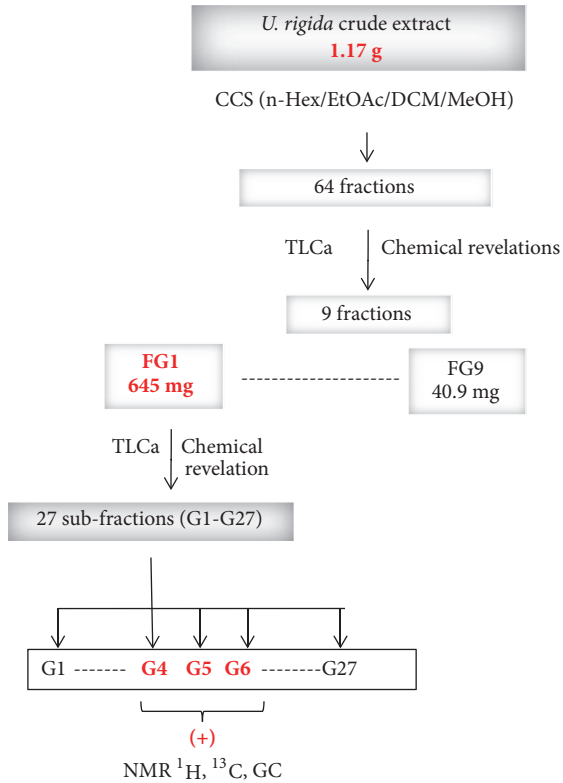

Figure 1: *U. rigida* (Ghar El Melh) crude extracts purification steps. CCS: column chromatography silica gel; n-Hex: n-hexane; EtOAc: ethyl acetate; DCM: dichloromethane; MeOH: methanol; TLCa: analytic thin layer chromatography; GC: gaz chromatography; ^{1}H NMR: nuclear magnetic resonance of proton; RMN^{13}C: nuclear magnetic resonance of carbon; (+): active against at least one indicator microorganisms.

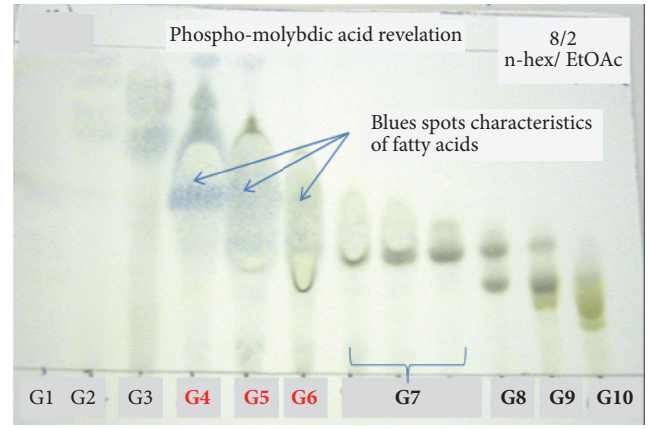

Figure 2: Phosphomolybdic acid revealed TLC of *U. rigida* (GEM) purified fractions (as explained in Figure 1).

activity in their subfractions G4 (4), B4, and G5 (5). This indicates that this FA is involved in the observed activity. Moreover, when comparing the FA profile of the G13 fraction (which is an inactive fraction) to those of the other active ones we notice that the absence of the stearic acid and palmitoleic acid in G13 could partially explain the lack of activity.

To determine the correlation rates between the observed antibacterial activity and the FA composition (SFA, MUFA, and PUFA) a statistical analysis in principal components (PCA) was made (Figure 6). This representation allows distinguishing clearly 3 groups of fractions. The first group consists of G4, G5, and G6 fractions which present an important activity towards *S. aureus* and *E. feacalis* and which are rich in SFA. The second group is composed of G7, G8, and G9 fractions containing high amount in PUFA and MUFA and showing low antibacterial activities. The last group contains only the G11 fraction. Indeed G11 fraction is characterized by its activity against *A. salmonicida* and relatively low amount of FAs (not exceeding the 55 %) compared to the other fractions. This fact suggests that observed activity against *A. Salmonicida* was probably caused by different non FAs substances.

4. Discussion

U. rigida samples were collected from two different geographic locations to determine the effect of geographical site on the antimicrobial activity. *U. rigida* (CZ) is mainly active against Gram+ve bacteria and only inhibits 16% of Gram-ve bacteria, whereas *U. rigida* (GEM) has a broader spectrum of activity with an inhibitory effect against 5 of the 6 Gram+ve bacteria and 58% inhibition of Gram-ve ones.

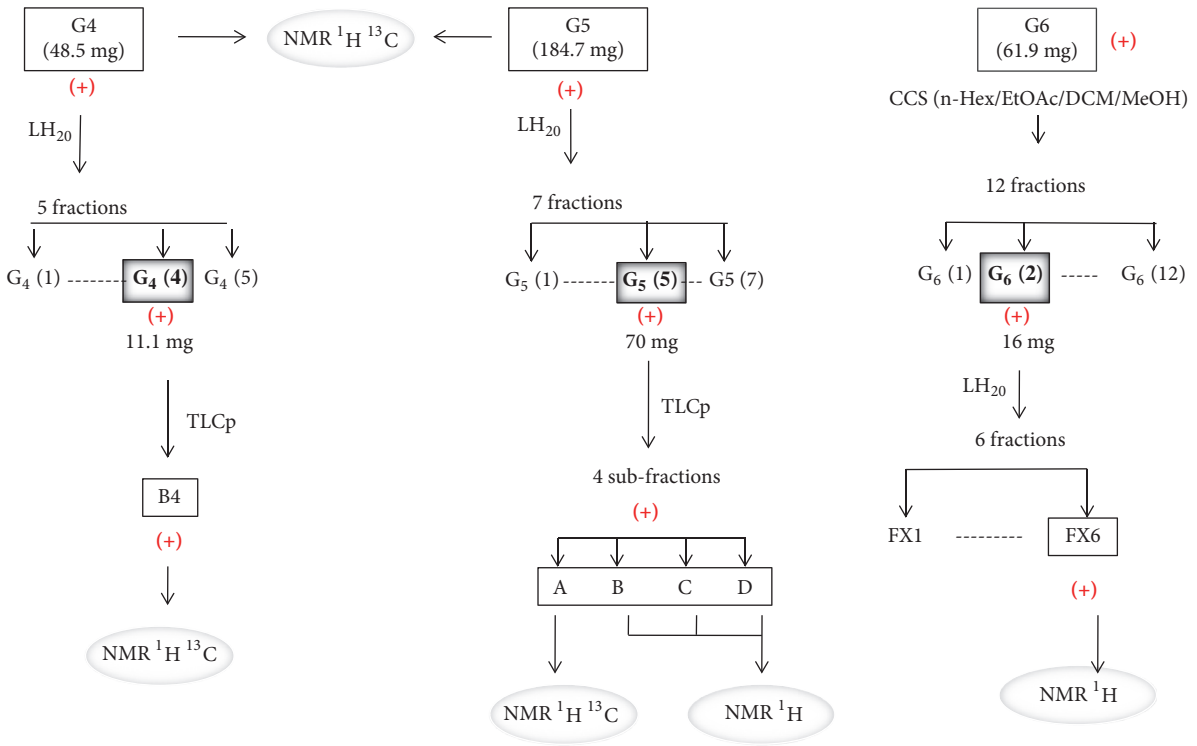

Figure 3: Purification steps of G4, G5, and G6 fractions obtained from *U. rigida*. CCS: column chromatography silica gel; TLCp: preparative thin layer chromatography; ^{1}H NMR: nuclear magnetic resonance of proton; ^{13}C NMR: nuclear magnetic resonance of carbon; (+): active against at least one indicator microorganisms.

Table 5: Antimicrobial activity of *Ulva rigida* (GEM) purified fractions (as explained in Figure 1).

Sub-fractions from FG1	Indicator bacteria					
	S. aureus ATCC 25923		*E. faecalis* ATCC 29212		*A. salmonicida*	
	ID (mm)	MIC (μg/ml) ((μg/ml)	ID (mm)	MIC (μg/ml) (μg/ml)	ID (mm)	MIC (μg/ml) ((μg/ml)
G4	**21.6±0.5**	**62.5±0**	**22±0**	**125±0**	-	-
G5	**23±1.5**	**62.5±0**	**21±1.5**	**250±0**	-	-
G6	**18±0.5**	**62.5±0**	**16.3±0.3**	**250±0**	-	-
G7	10±0	250±0	-	-	-	-
G8	9.6±0.5	250±0	-	-	-	-
G9	11±0	250±0	-	-	-	-
G10	-	-	-	-	7.3±0.5	250±0
G11	15±1	250±0	-	-	7.3±1.1	250±0
G12	-	-	-	-	8.3±0.5	250±0
G13	-	-	-	-	10±0	250±0
G14	24±1.5	250±0	-	-	10.6±0.5	166.6±72
G15	22±0.5	250±0	nt	nt	nt	nt
G16	21±0.5	250±0	-	-	-	-
G23	7.3±0.5	-	nt	nt	nt	nt
G26	-	-	9.6±0.5	250±0	-	-

MIC: minimal inhibitory concentration, nt: nontested; -: no activity; ID: inhibition diameter of fractions tested at concentration of 250 μg/disc.

Gram-ve bacteria *E. coli* was inhibited only by *U. rigida* (GEM).This bacterium is known to be resistant to the majority of seaweed extracts and most marine organisms in general [15, 16]. In addition to *E. coli*, the indicator bacteria; *V. tapetis*, *P. cepacia*, *P. aeruginosa*, *A. hydrophila,* and *S. typhimurium* were also inhibited by *U. rigida* (GEM). They were resistant to the extracts of samples collected from CZ. The susceptibility of Gram-ve bacteria to *U. rigida* (GEM) extracts can be explained by the effect of factors related to the type and biochemical characteristic of sediment and water of the

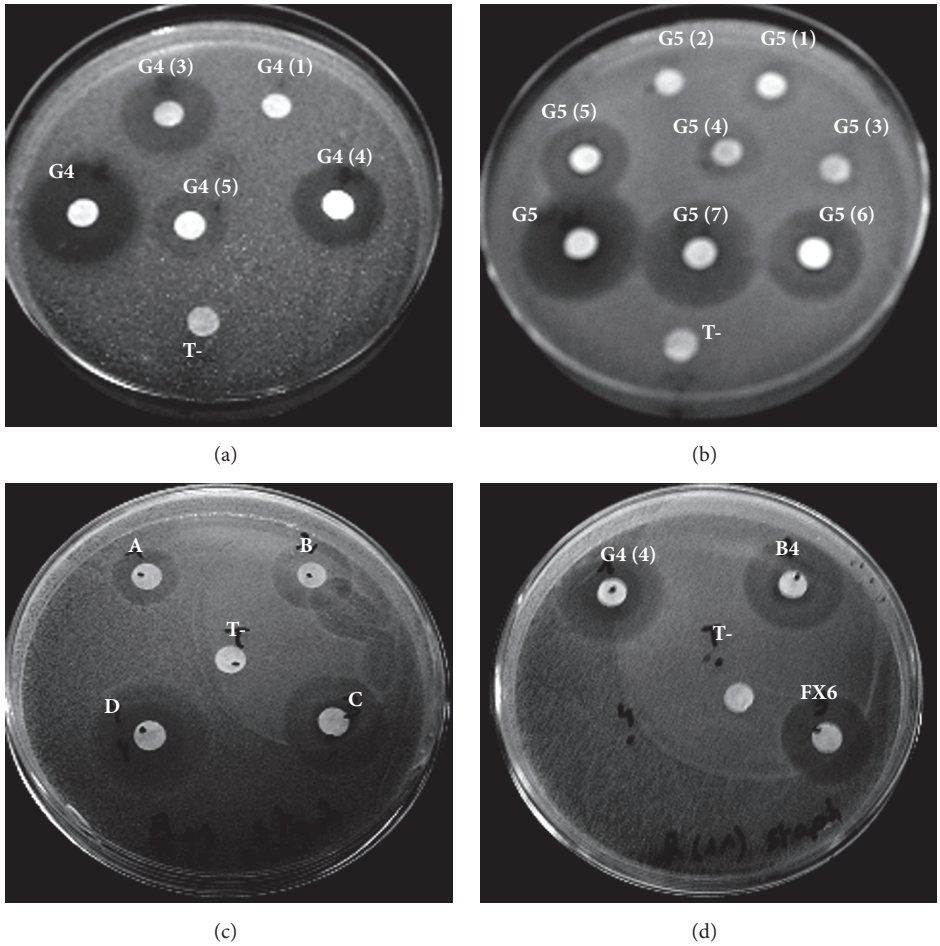

Figure 4: Antibacterial activity of *U. rigida* G4, G5, and G6 fractions and subfractions against *S. aureus* ATCC 25923.

Table 6: Antibacterial activity against *S. aureus* ATCC 25923 of sub-fractions obtained from G4, G5, and G6 of *U. rigida* (Ghar El Melh).

Sub-fractions obtained by TLCp or LH20	Diametre (mm)	MIC (μg/ml)
G4 (4)	17.3±0.5	20
G5 (5)	17.6±0.5	40
G6 (2)	18±0.5	20
B4	20.3±0.5	20
A	16.6±0.5	10
B	13±0	40
C	20.6±0.5	10
D	24.3±1.1	20
FX6	18.6±0.5	10

LH20: Liquid Sephadex chromatography; TLCp: preparative thin layer chromatography; MIC: minimal inhibitory concentration. Fractions were tested at a concentration of 40 μg/disc; +/-: represents the standard deviation; the number of independent replicates was n=3.

lagoon and other factors probably related to the interaction between seaweed and several micro and macroorganisms living in the same environment.

Physicochemical characteristics and hydrobiological properties of the two collections sites are different; in particular the concentrations of ammonium, nitrate, total nitrogen, and chlorophyll *a* were markedly different in GEM and CZ. Concentrations recorded from the lagoon water were higher. The water of the lagoon was concentrated with nitrate and ammonium, when compared to the coast of CZ. These nutrients (from agricultural sources or from urban wastewater discharges) are indicators of environmental pollution leading to eutrophication and causing the excessive proliferation of green algae, especially *Ulva*. Moreover, it is also worth mentioning that GEM lagoon water was charged with chlorophyll *a*. The latter is considered as

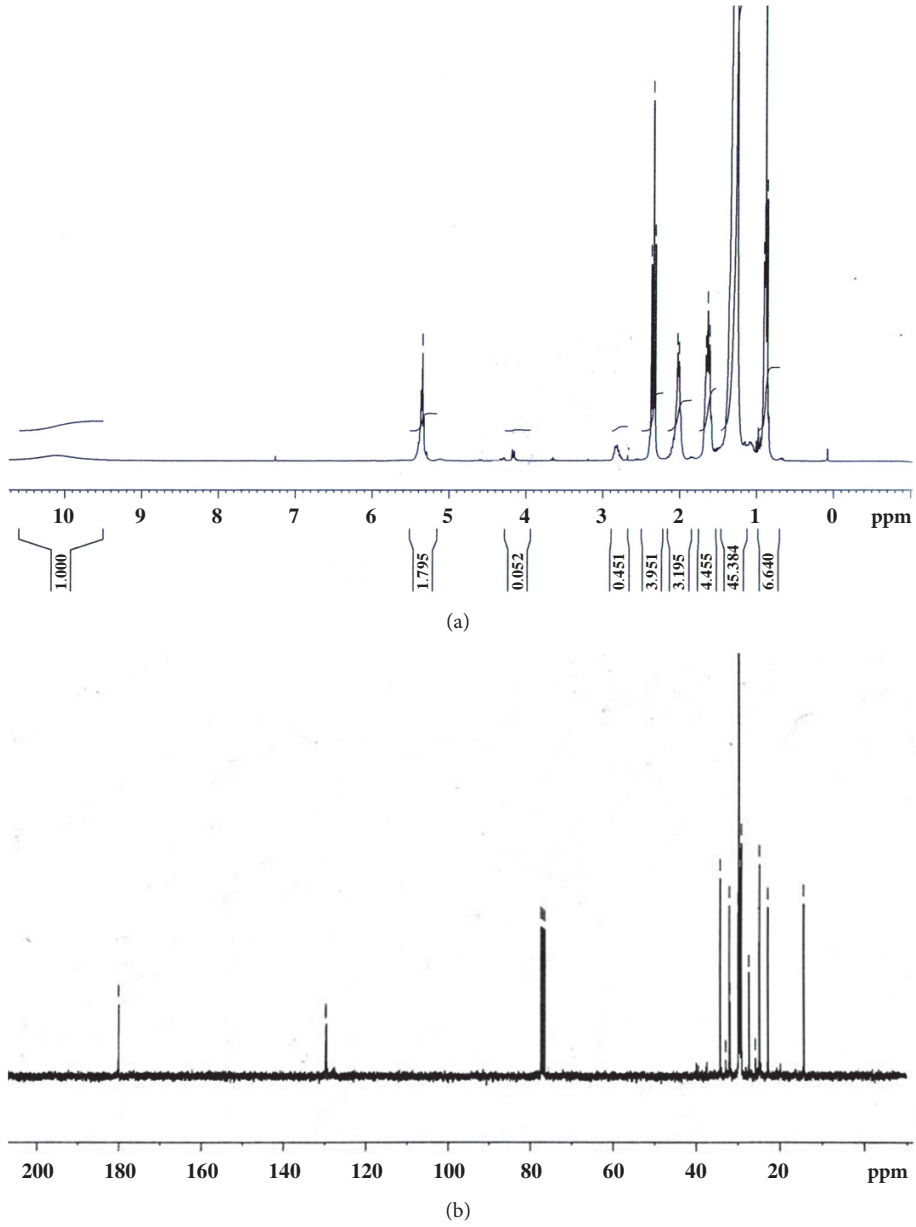

Figure 5: NMR ^{1}H (a) and ^{13}C (b) spectra of G4 fraction, in $CDCl_3$, obtained from *U. rigida* (Ghar El Melh) purification.

an indicator of the abundance of microscopic algae. The antibacterial activity of *U. rigida* (GEM) with regard to Gram-ve bacteria can be explained by the fact that the algae growing in a polluted environment (characterized by the presence of unhealthy fish and invertebrates and a low oxygen concentration) tend to defend themselves by the production of secondary metabolites that would not found in the same specie collected from an unpolluted marine zone.

It is conceivable that the geographical site plays an important role in the production of secondary metabolites. These results and observations support the hypothesis of the impact of collection site on the secondary metabolites produced by algae. This is confirmed by Martí et al. [17], Maréchal et al. [5], and Salvador et al. [18], who emphasized that the geographical site is among the factors affecting algae toxicity. This variation related to the collection site might be due to the nature of the site, whether exposed to shear forces or quiet mode, in the sea, or in protected bays. Various biotic and abiotic environmental factors may impact the algae biology and physiology and thus influence their secondary metabolites production. Marti et al. [17] have noted that also various ecological parameters such as nutrients and photoperiod can determine the production of secondary metabolites.

Among the fractions obtained from *U. rigida* purification, 16 fractions contain FAs in high concentrations.

FAs were previously incorporated into food with the aim to prevent the action of human pathogenic microorganisms such as those of genus *Salmonella, Listeria,* and *Staphylococcus* [19]. The antimicrobial effect of the FAs isolated from *U. intestinalis* was tested by Horincar et al. [20] against four

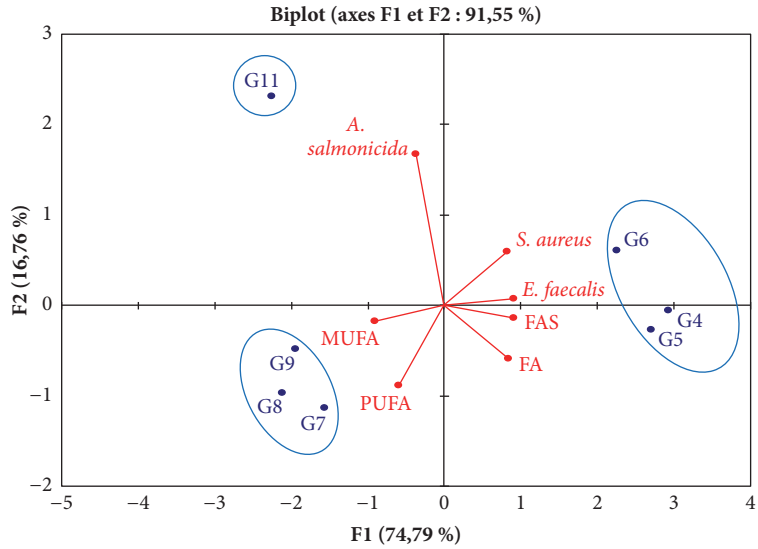

Figure 6: Correlations between fractions type, fatty acids composition, and antibacterial activity.

Table 7: Fatty acids composition of *U. rigida* (Ghar El Melh) fractions.

		Proportion (%)										
STD	STD	G4	G5	G6	G7	G8	G9	G11	G13	G4 (4)	G5 (5)	B4
SFA												
(C14:0)	7,70	2,99	3,39	1,17	1,21	0,86	1,16	1,44		0,77	0,96	2.87
(C15:0)	0,40	1,27	2,09	1,25	0,92				3,69	0,79	0,88	1.43
(C16:0)	10,59	**75,45**	**71,70**	**68,76**	31,90	21,64	21,14	14,89	23,57	**55**	**53,32**	**67.84**
(C18:0)	1,33	3,25	1,35	3,33	0,81	0,60		0,47	6,01	1,72	2,08	1.32
MUFA												
(C16:1 w7)	15,31	3,68	7,11		5,97	9,70	5,80	12,03		7,25	8,51	6.41
(C18:1 w9)	10,58	**11,98**	**12,32**	2,92	18,51	14,43	14,03	6,98	6,66	**20,52**	**26,46**	**17.28**
(C18:1 w7)	6,62			3,86					6,55			
(C20:1 w9)	2,36			1,37					3,76			
PUFA												
(C16:2 w4)	1,83		0,32									0.46
(C18:2 w6)	1,00	1,35	0,41		3,38	3,46	3,77	2,14		0,42		0.91
(C16:3 w4)	2,73		0,32									0.42
(C18:3 w4)	0,85											
(C18:3 w3)	1,91			2,15	5,88	6,72	7,21	6,46		0,16		
(C18:4 w3)	2,82				6,04	7,56	8,05	8,88	3,41			
(C20:4 w6)	1,11											
(C20:4 w3)	1,87				0,37	0,44	0,46					
(C20:5 w3)	16,81					0,24	0,25					
(C22:5 w3)	2,816				0,71	0,89	0,91					
(C22:6 w3)	10,79											
Total	99,50	100	99,05	84,85	75,75	66,59	62,82	53,33	57,10	87.24	92.22	98.97
PUFA	44,57	1,35	1,06	2,15	16,4	19,34	20,67	17,49	3,41	0.58	0	1.80
MUFA	34,89	15,66	19,43	8,16	24,49	24,13	19,83	19,01	16,98	27.77	34.97	23.69
SFA	20,03	82,97	78,55	74,53	34,86	23,11	22,30	16,81	33,27	58.3	57.25	73.47
w3	37,03	0	0	2,15	13,01	15,88	16,89	15,35	3,41	0.16	0	0
w6	2,12	1,35	0,41	0	3,38	3,46	3,77	2,14	0	0.42	0	0.91

pathogenic bacteria (*Bacillus cereus, L. monocytogenes, E. coli,* and *S. enteritidis*). The MIC of the *U. intestinalis* extracts containing FAs was 3.8 mg/ml. In the present work, the MIC of the active fractions containing a set of FAs is relatively low (10-250 μg/mL). This clear difference could be explained by a difference in the composition or the amounts of the active FAs. The activity can also be variable with the target bacterium.

Stabili et al. [21] demonstrated that the alpha linolenic acid isolated from the green alga *Cladophora rupestris* collected from the Mediterranean Sea is the most dominant FA in April (Spring), which confirm its role in the observed activity against *Vibrio* spp. during this month, with a MIC value of 18 μg/ml. Antibacterial and antifungal properties were previously attributed to linoleic and oleic acids. The latter is also known to have a bactericidal activity towards several pathogenic microorganisms, including *S. aureus, Helicobacter pylori, V. Parahaemolyticus*, and *Mycobacterium* [21–24].

In this study, both G4 and G5 fractions are the most active compared to the other fractions (G7, G8, G9, and G11) obtained from the first purification of FG1. G4 and G5 contain mainly saturated fatty acids and have a low PUFA amount. On the other hand, G7, G8, G9, and G11 which showed high PUFA proportions gave low antibacterial activity. This suggests that G7, G8, G9, and G11 fractions contain besides the FAs, other compounds which may have antagonistic effect on these PUFA known to have a power interesting bioactive effect [25]. This hypothesis also leans on the fact that in these fractions the global proportion in FA does not exceed the 75 % contrary to the other fractions where the FA proportions are between 85% and 99%. Thus, this could explain that, despite the high quantity in PUFA in these fractions, their inhibition activity was not remarkable.

The correlation rates between the observed antibacterial activity and the FA composition (SFA, MUFA, and PUFA) determined by CPA show that G11 fraction is characterized by its activity on *A. salmonicida*. This fraction has relatively low FA proportions compared to the other fractions. This fraction is characterized by the fact that its total FA proportion does not exceed 55% suggesting that the observed activity on *A. Salmonicida* is caused by substances other than FA. Furthermore, the G7, G8, G9, and G11 fractions being characterized by their relatively high PUFA proportion (and low FA proportion (between 53 % and 75 %)) compared to the other fractions and a low activity towards *S. aureus*. This suggests that the observed activity on *S. aureus* is probably due to the effect of other substances which act by decreasing or by blocking the PUFA activity.

We also noted that the oleic acid proportion (C18: 1 w9) in G4 and G5 fractions increased in their sub-fractions G4 (4) and G5 (5). This increase is proportional with the antibacterial activity observed for these fractions. This lets deduce that the oleic acid is totally or partially responsible for the observed activity. Although the G6 contains low oleic acid proportion, this fraction showed significant inhibition effect. This fraction may contain other active substances than FA. Moreover, the fractions having high palmitic acid proportion (G4, G5, G6, G4 (4), G5 (5), and B4) are the most active fractions. This suggests that the palmitic acid even not known for its antibacterial properties could act in synergy with the oleic acid to give a bacterial inhibitive activity. In addition, the stearic acid (C14:0) also seems to have a role in the observed activity especially towards *S. aureus* ATCC 25923.

The PUFA: C20:4 w3, C20:5 w3, and C22:5 w3 are known to have antibacterial properties [25]. In this study their effect was not pronounced in the G7, G8, and G9 fractions since they are present in very small quantities (0.2% to 0.9 %). Also alpha linolenic acid (C18:3 w3) and stearidonic acid (C18:4 w3) antibacterial affects were not observed in G7-G11 fractions which are weakly active. This could be explained by the fact that the action of these FAs were inhibited by the interference of others metabolites in the same fraction. Knapp and Melly [26] demonstrated that the PUFA and MUFA are particularly active towards Gram+ve bacteria. These authors indicated that the toxicity of the PUFA towards *S. aureus* depends on incubation time, concentration, and FA insaturation.

The antibacterial action of FAs is always attributed to long chains of PUFA as the oleic, linoleic, and linolenic acid and their mechanism of action is to inhibit the synthesis of bacterial FAs [25]. FAs are known not to be able to inhibit the Gram-ve bacteria such as *E. coli* [27]. This could be a consequence of the external membrane impermeability of the Gram-ve bacteria, which acts as a barrier against hydrophobic substances [27]. Even if relation between oleic acid structure and antimicrobial activity is not clear, it seems that the number and the position of double bond, as well as presence of hydrophilic head and a hydrophobic tail, can influence the antimicrobial activity affecting the bipolar membrane of the bacterial cell wall.

5. Conclusions

U. rigida collected from Tunisian coasts displayed antibacterial activity throughout the year. Algae collected from the lagoon possess the widest antibacterial activity spectrum. *A. Salmonicida, A. Hydrophila, S. typhimurium, Str. agalactiae, S. aureus,* and *E. feacalis* pathogens are the most sensitive to *U. rigida* collected from lagoon. The difference between nitrate, ammonium, total phosphorus, and chlorophyll *a* values in the two collection sites seems to have an effect on antibacterial activity variation of *U. rigida* extracts. Oleic, palmitic, and stearic acids seem to be responsible for the observed activity in the seaweed collected from the lagoon with low MIC values. Indicators pathogens inhibited by *U. rigida* compounds present several resistances to antibiotics. They are often associated with many infections as the meningitis, sepsis, and endocarditis (the case of *S. aureus*). *A. salmonicida* and *A. hydrophila* are responsible for furunculosis and "Motile *Aeromonas* Septicemia" affecting shellfish, amphibians, crustaceans, clams, and various fish such as salmon and sea bream and are responsible, for serious economic losses around the world. Therefore, fatty acids from *U. rigida* collected from Ghar El Melh lagoon might be potential source for use in the development of new antibacterial substances against human and marine organisms-diseases.

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 they have no conflicts of interest.

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Research Article

Pelargonium graveolens Aqueous Decoction: A New Water-Soluble Polysaccharide and Antioxidant-Rich Extract

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Background. The decoction of *Pelargonium graveolens* yields an antioxidant-rich extract and a water-soluble polysaccharide. This study aims (1) to investigate the effect of process parameters (extraction time and temperature) on the antioxidant activity of the decoction and the extraction yield of CPGP by response methodology and (2) to study the chemical properties of the optimized decoction and rheological properties of the corresponding extracted polysaccharide. *Results*. The antioxidant-rich decoction contained about 19.76 ± 0.41 mg RE/g DM of flavonoids and 5.31 ± 0.56 mg CE/gDM of condensed tannins. The crude *Pelargonium graveolens* polysaccharide (CPGP) contained 87.27 % of sugar. Furthermore, the CPGP solutions (0.5%, 1%, and 2%) exhibited shear-thinning or pseudoplastic flow behavior. A central composite design (CDD) was applied to assess the effects of temperature and time on the antioxidant activity of the decoction, on the one hand, and on water-soluble polysaccharide yield, on the other. The decoction optimization of *Pelargonium graveolens* aimed to use less energy (93°C for 11 minutes) leading to the highest values of decoction phenolic content (33.01 ±0.49 mg GAE/gDM) and DPPH scavenging activity (136.10 ± 0.62 mg TXE/gDM) and the highest values of CPGP yield (6.97%). *Conclusion*. The obtained results suggest that the CPGP rheological characteristics are suitable for applications in many industries, especially food. The values of optimal conditions showed that *Pelargonium graveolens* decoction operation could have multiple uses, especially for consuming less energy.

1. Introduction

Pelargonium graveolens is a herb belonging to the Geraniaceae family and it has good aromatic properties. It is cultivated worldwide [1, 2], mainly for its essential oil fraction, which is extensively used in many industries. The essential oil of the fresh plant is widely used in perfume industry thanks to its desirable scent [3]. Besides, many studies on active molecules in essential oil and organic extracts of *Pelargonium graveolens* have shown good antioxidant activity and antimicrobial effect, especially against *B. cereus*, *B. subtilis,* and *S. aureus* [4–6]. However, because of the toxicity of essential oils and organic extracts, their application in food against spoilage pathogens is limited, and more interest in safety matters should be shown [7].

In Tunisia, rose-scented geranium is broadly used to produce a food flavoring hydrosol used in traditional pastries. Some studies have proven its good antioxidant activity and capacity to heal throat pains [8]. Nevertheless, after distillation, the used plant is considered as waste. The decoction uses the whole plant and exhibits the presence of many active compounds such as phenolics [9, 10]. Yet, to our knowledge, little is known about the phytochemical composition and biological activities of *Pelargonium graveolens* decoction even though it is an ancestral practice used for its extract digestibility and safety compared to essential oils [10]. Moreover, decoctions are still used and even optimized to improve their added-value products [9, 11]. Decoction optimization parameters include temperature extraction, time extraction [9], pH, and the ratio of water to raw material [11]. Optimization was performed using experimental designs to produce response surfaces that were also widely used to determine optimal conditions to extract polysaccharides from different sources [12–14] and different processes [15]. Although most of new

water-soluble polysaccharide extraction operations start with a decoction [14, 16, 17], no special interest has been given to both optimal extraction parameters leading to an added-value decoction and a maximum yield of its water-soluble polysaccharide.

This study aims (1) to investigate the effect of process parameters (extraction time and temperature) on the antioxidant activity of the decoction and the extraction yield of CPGP by response methodology and (2) to study the chemical properties of the optimized decoction and rheological properties of corresponding extracted polysaccharide.

2. Material and Methods

2.1. Plant Material and Sampling. Geranium aerial parts were harvested from a random sample of a plant growing in Ariana (North of Tunisia: latitude 36°51′36″ N, longitude 10°11′36″ E, altitude 10 m) in April 2014. Leaves, flowers, and stems were manually isolated from the branches to obtain a weight of 1.00 kg and dried at 20°C for 2 weeks. A specimen was kept in our unit as a reference.

2.2. Chemicals. All chemicals were purchased from Sigma, Tunisia.

2.3. Decoction Operation. Dried ground *Pelargonium graveolens* whole plant (stems, flower, and leaves) (10 g) was extracted with distilled water (ratio of water to raw material (ml/g) was 10:1), while the water temperature was maintained at a given temperature (within ± 2°C, extraction temperature ranging from 78 to 98°C) for a given time (extraction time ranging from 8 to 20 min) (Figure 1). The flask was then cooled and the mixture was then filtered over a Buchner funnel. The decoction was prepared in triplicate. The resulting decoctions were stored at 4°C for future use.

2.4. Crude Polysaccharide Extraction. After decoction centrifugation, a volume of ethanol was added to the supernatant. The mixture was shaken overnight at room temperature (400 mo./min). Then, the solution was centrifuged for a quarter of an hour (4°C; 3500 rpm); the resulting precipitate was collected; the crude *Pelargonium graveolens* polysaccharide (termed CPGP) was obtained. The extract was air dried at 40°C until constant weight (Figure 1). The CPGP yield (%) was calculated by the equation [18]:

$$\text{CPGP yield}\,(\%) = \frac{m_0}{m} \text{ x } 100 \quad (1)$$

where m_0 (g) is the dried CPGP weight and m (g) is the dried raw material (DM) weight.

2.5. Optimization of Decoction. The central composite design (CDD) was applied to study the effect of temperature (X1) and time (X2) on the total phenolic content (Y1), DPPH essay (Y2), and yield of CPGP (Y3) as responses. The experimental factors and levels are shown in Table 1. The coded levels and experimental value of each factor, in each experience, are shown in Table 2.

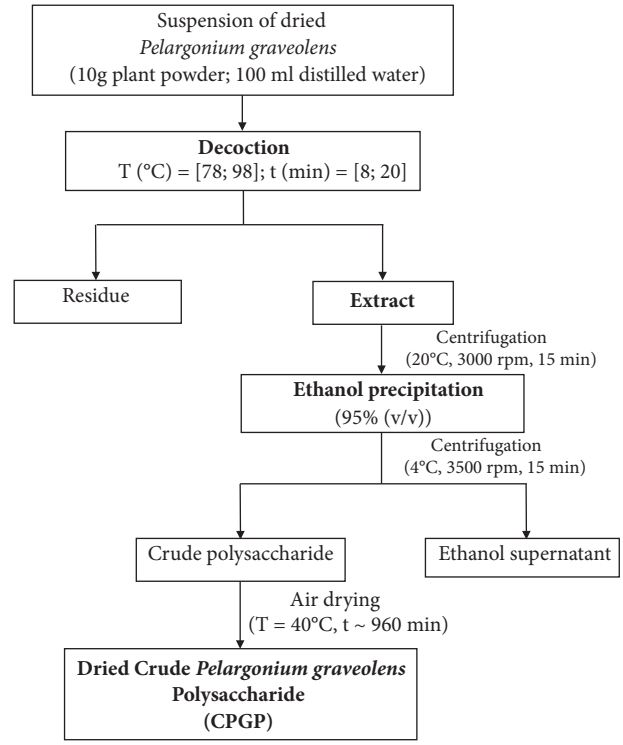


Figure 1: Process of *Pelargonium graveolens* decoction and polysaccharide extraction.

The polynomial model used to express the responses was

$$\mathbf{Y = b0 + b1 * X1 + b2 * X2 + b11 * (X1 * X1) + b22 * (X2 * X2) + b12 * (X1 * X2)} \quad (2)$$

where Xi represents the level of the factor i, Y is the experimental response, and b is a parameter of the model (regression coefficient).

Every model parameter has a precise meaning: b0 represents the response analyzed at the domain center; the values of b1 and b2 indicate the importance of the effects of the factors (temperature and time, respectively) on the responses; b12 is an interaction parameter between the two factors. The values of b11 and b22 determine the movement of the response surface (upward for positive values or downward for negative values) [9].

2.6. Physical Chemistry of the Decoction

2.6.1. Total Phenolic Content. The Folin–Ciocalteu method [19] was used to assess the total phenolic content. The phenol contents were expressed as milligrams of Gallic acid equivalent per gram of dry matter (mg GAE/gDM).

2.6.2. Flavonoid Contents. The colorimetric method [20] was used to measure the flavonoid contents. Briefly, 0.5 mL of each diluted extract was mixed with 0.5 mL of 2% $AlCl_3$ methanol solution. After 30 min incubation, the absorbance was read at 430 nm. Flavonoid contents were calculated from a calibration curve of rutin and expressed as milligrams of

Table 1: Experimental factors and their levels in CCD.

		Surface				
		Levels			Star points*	
Factor	Unit	-1	0	1	$-\alpha$	$+\alpha$
(X1) Extraction temperature	°C	81	88	95	78	98
(X2) Extraction time	min	10	14	18	8	20

*$\alpha = 1.41$.

Table 2: Central composite design for the decoction: temperature and time, with observed responses (total phenolic content, DPPH scavenging activity, and crude *Pelargonium graveolens* polysaccharide yield).

TEST	*Factors*				*Responses*		
	X1		**X2**		**Y1**	**Y2**	**Y3**
	Temperature (°C)	Coded level	Time (min)	Coded level	Total phenolic content (mg GAE/gDM)	DPPH scavenging activity (%)	CPGP yield (%)
1	81	-1	10	-1	28.24	72.9	3.48
2	95	1	10	-1	36.04	81.93	8.97
3	81	-1	18	1	30.37	65.13	4.11
4	95	1	18	1	36.36	85.92	7.78
5	88	0	14	0	30.79	68.7	7.25
6	88	0	14	0	33.8	77.2	4.99
7	78	-1.41	14	0	27.05	60.5	4.06
8	98	1.41	14	0	36.54	88.44	8.21
9	88	0	8	-1.41	32.27	79.41	5.64
10	88	0	20	1.41	32.4	76.47	7.33
11	88	0	12	-0.5	31.64	73.11	4.68
12	88	0	16	0.5	31.63	83.19	5.48
13	84	-0.5	14	0	26.86	67.65	3.69
14	92	0.5	14	0	34.42	81.3	5.95
15	79	-1.3	12	-0.5	28.47	65.69	3.43
16	97	1.3	16	0.5	35.54	83.82	6.93

rutin equivalent per gram of dry matter (mg RE/gDM). The results are means of triplicates.

2.6.3. Total Condensed Tannins. To measure the condensed tannins, the vanillin assay [21] was performed. To 50 μl of diluted sample, a volume of methanol vanillin solution (3 ml, 4%) and a volume of H_2SO_4 (1.5 ml) were added. After a 15 min reaction, the absorbance was read at 500 nm. The methanol was used as a blank. The amount of total condensed tannins was expressed as milligrams of catechin equivalent per gram of dry matter (mg CE/gDM). All samples were analyzed in three replications.

2.6.4. Free Radical Scavenging Activity. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity was measured [22]. Briefly, 50μL of a double serial dilution of the aqueous extracts were mixed to 0.95 mL of 60 μM DPPH radical solution and left away from light for 30 min. The spectrophotometer was set at 517 nm. The percentage of radical inhibition (I %) was estimated as

$$I\% = 100 \times \frac{(A_0 - A_1)}{A_0} \quad (3)$$

where A_0 is the control absorbance and A_1 is the sample absorbance. All tests were triplicated. For the optimized decoction extract, the DPPH activity was calculated from a calibration curve of Trolox and expressed as milligrams of Trolox equivalent per gram of dry matter (mg TXE/gDM).

2.6.5. Color Measurement. The CIELAB coordinates (L∗, a∗, b∗) were measured in a Minolta colorimeter (Minolta, Model CM-3600 d, UK) controlled by a computer that calculated color from the reflectance spectrum [23]. The L∗ parameter (lightness index) ranges from 0 (black) to 100 (white). However, the a∗ parameter indicates the degree of red (+a∗) or green (-a∗) colors, whereas the b∗ parameter measures the degree of the yellow (+b∗) or blue (-b∗) colors. Samples were poured in Petri dishes till the brim and placed on the device sensor.

2.7. Sugar Content, FTIR Spectra, and Rheology of CPGP Solutions. The *Pelargonium graveolens* decoction was first treated with the Sevag reagent to eliminate any resulting proteins [24]. Next, the supernatant was dialyzed for three days and, finally, the CPGP was precipitated using ethanol

Table 3: Parameters of the polynomial models representing the studied responses (Y1-Y3).

Model	Y1		Y2		Y3	
Model parameters	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
b0	32.524	* * *	77.720	* * *	4.937	* * *
b1	3.911	* * *	8.227	* * *	1.702	* * *
b2	0.224	n.s	-0.503	n.s	0.195	n.s
b11	-0.161	n.s	-1.976	n.s	0.446	n.s
b22	0.032	n.s	0.277	n.s	0.738	*
b12	-0.442	n.s	2.508	n.s	-0.562	n.s
Model validation						
Significance level (%)	* * *		* * *		* * *	
Df	13		13		13	
Sum of squares	1.28E+02		9.16E+02		42.341	
Mean square	24.04		1.66E+02		7.673	
$\mathbf{R^2}$	0.949		0.910		0.902	
Adjusted $\mathbf{R^2}$	0.918		0.854		0.847	

* * *: Significant at the level 99.9%
**: Significant at the level 99%
*: Significant at the level 95%
n.s: not significant
Df: degrees of freedom.

(V/V). The obtained crude polysaccharide was dried at 40°C until constant weight, then suspended in distilled water to measure the sugar content [18]. For the FTIR test, the CPGP was rather lyophilized than dried before spectroscopy experiment.

2.7.1. Sugar Content. The phenol-sulphuric method was used [25]. The purity (%) of CPGP is calculated as the sugar content of extraction per dried crude polysaccharide weight.

2.7.2. Fourier Transform Infrared Spectroscopy. The Fourier Transform Infrared (FTIR) method was used to characterize CPPG by a VERTEX 70 (Bruker Optics, USA) spectrometer. The decoction was further deproteinized by the Sevag reagent (a mixture of $CHCl_3$ and n-butanol, v/v = 4:1). The aqueous fraction was precipitated by adding ethanol. The mixture was centrifuged and the crude polysaccharide (the precipitate) was then suspended in water and dialyzed for three days. The precipitate was lyophilized. The FTIR spectral bonds ranged from 500 to 4000 cm^{-1}.

2.7.3. Viscosity Measurement. The flow behavior of different CPGP water solutions (0.5; 1 and 2%) was measured by a strain-controlled rheometer (AR 2000, TA Instruments, Ltd., Crawley, UK). The viscosity was measured at a temperature of 20°C and shear rates between 10 s^{-1} and 1000 s^{-1}. Flow behavior was determined by the power law model:

$$\sigma = k\gamma^{n} \quad (4)$$

where σ is the shear stress (Pa), γ is the shear rate (1/s), n is the flow index, and k is the consistency index.

2.8. Statistical Analysis. The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's test for the means comparisons and a p value of less than 0.05 was considered significant.

3. Results

3.1. Optimization of the Decoction of Pelargonium graveolens. There were 16 runs for optimizing the five individual parameters in the current CDD design (Table 2). The data were analyzed by multiple regression analysis using NEMRODW (9901- by LPRAI_Marseille, France) and each response was studied separately.

3.1.1. Model Validation. The statistical analysis checks the existence of coefficients which do not influence responses. A good and correct description of the model variation of the test results can be affirmed when the significance shown in the tables of the analysis of variance is superior to 95% [26–28].

Table 3 lists the obtained results of the statistical test, the estimated values of the model coefficients, and the model validation parameters. For response Y1, only 2 parameters were significant in decoction process. Thus, total phenolic content of the decoction could be given by the equation:

$$Y1 = 32.524 + 3.191\ X1 \quad (5)$$

According to this equation, the temperature may have a linear effect on the total phenolic content. For response Y2, 2 out of the 6 model parameters were significant in decoction process. Similarly to the case of the response Y1, the coefficients b2-b12 do not influence the response since these coefficients' value of

significance is less than 95%. Thus, the decoction antioxidant activity could be given by the equation:

$$Y2 = 77.72 + 8.227\ X1 \quad (6)$$

According to this equation, the temperature may have a linear effect on the DPPH scavenging activity. The statistical analysis for response Y3 shows that only three parameters were significant in decoction process. Thus, polysaccharide yield extract from the decoction could be given by the equation:

$$Y3 = 4.937 + 1.702\ X1 + 0.738\ (X2.X2) \quad (7)$$

According to this equation, the temperature may have a linear effect on CPGP yield. However, the extraction time may have a quadratic effect on the same response.

3.1.2. Extraction Parameters Influence on Decoction Total Phenolic Content. According to the positive linear coefficient of (5) (+3.191), the phenolic content reaches higher values with increase in extraction temperature. This can be observed in Figure 2(a), showing the contour plots for phenolic content. The area of the experimental domain shows that, independent of time, the amount of total phenolics reaches its maximum for a temperature interval between 88°C (X1=0.5) and 98°C (X1=+ α). Within this interval, about 37% of phenolic content is obtained (Figure 2(a)_3D surface plot).

3.1.3. Extraction Parameters Influence on Decoction Scavenging Activity. As time is kept constant, a difference in temperature increases the scavenging activity. Figure 2(b) shows that the scavenging activity reaches its maximum for a temperature extraction interval between 88°C (X2=0.5) and 98°C (X2=+ α). The time of extraction would have an impact only for extreme temperature.

3.1.4. Extraction Parameters Influence on Crude Pelargonium graveolens Polysaccharide Yield. According to (7), the time and temperature of the extraction increase the CPGP yield. Figure 2(c) shows that the yield of polysaccharide CPGP reaches its maximum at an interval of temperature between 95°C (+1) and 98°C (+ α), regardless of decoction time.

3.1.5. Predicted and Experimental Optimal Condition of Total Phenolic and CPGP Contents. The main objective of this study is to look for the optimum parameters values which help to enhance the decoction antioxidant activity and the corresponding polysaccharide yield. Tables 4 and 5 present optimal conditions and predicted variables. In an experiment with X1 of about 0.773 (temperature of the order of 93°C) and X2 of -0.634 (time of 11 minutes), the desirability for phenolic content (Y1 close to 34.98 mg GAE/gDM), scavenging activity (Y2 close to 82.10%), and the yield of water-soluble polysaccharide (Y3 close to 6.97%) is maximal (98.84%). Hence, the values of the parameters mentioned here before strengthen the probability of having an optimum.

To ensure the predicted result, test rechecking was conducted using the modified optimal conditions of temperature extraction of 94°C and decoction time of 10 minutes. Table 6 showed that the experimental results did not vary a lot from the predicted value.

Table 4: Optimal conditions for the extraction process.

Variable	Value	Factor	Value
X1	0.773326	Temperature	93
X2	-0.634008	Time	11

3.2. Physical Chemistry of Optimal Decoction. The aqueous extract represented 51.41% of the starting mixture (into-water dry material) which was nearly six times the weight of the used dry material (DM). The decoction had an acid pH, about 4.32. It had a liquid appearance (viscosity about 1.33 Pa.s) and a color similar to dark tea infusion, whose characteristics are presented in Table 7.

The total phenolic content, flavonoids, and condensed tannins of the optimal *Pelargonium graveolens* decoction were measured to reflect their biological property as expressed in DPPH scavenging activity (Table 8).

3.3. Pelargonium graveolens Crude Polysaccharide (CPGP) from Optimal Decoction. In this work, the rheological properties of the CPGP and its preliminary structural analysis were studied.

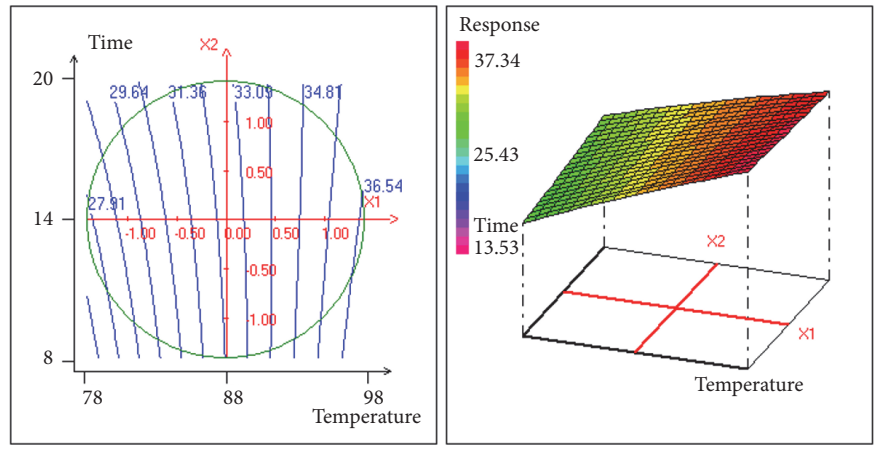
3.3.1. Rheological Properties of Crude Pelargonium graveolens Polysaccharide. The rheology of *Pelargonium graveolens* water solutions was studied using the behavior of the apparent viscosity versus the shear rate (Figure 3). The graph showed two areas: the apparent viscosity decreased until a shear rate of 400 s^{-1}, then a constant «unlimited» viscosity was established. This rheological property characterizes the pseudoplastic or shear-thinning behavior (n<1).

The flow behavior index (n) and consistency index (k) values (Table 9) were obtained from the representation of the shear stress versus shear rate according to the power law model (Equation (4), Figure 4). The results showed that the increase in concentration increases the shear stress. Table 9 shows that the flow index decreases with the increase in the concentration of polysaccharide. The statistical analysis revealed that there are no significant differences between all concentrations in terms of index flow.

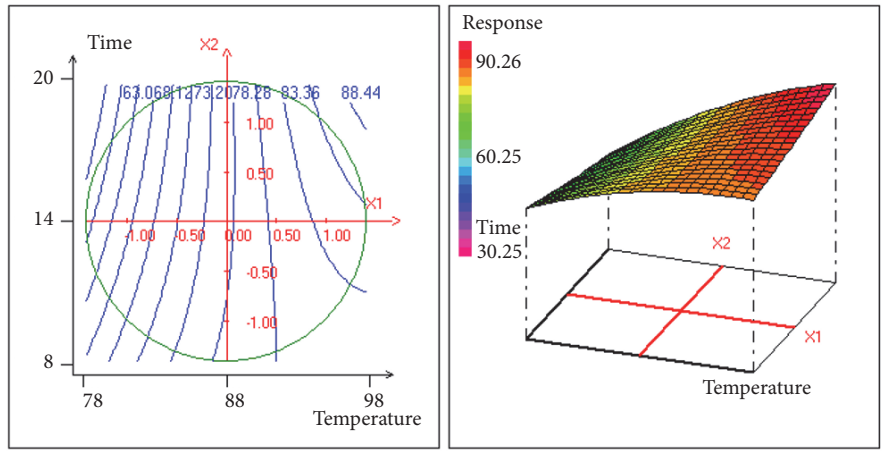
However, the consistency index values ranged from 322.60 to 382.70 $Pa{\cdot}s^n$. There were significant variations of index consistency of all samples.

3.3.2. Preliminary Structural Analysis. For preliminary CPGP structural analysis purpose, the sugar content by the phenol-sulphuric method and the Fourier Transform Infrared spectra were measured.

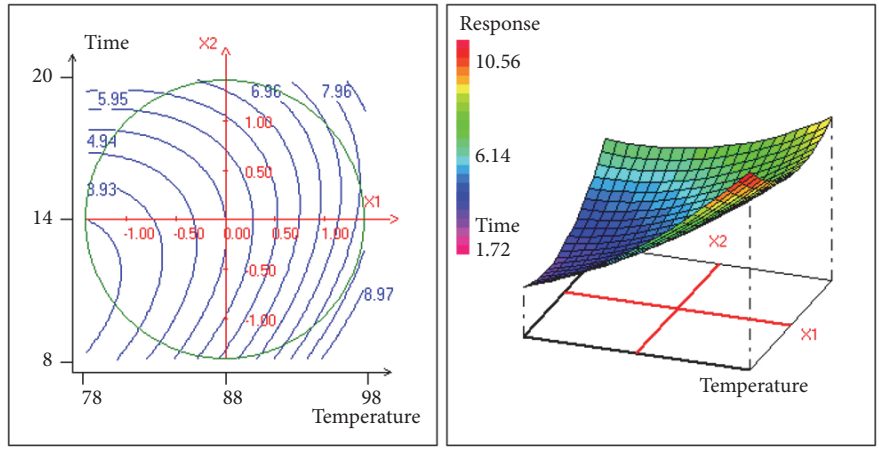
The total sugar content of CPGP was estimated to be 87.27% and Figure 5 shows the FTIR spectra of CPGP, exhibiting a large absorption band at around 3350 cm^{-1}, four weak peaks at 2981 cm^{-1}, 2370 cm^{-1}, 1633 cm^{-1} and 1410 cm^{-1}. Two intense absorption bands were also observed at 1043 cm^{-1} and 1087 cm^{-1}. Finally, an absorption band at



(a)



(b)



(c)

Figure 2: Contour plots and 3D-response surfaces for (a) total phenolic content, (b) DPPH scavenging activity, and (c) CPGP yield, as a function of time and temperature of decoction.

Table 5: Predicted values of the responses at optimal conditions.

Response	Name	Value	di %	Weight	di min %	di max %
Y1	Total phenolic content **(mgGAE/gDM)**	**34.98**	99.65	1	42.51	99.65
Y2	DPPH scavenging activity **(%)**	**82.10**	98.43	1	48.69	98.43
Y3	CPGP yield **(%)**	**6.97**	98.45	1	63.20	98.45
	DESIRABILITY		98.84		50.76	98.84

di: percentage of calculated desirability.

Table 6: Predicted and experimental values of responses at optimal and modified conditions.

	Extraction temperature (°C)	Extraction time (min)	Total phenolic content (mg GAE/gDM)	DPPH scavenging activity (%)	CPGP yield (%)
Predicted values	93	11	34.98	82.10	6.97
Modified conditions	94±2	10	33.02 ±0.58	68.05±0.74	6.43±0.31

Table 7: Color (L, a, b) index of the *Pelargonium graveolens* optimized extract.

Sample	L∗	a∗	b∗
Decoction extract	18,74 ± 0,68	2,17 ± 0,33	11,07 ± 0,63

L∗: Lightness, a∗(-green/+red), b∗(-blue/+yellow).

Table 8: Chemical content and antioxidant properties of *Pelargonium graveolens* optimized decoction extract.

Sample	Total phenolic content (mgGAE/gDM)	Flavonoids (mg RE/gDM)	Condensed tannins (mg CE/gDM)	DPPH scavenging activity (mg TXE/gDM)
Optimal decoction extract	33.01 ± 0.49	19.76 ± 0.41	5.31 ± 0.56	136.10 ± 0.62

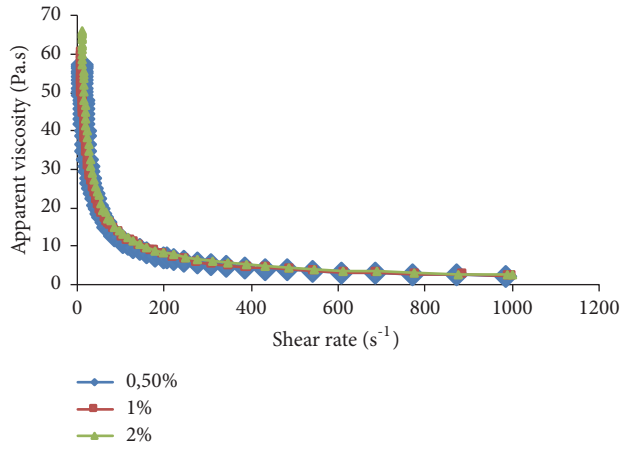


Figure 3: Flow behaviors of crude *Pelargonium graveolens* polysaccharide at different concentrations (0.5, 1, and 2 %; w/v).

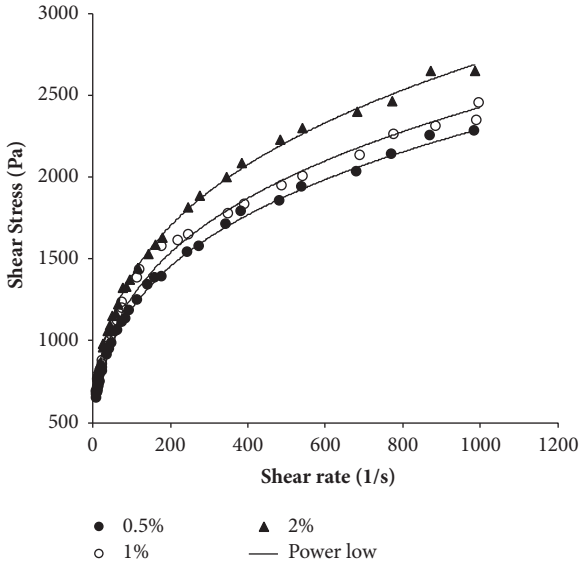


Figure 4: Shear stress versus shear rate of crude *Pelargoinum graveolens* polysaccharide water solution at different concentrations.

around 877 cm^{-1} marks the beginning of the «fingerprint» area.

4. Discussion

The central composite design was applied to assess the effect of temperature and time on the phenolic content and the antioxidant activity of the decoction. The response surface analysis revealed that the temperature was the most impacting factor. Indeed, the response reached the maximum value with the increase in temperature. This suggests that the temperature has an effect on plant tissue in improving the phenolic compound extraction [9]. Such finding was recurrent in many studies, stating that the increase in extraction time and temperature enhances the material particles solubility [29] and the diffusion coefficient [30].

Table 9: Flow behavior index (n) and consistency index (k) of CPGP at different concentrations.

Concentration (w/v) (%)	n	K (Pa·s^n)	R^2
0,5	0.283±0.01[a]	322.60±5.80[a]	0.998
1	0.277±0.02[b]	354.60±7.50[b]	0.995
2	0.279±0.01[c]	382.70±6.94[c]	0.999

The different letters indicated significant difference at p<5%.

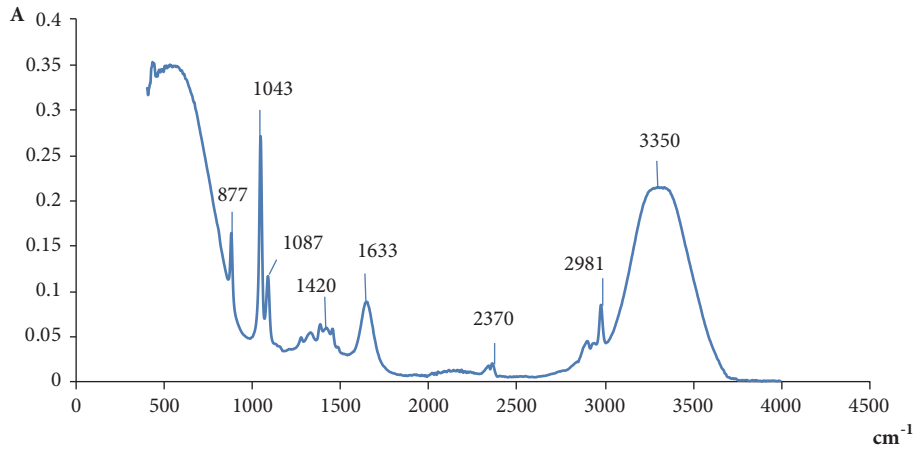


Figure 5: Fourier transform infrared spectra of extracted CPGP.

Besides, maintaining a relatively high temperature would increase the yield of the crude *Pelargonium graveolens* polysaccharide (CPGP). Indeed, different herbal water-soluble polysaccharides extraction studies showed that the extraction temperature varied from 48,7°C to 100°C [4, 12–14, 31–34]. However, to enhance the macromolecules yield, the time of extraction varied from 29 minutes to 4 hours [15, 33]. At these extraction conditions, the polysaccharide yield varied from 5,37% to 18,88 % [12–14, 32–34]. But, since the yield of polysaccharide was the unique matter of these studies, no attention was paid to the activity of other decoction molecules at prolonged heat treatment.

After the validation of optimal extraction conditions, the *Pelargonium graveolens* decoction at 94°C for 10 minutes presented a particular brown color. For tea, this color is desirable [35] and it is influenced by the polyphenol oxidase activity which oxidizes polyphenols to flavonoids, catechins, and brown colored compounds [35, 36]. For tea infusion, the L∗ value is about 59.03; however, for fermented tea infusion it is about 40.25, whereas the lightness of the decoction is much lower (18.74), which may correspond to higher dark colored compounds like those developed through tea fermentation [36]. The redness (a∗) and yellowness (b∗) of tea infusions (26.17-59.03 and 15.17-25.71, respectively) are, however, higher than color parameters of the optimal decoction (2.17 and 11.07). These parameters could help to differentiate between various *geranium* species extracts using different processes [36] (infusion, decoction, cold extraction...).

The value of the radical scavenging activity of the optimal *Pelargonium graveolens* decoction was about 136.1 mgTXE/gDM (68.05%). This finding was explained by the presence of phenolic components (flavonoids and condensed tannins) and mostly flavonoids (19.77 mgRE/gDM) [10, 37]. In addition, the studies of the antioxidant and phenolic profiles of *Pelargonium graveolens* hydrosols, aqueous extracts, methanol extracts, and essential oils exhibited phenolic contents varying from 54.71 mgGAE/gDM to 102.44 mgGAE/gDM and scavenging activity up to 83% [2, 38, 39].

Roseiro et al. (2013) [9] reported that, under optimum extraction temperature and time (98.5°C and 17 min, respectively), carob kibbles decoction exhibited a DPPH scavenging activity of about 85% and total phenolic content of about 39.5 mg GAE/gDM.

The *Pelargonium graveolens* water solutions exhibited a pseudoplastic or shear-thinning behavior. Adeli and Savmati (2014) [33] reported that the flow behavior index (n) of 1.5 % w/v Ziziphus lotus fruit polysaccharide solution (WPZL) was about 0.77, while the same index for the CPGP at 1% was about 0.27. This can be explained by the fact that the viscosity of CPGP is higher than that of WZPL at 1% w/v concentration.

The novel water-soluble polysaccharide had a total sugar content of 1.42 time higher than boat-fruited sterculia seeds (61.17%) [40] and 1.19 times higher than chickpea polysaccharide [41].

An attempt to a structural analysis of the CPGP by the Fourier Transform Infrared has revealed the presence of a large absorption band at around 3350 cm^{-1}, which may be associated with a hydroxyl group [14]. In fact, as reported by Chien et al. (2015) [42], the peaks from 3200 to 3600 cm^{-1} may be associated with O-H groups. Furthermore, the band detected at 2981 cm^{-1} indicated the stretching vibration of C-H groups [34, 42]. The presence of an absorption band at 1633

cm^{-1} suggests the presence of carboxylate stretching group (COO^-) [14] for a band peak around 1605 cm^{-1}. Nevertheless, other studies associated the absorption bands from 1640 cm^{-1} to 1651 cm^{-1} with C=O groups [42]. However, at 1642 cm^{-1}, the band was associated with water [31]. Moreover, the peak at 1420 cm^{-1} was assigned to C-O stretching vibration [31] and suggests the presence of uronic acid content [13, 43]. A strong absorption band was also observed at 1043 cm^{-1} that could be associated with the C-O-C stretching vibration of glycosidic structure [14] and might suggest the presence of pyranose ring (1043 to 1087 cm^{-1}) [31] or even furanose [34, 44].

The obtained bands at 890 cm^{-1} suggest the presence of the β-glycosidic bond [42] or β-D-glucan [45]. It was also reported that the absorption bands between 810 and 870 cm^{-1} could suggest the presence of mannan in the studied sample [42, 46].

5. Conclusion

The response surface methodology was used in this study to improve the antioxidant potential of the decoction of *Pelargonium graveolens* and to enhance the yield of the polysaccharide extraction. The extraction temperature had a linear effect on the different responses and the extraction time had a quadratic effect only on the polysaccharide yield. Besides, there was no interaction between the two extraction parameters. The optimal extraction conditions were obtained: extraction temperature (93°C) and extraction time (11 min). Under these conditions, different process responses were as follows: the phenolic content was 33.02%, the radical scavenging activity was 68.05%, and the CPGP yield was 6.43%. These results are in good agreement with the predicted values.

The crude *Pelargonium graveolens* polysaccharide solutions were found to exhibit shear-thinning non-Newtonian flow behavior for concentrations above 0.5% (w/v). The obtained results suggest that the CPGP rheological characteristics are suitable for applications in many industries, especially food. Moreover, the values of optimal conditions showed that decoction operation could have multiple uses, especially for consuming less energy.

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 they have no conflicts of interest.

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Research Article

Antagonism of Bacteria from Dog Dental Plaque against Human Cariogenic Bacteria

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Dental caries are a process of demineralization and destruction of human teeth. They originate through many factors and are associated with biofilm formation, which consists of bacteria adhered to the teeth that form a structurally and functionally organized mass called dental plaque. Both the presence of *Streptococcus mutans* and the frequent consumption of sucrose correlate with a higher prevalence of caries in humans. In dogs, however, the incidence of this disease is low, due to factors such as differences in dental microbiota and/or their low consumption of sucrose. This work evaluated the antagonism of bacteria from dog's dental plaque against *S. mutans*, for the identification of producing strains of biotechnological products for use in preventing caries. This study used 95 bacterial isolates of canine dental plaque from the Veterinary Department at the Federal University of Viçosa, Minas Gerais, Brazil. A spot-on-the-lawn method was performed using Brain Heart Infusion agar with catalase for an initial identification of the antagonistic activity. Additional tests were conducted on the isolates classified as antagonists for confirmation of the activity, using modified Mann-Rogosa-Sharpe medium containing low dextrose concentration. These isolates were incubated at 37°C for 24 hours in anaerobiosis. The peptide nature of inhibition was evaluated using the following proteinases: proteinase K from *Tritirachium album*, bovine pancreatic trypsin, and type XII-A α-amylase from *Bacillus licheniformis*. In the initial identification of those strains exhibiting antimicrobial activity, 14 were classified as antagonists. One of the isolates (*Bacillus* sp.) indicated bacteriocinogenic activity, with a deformed inhibition halo on *S. mutans* by the addition of trypsin. These results suggest that this bacterial isolate may be applicable to biotechnological use to combat the main etiological agent of caries in humans. Further studies are needed to evaluate the bacteriocinogenic nature of the antimicrobial activities of the other 13 antagonistic bacterial isolates.

1. Introduction

Dental caries is a multifactorial disease, where microbial involvement and the host response are both of fundamental importance. The genesis of caries is associated with the formation of a biofilm consisting of bacteria that adhere to the surface of the tooth. These bacteria form a structurally and functionally organized mass called dental plaque [1–4]. Caries is the result of a chronic process that, according to Newbrun [5], appears after the interaction and presence of four factors: a susceptible teeth, microorganisms, diet, and time [6]. The aetiology of caries disease involves *Streptococcus mutans*, an acidophilic and acidogenic microorganism important in the production of acid in human dental plaque [7, 8]. The presence of *S. mutans* and the frequent consumption of sugars are directly correlated with a higher prevalence and incidence of caries [9].

In dogs, dental caries are somewhat unusual. The differences in a dog's dental microbiota, their poor sucrose consumption [10, 11], or the possibility of microorganisms present in the oral cavity could establish antagonism on *S. mutans* and other cariogenic bacteria. This antagonism results from competition for nutrients or production of compounds inhibitory to these bacteria, bacteriocins, which would develop a critical barrier against colonization by pathogenic species [1]. Pieri and colleagues [12] investigated the microbiota of the dental plaque of dogs through isolating and identifying its bacterial components. They found that one

of the most present genera in dog plaque is *Streptococcus*; however, none of the isolates presented genetic similarity of the 16S rDNA gene with *Streptococcus mutans*, indicating the absence of this species in dogs [12–14].

Bacteriocins are peptides produced by bacteria to give them a competitive advantage [1]. *S. mutans* may produce bacteriocins to antagonise other bacteria in the dental plaque of humans. The species of *Streptococcus* present in the dental plaque of dogs could perform this same mechanism against *S. mutans* in the dental plaque of humans [1, 12]. Numerous lactic acid bacteria are consistently found in the dental plaque of dogs; many of these have the capacity to produce bacteriocins against similar bacteria in order to establish their colonization sites [7–9]. This ability to inhibit target strains is potentially useful in food preservation and the production of alternative antimicrobial therapeutic agents for diseased sites [15–18].

The most effective way to prevent caries is the mechanical removal of biofilm by brushing and using dental floss. However, this physical removal of biofilm is typically not enough to control the disease for the majority of the population. It is important to identify additive resources to combat dental biofilm, such as chlorhexidine [4, 13]. Chlorhexidine is currently considered as an antiseptic reference in dentistry and is approved by the American Dental Association Council on Dental Therapeutics. However, the continuous use of this product in the oral cavity has numerous side effects, including burning in the oral cavity, ulcerations in the jugal mucosa, darkening of the dental enamel, and loss of taste [4, 19–21]. Consequently, there has recently been increased interest in the development of new antimicrobial chemotherapeutic agents with potential for incorporation into oral products that control cariogenic microbiota [1, 4, 9, 17, 22–26].

Caries is a major public health problem, reaching between 60 and 90% of school-age children and a large majority of adults in developed and developing countries [27, 28]. The work presented here furthers the development of anticaries products through evaluating the antagonism of bacterial isolates from canine dental plaques against *S. mutans* to identify strains that produce compounds with biotechnological potential.

2. Materials and Methods

All methods in this study were conducted at the Laboratory of Microbiology Studies, Institute of Life Sciences, Federal University of Juiz de Fora, Campus Governador Valadares (UFJF-GV). The antagonistic potential of 95 bacterial isolates from the dental plaque of 10 dogs was previously obtained by Pieri and colleagues [12], from January to December of 2009 (Table S1). These isolates were within the bacterial repository of the Veterinary Department at the Federal University of Viçosa, and were selected representing equal percentage of its genus in total isolates obtained from the dogs. *Streptococcus mutans* (ATCC UA159) was used as cariogenic target strain. *Staphylococcus aureus* (ATCC 25923) were used as the traditional target cultures for these assays. All cultures were stored in Brain Heart Infusion (BHI) broth, with 20% glycerol as cryoprotectant, at -80°C until use. The analysis of the antagonistic activity of the isolates was performed according to Moraes and colleagues [16], by using spot-on-the-lawn methodology for initial identification of antimicrobial activity.

Bacterial cultures of dog plaque were activated for 24 hours at 37°C. From these cultures, 2 μL were inoculated on the surface of Petri dishes containing BHI agar and a catalase solution (100 IU/mL) and subsequently incubated at 37°C for 24 hours [29, 30] (Figure 1(a)). After the formation of the colonies on the agar surface (Figure 1(b)), a 10 mL overlay of BHI semisolid agar was added (0.75% bacteriological agar), containing approximately 106 CFU/mL of the target bacteria: *S. mutans* (ATCC UA159) and *S. aureus* (ATCC 25923) (Figure 1(c)). After solidification of the overlayer, the plates were again incubated at 37°C for 24 hours. After that incubation step, the formation of any inhibition halo around the bacterial colony indicated antagonism (Figure 1(d)). All tests were conducted in triplicate.

The cultures that presented antagonistic activity were subjected to additional tests to confirm the protein nature of the produced antimicrobial substances [16]. A modified Mann-Rogosa-Sharpe (MRS) agar plate containing dextrose at a low concentration (5 g/L) was used, where 2 μL of one of the cultures of active isolates of the dog dental plaque was inoculated in the centre of the plate and incubated anaerobically at 37°C for 24 hours (Figure 1(a)). After incubation, four holes adjacent to the colonies formed (Figure 1(e)) were inoculated with 30 μL of one of the following solutions: sterile distilled water (negative control), *Tritirachium album* proteinase K (Sigma-Aldrich, Saint Louis, MI, USA), bovine pancreas trypsin (Sigma-Aldrich, Saint Louis, MI, USA), and *Bacillus licheniformis* type XII-A α-amylase (Sigma-Aldrich, Saint Louis, MI, USA). All inoculated enzymes held concentrations of 20mg/mL. The plates were then incubated at 37°C for two hours to allow diffusion of the inoculated solutions into the agar. After diffusion, a 10 mL overlay of BHI semisolid agar, seeded with approximately one million CFU/mL of the target microorganism, was added, followed by incubation for 24 hours at 37°C. Sensitivity of the antagonistic culture to the proteolytic enzyme solutions was observed as interference in the inoculated regions (Figure 1(f)), thus confirming the protein nature of the inhibitory substance.

3. Results and Discussion

From the 95 isolates tested in the initial evaluation of the antagonistic activity, 14 formed inhibition halos around the colonies in the BHI agar medium containing a catalase solution. These isolates were initially classified as antagonists against *S. mutans* and/or *S. aureus*.

Of the 14 isolates initially classified as antagonists, only one did not show growth in the dextrose-modified MRS medium and was subsequently discarded. The other 13 strains showed growth and formation of inhibition halos around the colonies when incubated with the target strains. These 13 isolates which were confirmed as antagonists and the diameters of their inhibition halos were evaluated (Table 1).

Table 1: Inhibition of pathogenic bacteria by bacterial isolates from dog's dental plaque. The 16S RNA Genbank access codes, genus/species, target strain, and diameter of the inhibition halo for the 13 bacterial isolates, initially classified as antagonistic against *S. mutans* and *S. aureus*.

Genbank access code	Genus/species	Target strain	Inhibition Halo (mm)
HQ717194	*Aerococcus viridans*	*S. mutans*	25
		S. aureus	-
HQ717297	*Lactococcus lactis*	*S. mutans*	12
		S. aureus	12
HQ717208	*Actinomyces* sp.	*S. mutans*	20
		S. aureus	-
HQ717211	*Bacillus* sp.	*S. mutans*	22
		S. aureus	-
HQ717189	*Enterococcus faecalis*	*S. mutans*	23
		S. aureus	7
HQ717204	*Enterococcus faecalis*	*S. mutans*	18
		S. aureus	-
HQ717193	*Enterococcus faecalis*	*S. mutans*	17
		S. aureus	-
HQ717209	*Enterococcus faecalis*	*S. mutans*	22
		S. aureus	14
HQ717177	*Enterococcus faecalis*	*S. mutans*	22
		S. aureus	-
HQ717206	*Actinomyces sp.*	*S. mutans*	21
		S. aureus	-
HQ717183	*Enterococcus faecalis*	*S. mutans*	19
		S. aureus	-
HQ717203	*Enterococcus faecalis*	*S. mutans*	24
		S. aureus	12
HQ717328	*Actinomyces* sp.	*S. mutans*	25
		S. aureus	11

Of the 13 isolates, only *Bacillus* sp. (HQ717211; Table 1) had an inhibition halo that was modified by proteolytic enzyme solution (bovine pancreas trypsin). These results indicated the sensitivity of the culture to the enzyme solution, thereby confirming the protein nature of the inhibitory substance.

The spot-on-the-lawn methodology is widely used to detect the protein character of antimicrobial substances produced from bacteria. It is considered advantageous, as proteins are distinguishable even in cultures that produce small inhibition halos [16, 30, 31]. This methodology was performed using BHI medium with catalase (100 IU/mL) incubated at 37°C for 24 hours for the initial identification of the antimicrobial activity. The catalase solution was added to hydrolyse any possible hydrogen peroxide produced by the cultures [16, 32, 33]. Hydrogen peroxide has antimicrobial potential and could thus interfere with the identification of isolates with antagonistic activity derived from the production of bacteriocins [16, 34]. The inhibition halos identified in the 14 isolates classified as antagonists had no relation to hydrogen peroxide production.

Although any one acidogenic bacterium may contribute to enamel demineralization that results in caries, the *S. mutans* strain presents additional characteristics that may initiate and exacerbate the disease [1, 35]. In addition to the fermentation of sucrose in organic acids, *S. mutans* hydrolyses substrate forming polymers, allowing them to coaggregate with other bacteria, thus forming an extracellular matrix with greater biodiversity [1, 35]. Although other human oral bacteria such as *S. sanguis*, *S. salivarius,* and *S. gordonii*, can synthesize these polysaccharides, only *S. mutans* presents a preference to the presence of sucrose in the infection site [1, 35]. In addition, *S. mutans* has the ability to store amylopectin intracellular polysaccharides for fermentation in the absence of extracellular carbohydrates, allowing continuous fermentation between host meals. *S. mutans* also shows greater release of acid when compared to other bacteria of the genus *Streptococcus* [1]. Therefore, the reduction of *S. mutans* levels in the plaque microbiota could become a desirable strategy for the prevention and treatment of the disease [9].

Streptococcus spp., obtained from dental plaques of dogs (supplementary data), were expected to act as potential inhibitors of *S. mutans*, as bacteriocins are produced by a microorganism to antagonise those with high genetic similarity [1, 29]. However, of the eight isolates of *Streptococcus* spp. tested in this study, none presented antagonistic activity. Our data showed a large variety of bacteria presenting antagonism against *S. mutans*. These species include *Enterococcus faecalis*, *Actinomyces* sp., *Aerococcus viridans*, *Bacillus* sp., and *Lactococcus lactis*. Among the isolates classified at the end of

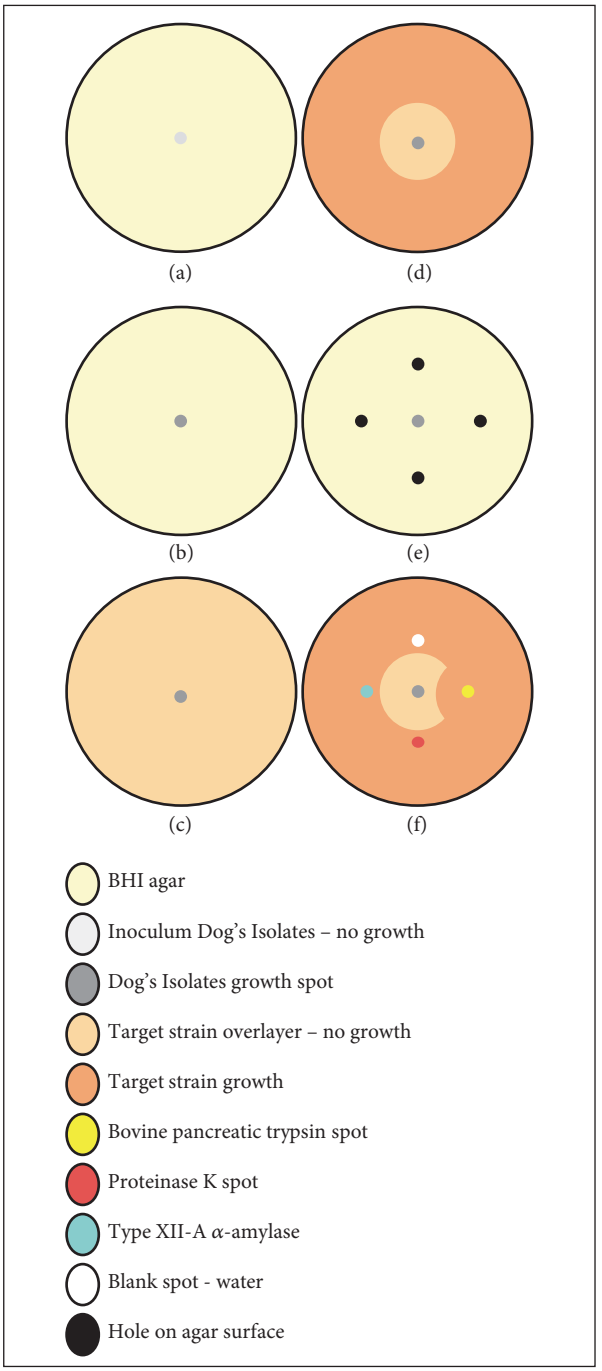

Figure 1: Sequence of spot-on-the-lawn surface to establish antagonistic activity of one strain of bacteria against a target strain and to confirm the protein nature of this inhibition; (a) 2 μL of test strain is inoculated on the surface of Petri dishes containing agar; (b) formation of the colonies of test strain on the agar surface; (c) addition of a semisolid agar overlayer with the target bacteria; (d) inhibition halo after the incubation of target strain, confirming antagonistic activity; (e) four holes adjacent to the colonies made to inoculation of different proteinases; (f) interference of bovine pancreatic trypsin on inhibition halo of test strain against target strain, confirming the protein nature of antagonistic activity.

the second stage of the incubation, 53.9% of the antagonists were *Enterococcus faecalis* (Table 1). This bacterial species was previously classified as *Streptococcus faecalis*, due to its high genetic and phenotypic similarity with the *Streptococcus* genus [36]. Considering that similarity, the bacteria of the genus *Enterococcus* could potentially antagonise those species within the genus *Streptococcus*.

With regard to the second phase of evaluating antagonistic activity of the bacterial isolates, the use of the MRS medium under anaerobic conditions tends to inhibit the

production of hydrogen peroxide [31, 33]. In addition to the exclusion of the possible hydrogen peroxide activity on target strains, this medium and culture condition caused an increase in the diameter of the halos of some test strains, indicating antagonistic activity.

Following the aim of this work, the protein character of the antagonist activity of isolates was evaluated against *S. mutans*. The *S. aureus* overlay was used as a comparison strain in the initial phase of evaluation of the antagonistic activity, as it is used often with the spot-on-the-lawn method. However, as *S. aureus* is a recognized pathogen for both human and animal health [24], new studies evaluating the protein nature of isolates from canine dental plaque should be carried out for a possible identification of bioproducts for the treatment of diseases caused by this pathogen.

While 13 strains showed the formation of an inhibition halo in the overlay of *S. mutans*, only one isolate (HQ717211, *Bacillus* sp.) showed deformation of the halo in the deposit site of proteolytic enzyme bovine pancreatic trypsin, suggesting the loss of antagonistic activity by the interaction of the enzyme and the protein-based bacteriocin produced by this isolate.

4. Conclusions

This study shows that the isolate of *Bacillus* sp. (HQ717211), obtained from canine dental plaque, has biotechnological potential in combatting a major etiologic agent of caries in humans. In addition, 13 other bacterial isolates were identified as potential antagonists of *S. mutans*. Future work confirming the bacteriocinogenic nature of these isolates should be considered for use in preventive therapy and treatment of caries in humans.

Data Availability

The 16S rDNA sequences for all isolates from canine dental plaque used to identify the bacteria involved on the findings of this study have been deposited in the Genbank repository, (Genbank access codes are located in Tables 1 and S1).

Conflicts of Interest

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

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Supplementary Materials

Table S1: all isolates from dog's dental plaque used for the study of bacteriocinogenic activity against *Streptococcus mutans* and their respective Genbank access number for 16S rDNA sequences. *(Supplementary Materials)*

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Research Article

Enhanced Production of Polymyxin E in *Paenibacillus polymyxa* by Replacement of Glucose by Starch

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Polymyxin E or colistin, produced by *Paenibacillus polymyxa*, is an important antibiotic against Gram-negative pathogens. The objective of this study is to evaluate the effect of starch in fermentation medium on colistin biosynthesis in *P. polymyxa*. The results indicated that replacement of glucose by starch stimulated colistin production and biosynthesis rate. Overall, the stimulation extent was starch concentration-dependent. As expected, addition of starch induced the expression of *amyE* encoding amylase and increased amylase activity in fermentation solution. Additionally, replacement of glucose by starch resulted in residue reducing sugar and pH of fermentation mixture low relative to glucose as the sole sugar source. At the molecular level, it was found that replacement of glucose by starch has enhanced the relative expression level of *ccpA* encoding catabolite control protein A. Therefore, the repression of starch utilization by glucose could be probably relieved. In addition, use of starch stimulated the expression of regulatory gene *spo0A* but repressed the expression of another regulatory gene *abrB*. As a result, the expression of genes directly involved in colistin biosynthesis and secretion increased, indicating that at the transcriptional level *spo0A* and *abrB* played opposite roles in regulating colistin biosynthesis in *P. polymyxa*. Taken together, our data demonstrated that starch instead of glucose can promote colistin production probably by affecting the expression of colistin biosynthesis-related genes, as well as reducing the repression of glucose to a secondary metabolic product.

1. Introduction

Polymyxin E, also called colistin, is an important old antibiotic known for around six decades for treatment of infection caused by Gram-negative pathogens [1, 2]. Later studies showed that colistin can also kill Gram-positive bacteria [3, 4]. Currently, its clinical use is broadly restricted regarding its toxicity mainly to the kidney and nervous system [5]. In recent, the occurrence of Gram-negative multidrug-resistant pathogens which are resistant to many available antibiotics has revived its clinical application in healthcare centers, since colistin is broadly considered as one of the last-line options of antibiotic therapy for multidrug-resistant bacteria. Therefore, its market demand is increasing [6, 7].

Colistin is composed of two parts: a cyclic heptapeptide and a tripeptide side chain which is acylated by a fatty acid at the amino terminus [8, 9]. In total, colistin has ten orderly assembled amino acid residues [10]. Among them, six are L-2,4-diaminobutyric acids (L-Dabs). L-Dab is biosynthesized by 2,4-diaminobutyrate aminotransferase (EctB) which is encoded by *ectB* [11]. Colistin can be biosynthesized by a multienzyme nonribosomal peptide synthetase system (NRPS) in *Paenibacillus polymyxa* [3, 4]. The phosphopantetheinyl transferase (Sfp) encoded by *sfp* is important for colistin biosynthesis due to its activation function on NRPS [12, 13]. A gene cluster including five open reading frames, *pmxA*, *pmxB*, *pmxE*, *pmxC*, and *pmxD*, encoding three synthetases PmxA, PmxB, and PmxE, and two membrane transporters PmxC and PmxD, respectively, has been characterized for biosynthesis and secretion of colistin in *P. polymyxa* [10, 14]. It has been determined that colistin biosynthesis is negatively regulated by AbrB, a DNA-binding protein, by directly binding to the upstream region of *pmxA* [11]. The expression of *abrB* itself is negatively controlled by Spo0A, another DNA-binding protein, encoded by *spo0A* [15]. These two genes play opposite roles in regulation of colistin production.

So far, colistin has been best characterized with respect to its structure and biosynthesis, antibacterial mechanism and bacterial resistance, and toxicity and derivatives. In contrast, extremely little is known about medium optimization for its

fermentation output. It has been reported that colistin production depends on the inorganic phosphate concentration [16]. Further optimization showed that addition of L-Dab as well as its precursor aspartic acid to fermentation medium containing appropriate PO_4^{3-} stimulates colistin production [16]. However, other studies showed that addition of either L-Dab or aspartic acid to medium after 35 h fermentation significantly inhibits colistin production by suppressing the expression of *pmxA* and *pmxE*, as well as *ectB* in another producer strain [17]. Although it has been found that corn meal in the medium is essential for the better production of colistin [16], glucose is the most widely used carbon source for colistin biosynthesis [18]. In our previous study, glucose was also used as a sugar source for colistin production in *P. polymyxa* C12 and its production reached around 6.2×10^4 U/mL (2600 μg/mL) in flask level [17]. As a concern, glucose has been widely found to repress the accumulation of secondary metabolic compounds in microbes through carbon catabolite repression (CCR) [19]. CCR in microbes is regarded as the mechanism in which bacteria preferentially utilize the rapidly metabolizable carbon source (normally glucose). As a result, the utilization of secondary carbon resource is repressed. CCR is considered to be a part of the global control system and therefore it affects many genes [20]. In Gram-positive bacteria, the catabolite control protein A (CcpA) is the master regulator of CCR. Various physiological processes in Gram-positive bacteria are regulated by CcpA [21–23].

In this study, we attempt to substitute starch for glucose and investigate the effect of the sugar source on colistin production in *P. polymyxa* C12. In addition, the effect of the sugar source on amylase activity and relative expression of genes associated with colistin biosynthesis was also evaluated.

2. Materials and Methods

2.1. Strain and Culture Conditions. Colistin-producer *P. polymyxa* C12 [17] used in this study was frozen at −80°C in our lab at Zhejiang University of Technology, China. Unless otherwise stated, *P. polymyxa* was firstly cultivated on a culture medium agar plate (10 g/L of beef extract, 15 g/L of peptone, 10 g/L of glucose, 2 g/L of yeast extract, 3 g/L of NaCl, 0.1 g/L of $FeSO_4 \cdot 7H_2O$, and 20 g/L of agar, pH 7.0) at 30°C for 2 d. Then, a ring of *P. polymyxa* was transferred to 50 mL of seed medium (30 g/L of soybean meal, 5 g/L of soybean oil, 0.1 g/L of $FeSO_4 \cdot 7H_2O$, 15 g/L $(NH_4)_2SO_4$, 0.77 g/L of KH_2PO_4, 0.7 g/L of $CaCO_3$, and 10 g/L of glucose, pH 7.0) in a 250 mL flask for incubation at 30°C for 24 h with a shaking at 200 rpm. Next, 5 mL of cell culture was transferred to 50 mL of fermentation medium (23.9 g/L of soybean meal powder (Zhejiang Qianjiang Biochemical Co., Ltd., China), 21.1 g/L of soybean cake powder (Zhejiang Qianjiang Biochemical Co., Ltd., China), 10 g/L of soybean oil, 0.1 g/L of $FeSO_4 \cdot 7H_2O$, 25 g/L $(NH_4)_2SO_4$, 0.77 g/L of KH_2PO_4, 1 g/L of $CaCO_3$, and 45 g/L of glucose, pH 7.0) in a 250 mL flask at 30°C with shaking at 200 rpm for fermentation. A certain amount of glucose from 20 g/L to 45 g/L in the fermentation medium was replaced by starch if necessary. Unless otherwise specified, *P. polymyxa* was fermented for 96 h and 0.5 mL of samples was collected per 12 h. The colistin concentration and relevant gene expression were determined by HPLC and quantitative real-time PCR (qRT-PCR), respectively.

2.2. Measurement of Cell Growth. Unless otherwise stated, the bacterial biomass of the cultured cells was determined based on the value of colony forming unit (CFU) [24, 25]. First, the cells were collected after centrifugation at 5,000 g for 5 min. After washing twice with 0.5 mL of fresh broth culture medium (10 g/L of beef extract, 15 g/L of peptone, 10 g/L of glucose, 2 g/L of yeast extract, 3 g/L of NaCl, and 0.1 g/L of $FeSO_4 \cdot 7H_2O$, pH 7.0), the cells were then resuspended in 0.5 mL of fresh broth culture medium. Then, the cells were tenfold gradiently diluted. Finally, 100 μL of cells was spread to a culture medium agar plate for growth. After cultivation at 30°C for 2 d, CFU were counted.

2.3. HPLC Analysis of Colistin. One milliliter of fermentation liquor and 9 mL of ultrapure water were mixed. Then, 1 mL of diluted fermentation liquor was centrifuged at 10,000 g for 10 min and the supernatant was collected and filtered with 0.45 μm hydrophilic microporous membrane (Millipore). Analysis of colistin was performed using binary gradient model of an HPLC system (SHIMADZU, Japan). Twenty μL of supernatant sample was injected into a reverse-phase column, YMC Pack ODS-A (150 × 4.6 mm I.D., 5 μm), eluted at 33°C, and analyzed in a mixed solvent of acetonitrile (22%) and water containing 0.223% Na_2SO_4 (78%), at a constant flow of 1 mL/min. The mixed solvent was prepared by mixing pure acetonitrile in A-pump with water containing 0.223% Na_2SO_4 in B-pump. Separation program was set as follows: 0~20 min, 22% A-pump and 78% B-pump; 21~30 min, 90% A-pump and 10% B-pump; 31~40 min, 22% A-pump and 78% B-pump. Colistin peak was determined at wavelength of 240 nm. Colistin concentration produced was calculated based on the extracted correlation between the concentration of standard colistin (Zhejiang Qianjiang Biochemical Co., Ltd., China) and the corresponding peak area in HPLC. One unit is equal to 0.0418 μg of colistin [17]. Colistin was dissolved in 1 mL of pure water to make 2×10^5 U/mL solution. Then, colistin was fivefold gradiently diluted with water to make serial colistin solutions. For HPLC analysis, 20 μL of standard colistin was injected.

2.4. Detection of Reducing Sugar and Assay of α-Amylase Activity. After fermentation, the cell mixture was centrifuged at 10,000 g for 10 min and the supernatant was collected. Unless otherwise specified, the amylase activity in the fermentation supernatant (crude enzyme solution) was determined by measuring the reducing sugar generated during the reaction [26]. In general, 0.2 mL of 0.1 M citrate-phosphate buffer with pH 7.0 containing 1% (w/v) soluble starch was preheated at 30°C for 5 min. Then, 0.05 mL of crude enzyme solution appropriately diluted with sterilized ultrapure water was added and mixed thoroughly. After incubation at 30°C for 30 min, the reaction was terminated by adding 1 mL of DNS reagent containing 182 g/L Rochelle salt,

Table 1: Sequences of primer pairs for PCR amplification of genes.

Genes	Nucleotide sequences (5′-3′)		Product sizes (bp)
	Forward primers	Reverse primers	
amyE	ATGGTCCACAATCCTGTT	CCTCATGTTCTTCCCTCA	1319
ccpA	TGGTCAGCAAACGCATCG	AAACCTCAGACCCGCAAG	832

Table 2: Sequences of primer pairs for real-time PCR analysis of gene expression.

Genes	Nucleotide sequences (5′-3′)		Product sizes (bp)
	Forward primers	Reverse primers	
pmxA	TCAACTCGCTCAGAAGCGTT	TTGTACGGAAACCGACGGAG	105
pmxB	ATGAAATCTTTGTTTGAAAA	CCAGGACGTACACCCTCAAC	111
pmxC	TATTCCCGAGCTCATCACGC	TCGGAAGCGAACGACCATTT	107
pmxD	TGTTCGTTCAACGCCTCGTA	GCTTGCAAACGCTCGGTAAA	118
pmxE	CACTTTGCCTGAAACGACCG	GCCAGAATGCGTTCATACCG	111
spo0A	TCGCAGAATCCCGCAACATA	CGGTTGTGGAGTCAGGTTCA	103
abrB	AAATACGGAACAGCCCGTCC	TCGCTCGCCTGTCTTCAAAT	114
ectB	CAGTGGATACGGTCTGCCAA	CTCCGACAAACGCTAGCTGA	113
sfp	GTACCTCCTGCGCAAAGTGA	CACGACAGAGGGCTTTACGA	110
amyE	TCTGGGCGGAACGATTTTGA	CGAGTGCCGCCCTATTGTAT	110
ccpA	ATCAATTCCGGCTGCTTCCA	CACCGCCAAATCGCAATGAT	102

21 g/L NaOH, 6.3 g/L dinitrosalicylic acid, 5 g/L crystalline phenol, and 5 g/L Na_2SO_3. Next, the mixture was placed in boiling water for 5 min, followed by cooling down to room temperature. Finally, the absorbance value of mixture at 540 nm was measured [26]. The concentration of reducing sugar was determined based on the extracted correlation between standard glucose over a range of concentrations and the corresponding absorbance values at 540 nm. One unit of amylase activity was defined as the amount of enzyme required to release 1 μg of glucose equivalent per minute under the assay condition using glucose as the standard.

2.5. PCR Amplification and Sequence Retrieval of Genes. The sequences of *pmxABCDE*, *spo0A*, *abrB*, *ectB*, and *sfp* have been amplified and collected in our previous study [17]. In this study, the primers (Table 1) for *amyE* encoding amylase and *ccpA* encoding CcpA in PCR reaction were designed based on the complete genome sequence of *Paenibacillus polymyxa* SC2 (GenBank access no. CP002213.2). Bacterial genomic DNA was extracted using a bacterial genomic DNA extraction kit (GE, USA). PCR reaction was performed as reported previously [27, 28]. In brief, the gene fragments were amplified in 50 μL containing 37 μL of ddH_2O, 5 μL of 10X EasyTaq buffer, 4 μL of 2.5 mM dNTPs, 100 nM forward primer, 100 nM reverse primer, 1 ng genomic DNA, and 1 U *Taq* DNA polymerase (TaKaRa, Dalian, China) with denaturation at 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 50 s at 55°C, 90 s at 72°C, and a final 10 min extension at 72°C. At the end of reaction, PCR product was cooled to 4°C for further use. After size confirmation on 1.0% agarose gel, the desired amplicons were purified using a gel extraction kit (Qiagen, CA, USA) for TA cloning with pMD19-T simple vector (TaKaRa, Dalian, China). After sequencing by Sangon Biotech (Shanghai, China), the gene sequences were collected and compared with the reference genes in GenBank for confirmation.

2.6. qPCR Analysis of Gene Expression. In brief, 0.5 mL of the bacterial cells was pelleted after centrifugation at 8,000 rpm for 10 min at 4°C and the total RNA was extracted using an RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. RNA purity was spectrophotometrically evaluated based on $OD_{260\,nm}/OD_{280\,nm}$ ratio. Then, 300 ng of DNA-free RNA was reversely transcribed to cDNA in a 10 μL volume using PrimeScript™ RT Master Mix (Perfect Real Time) kit (Toyobo, Tokyo, Japan). After appropriate dilution, the obtained cDNA was used for amplification of target gene fragment with primer sets (Table 2) [17] by using the SYBR green *Premix Ex Taq*™ (Tli RNaseH Plus) kit. A master mixture was prepared and each well of reaction contained the following reagents: 5 μL of SYBR Green Master Mix, 0.2 μL forward primer and reverse primer, respectively, and 3.6 μL of ddH_2O to a total of 9 μL. After addition of 1 μL of each diluted cDNA sample to each well, the PCR was run on CFX Connect Real-Time System (Bio-Rad, Hercules, CA) with an amplification protocol consisting of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 30 s. Immediately after the final cycle of PCR, melting curve was analyzed to retrieve the specificity of the reaction based on the observation of melting temperature from the product [29].

The cycle threshold (C_T) for each PCR was determined using StatView software which automatically set the threshold signal at the log phase of amplification curve. The amplification efficiency of gene was retrieved from the slope of

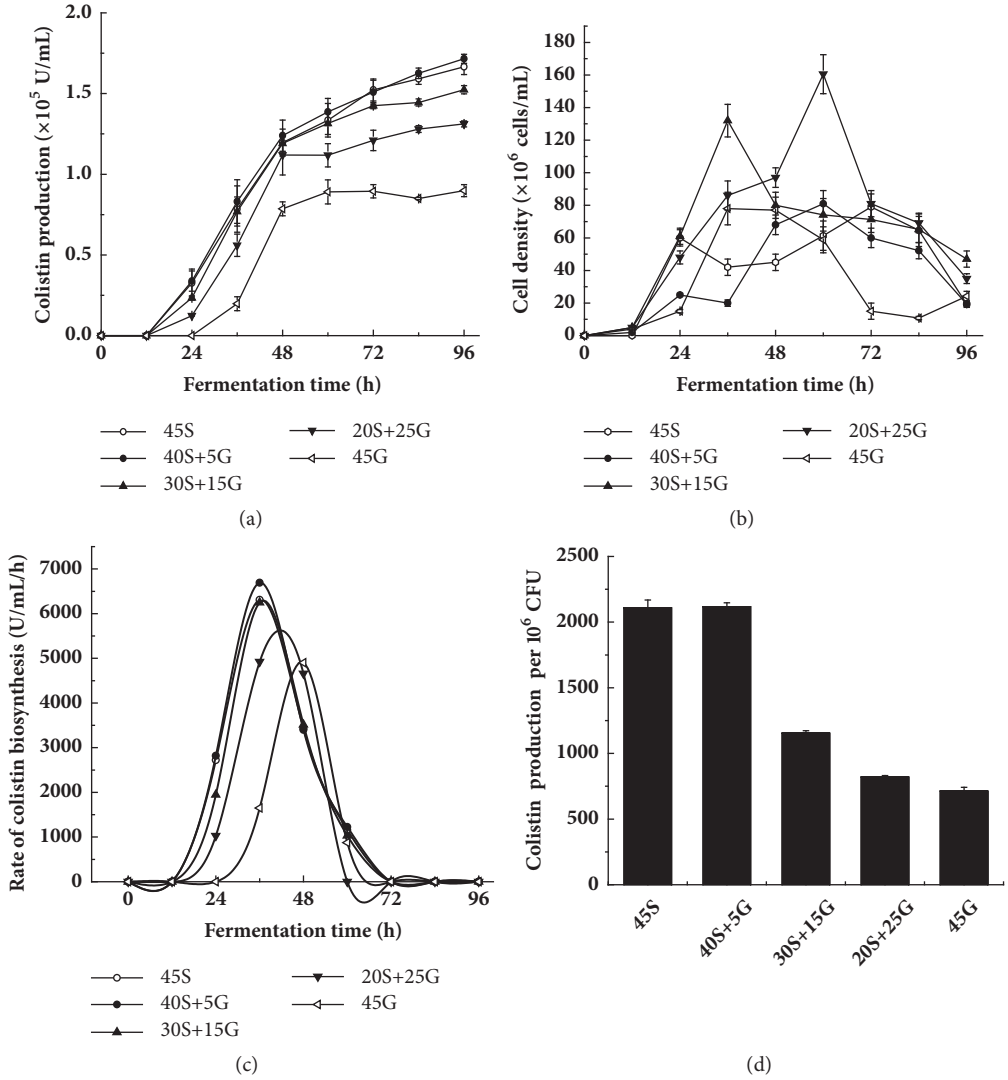

Figure 1: Influence of replacement of glucose by starch on colistin production in *P. polymyxa*. (a) Colistin production along fermentation; (b) growth curve of *P. polymyxa* along fermentation; (c) rate of colistin biosynthesis along fermentation; (d) colistin production per cell biomass after 96 h fermentation. 45S: 45 g/L starch; 40S+5G: 40 g/L starch plus 5 g/L glucose; 30S+15G: 30 g/L starch plus 15 g/L glucose; 20S+25G: 20 g/L starch plus 25 g/L glucose; 45G: 45 g/L glucose.

that linear regression according to the formula $E=10^{(-1/\text{slope})}$. Several dilutions of each cDNA sample were assayed for the gene of interest in order to obtain a linear regression between the C_T values (ranging from 15 to 35 cycles) and the log of cDNA. The 116 bp of 16S rRNA gene fragment ranging from 16SF (5′-GAGAAGAAAGCCCCGGCTAA-3′) to 16SR (5′-ACCAGACTTAAAGAGCCGCC-3′) was used as the internal control to verify that there was an equal amount of target cDNA in all samples. The expression of target gene relative to 16S rRNA gene was calculated as described in report [30].

2.7. Data Analysis and Availability. Unless otherwise specified, triplicate reactions per experiment were performed. All data were presented as mean ± standard error and tested for statistical significance based on analysis of variance (ANOVA) followed by Dunnett's post hoc test using StatView 5.0 program. When the probability (p) was less than 0.05 and 0.01, the values were considered significantly (∗) and very significantly (∗∗) different, respectively.

3. Results

3.1. Dependence of Starch Concentration on Colistin Biosynthesis. To investigate the effect of starch on colistin accumulation in *P. polymyxa* C12, different amounts of glucose in fermentation medium were replaced by starch. As shown in Figure 1(a), colistin was undetectable within the first 24 h using 45 g/L glucose as the sole sugar source. Then, its production rapidly increased up to 48 h, followed by almost a constant in the remaining period. The highest yield of colistin was around 8.5×10^4 U/mL. Instead, the replacement of

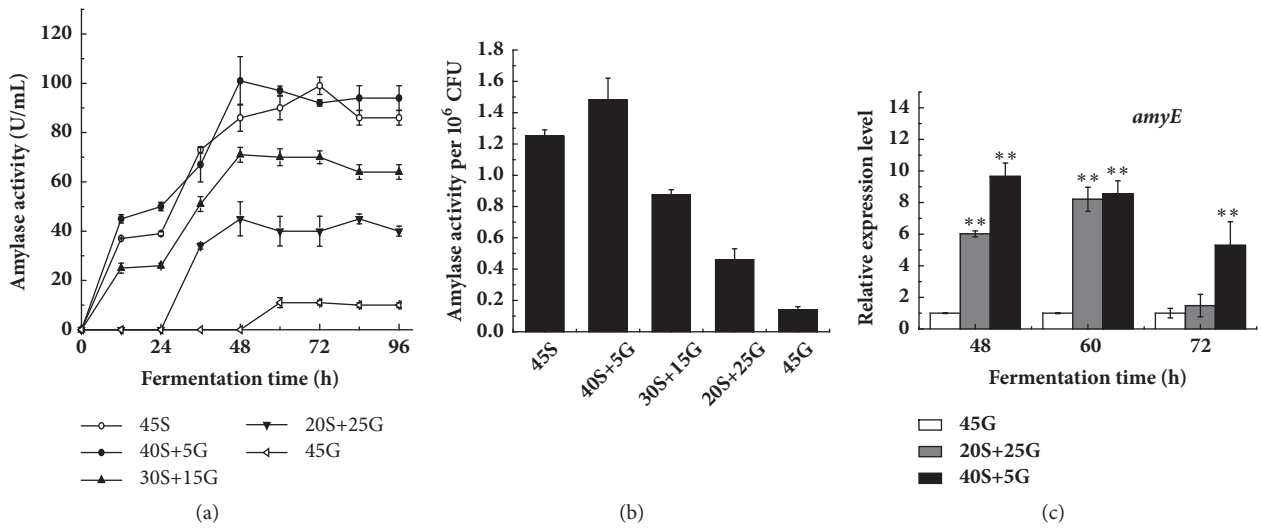

Figure 2: Influence of replacement of glucose by starch on amylase and gene expression. (a) Amylase activity along fermentation; (b) amylase activity per cell biomass after 96 h fermentation; (c) relative expression level of *amyE*. 45S: 45 g/L starch; 40S+5G: 40 g/L starch plus 5 g/L glucose; 30S+15G: 30 g/L starch plus 15 g/L glucose; 20S+25G: 20 g/L starch plus 25 g/L glucose; 45G: 45 g/L glucose. The statistically significant results are related to the condition of 45 g/L glucose.

glucose by starch in fermentation medium gave detectable colistin at 12 h. Next, colistin production rapidly increased also up to 48 h, followed by a moderate increase in the remaining period. Overall, the more the glucose was replaced by starch, the higher the colistin was produced. The highest yield of colistin with 40 g/L starch plus 5 g/L glucose was around 1.66 $\times 10^5$ U/mL, approximately one time higher than the one with 45 g/L glucose. The 45 g/L starch as the sole sugar source showed a similar result to 40 g/L starch plus 5 g/L glucose. Figure 1(b) indicated that the proportion of sugar source clearly affects the cell growth. Overall, the high proportion of glucose was beneficial to cell accumulation for early stage of fermentation. In contrast, the high proportion of starch was beneficial to cell accumulation for later stage of fermentation. Most probably, the use of glucose is faster than that of starch. Figure 1(c) showed that the rate of colistin biosynthesis with 45 g/L glucose as the sole sugar source rapidly increased and then decreased. The highest rate of colistin biosynthesis was 4.8×10^3 U/(mL·h) at 48 h. Similarly, the rate of colistin biosynthesis with all mixtures of glucose and starch rapidly increased and then decreased. Interestingly, the highest rate of colistin biosynthesis overall appeared earlier and higher with the increase of replacement of glucose with starch. The highest rate of colistin biosynthesis with 40 g/L starch plus 5 g/L glucose reached 6.7×10^3 U/(mL·h) at 36 h. Figure 1(d) further showed that the replacement of glucose with starch enhanced the colistin production per biomass. Overall, the more the glucose was replaced, the higher the colistin per biomass was produced. The highest yield of colistin per biomass with 40 g/L starch plus 5 g/L glucose was around 2.1×10^3 U/(mL·10^6 CFU), approximately two times higher than the one with 45 g/L glucose. The 45 g/L starch as the sole sugar source displayed a similar result to 40 g/L starch plus 5 g/L glucose. All these data congruously indicated that the replacement of glucose with starch stimulates the colistin accumulation in *P. polymyxa*.

3.2. Effect of Starch on Amylase Activity and Relative Expression of amyE. Starch should be decomposed by amylase before use in fermentation. Therefore, amylase activity was monitored. As shown in Figure 2(a), amylase activity with 45 g/L glucose as the sole sugar source can be detected at 48 h. Then, it increased to 11 U/mL at 60 h, followed by almost a constant in the remaining period. Amylase activities with both 40 g/L starch plus 5 g/L glucose and 45 g/L starch were around 100 U/mL at 48 h and 72 h, respectively, eight times higher than the one with 45 g/L glucose. Figure 2(b) further showed that amylase activity per 10^6 CFU with 40 g/L starch plus 5 g/L glucose was around 1.48 U/mL, eight times higher than 0.17 U/mL of amylase activity per 10^6 CFU with 45 g/L glucose. Figure 2(c) indicated that the relative expression level of *amyE* encoding amylase significantly increased with increase of starch replaced for glucose. All these results supported the reports that the transcription of *amyE* is strongly increased by starch [31] but repressed by glucose [32].

3.3. Effect of Starch on pH and Reducing Sugar Formation. Carbon source could affect reducing sugar and accordingly fermentation output [33, 34]. Figure 3(a) showed that the residue reducing sugars in fermentation with original glucose ranging from 15 g/L to 45 g/L decreased within the first 48 h and kept almost constant in the second 48 h. Most probably, the consumption of glucose in fermentation medium could result in the decrease of reducing sugar within the first 48 h. The higher the original concentration of glucose in fermentation medium was, the faster the reducing sugar decreased within the first 48 h. On the contrary, the residue reducing sugar in fermentation with original glucose ≤15 g/L

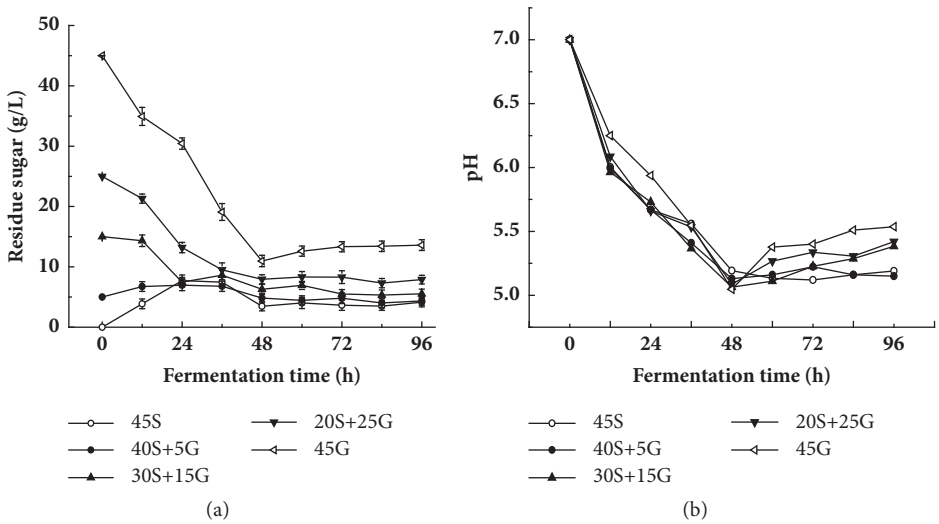

Figure 3: Influence of replacement of glucose by starch on residue reducing sugar (a) and pH (b) during fermentation. 45S: 45 g/L starch; 40S+5G: 40 g/L starch plus 5 g/L glucose; 30S+15G: 30 g/L starch plus 15 g/L glucose; 20S+25G: 20 g/L starch plus 25 g/L glucose; 45G: 45 g/L glucose. The statistically significant results are related to the condition of 45 g/L glucose.

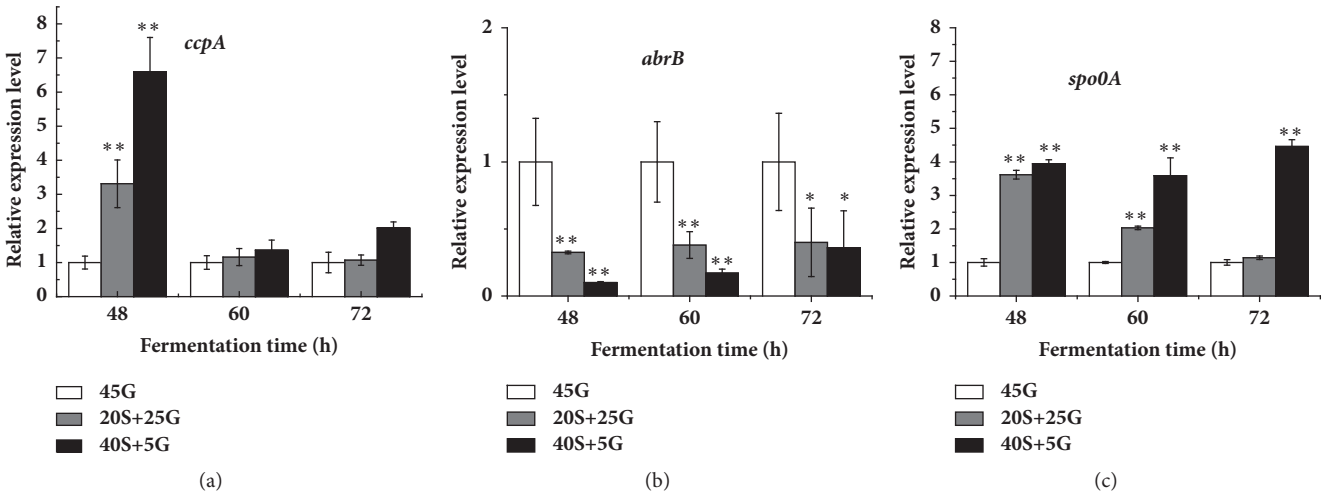

Figure 4: Effect of replacement of glucose by starch on relative expression level of genes involved in regulation of colistin biosynthesis. (a) *ccpA*; (b) *abrB*; (c) *spo0A*. 45G: 45 g/L glucose; 20S+25G: 20 g/L starch plus 25 g/L glucose; 40S+5G: 40 g/L starch plus 5 g/L glucose. The statistically significant results are related to the condition of 45 g/L glucose.

increased within the first 48 h and kept almost constant in the second 48 h. Most probably, the rate of decomposition of starch in fermentation medium would surpass the rate of reducing sugar consumption within the first 48 h, thus resulting in the increase of reducing sugar. Figure 3(a) further indicated that the higher the original concentration of glucose was, the higher the concentration of residue reducing sugar was at the end of fermentation. The residue reducing sugars in fermentation medium with 45 g/L glucose and 45 g/L starch as original sugar were 13.6 g/L and 4.1 g/L, respectively, at 96 h. Figure 3(b) showed that the pH of fermentation solution with different sugar sources displayed a similar pattern, rapid decrease within the first 48 h and slight increase within the second 48 h. Overall, the higher the original glucose concentration in fermentation medium was, the higher the pH of fermentation solution was within the second 48 h, which is negatively correlated with colistin production (Figure 1). The fact that the overall difference in pH of fermentation solution derived from proportion of sugar source is visible, but not remarkable, is worth noting.

3.4. Effect of Starch on Relative Expression of Genes for Regulation of Colistin Biosynthesis. CcpA encoded by *ccpA* is the master regulator of CCR in Gram-positive bacteria and it can affect the expression of the *abrB* [35]. Both *abrB* and *spo0A* are believed to be associated with colistin production [17]. Therefore, the relative expression of these three genes was investigated. Figure 4 showed that *ccpA* and *spo0A*

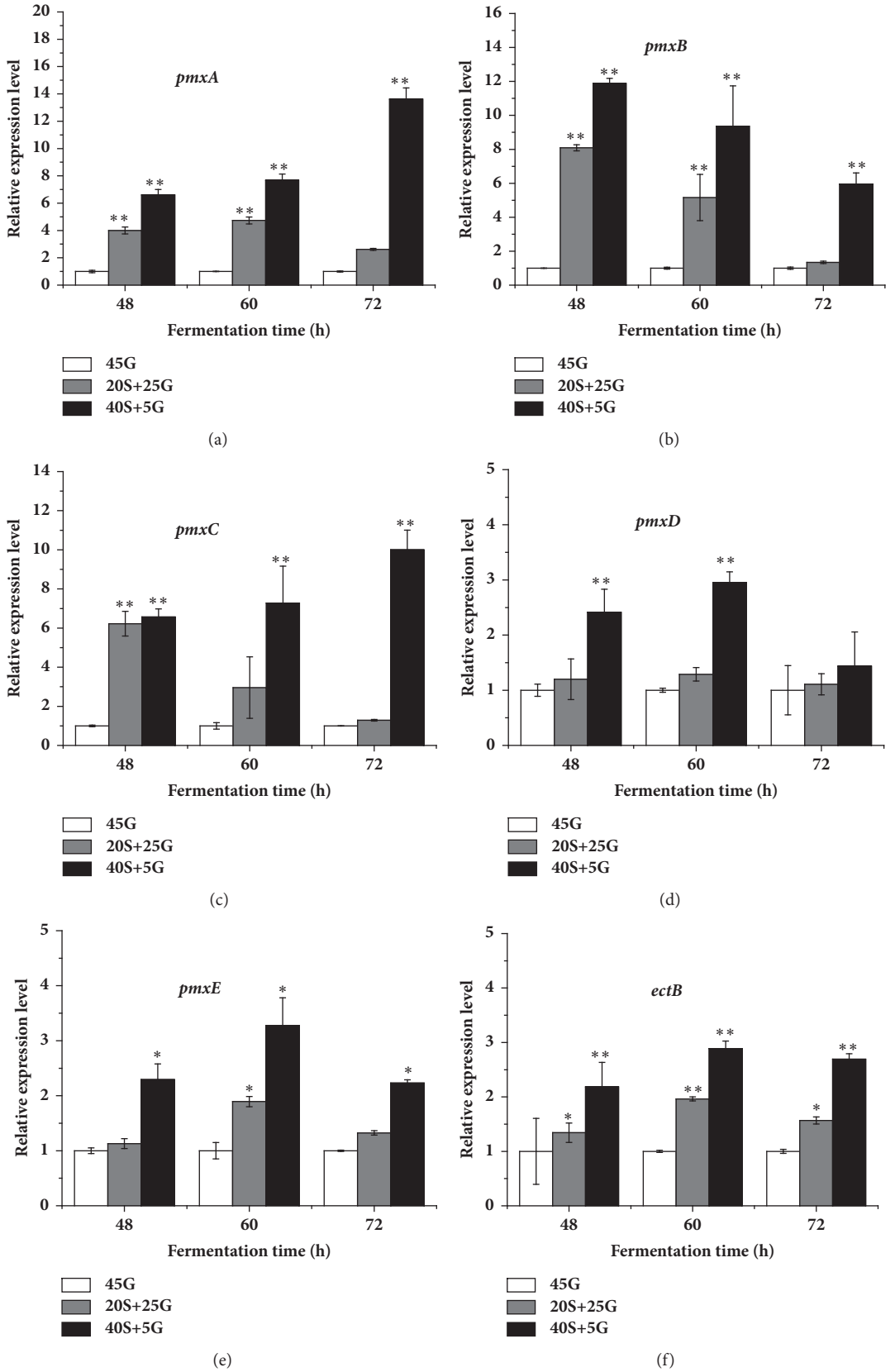

Figure 5: Continued.

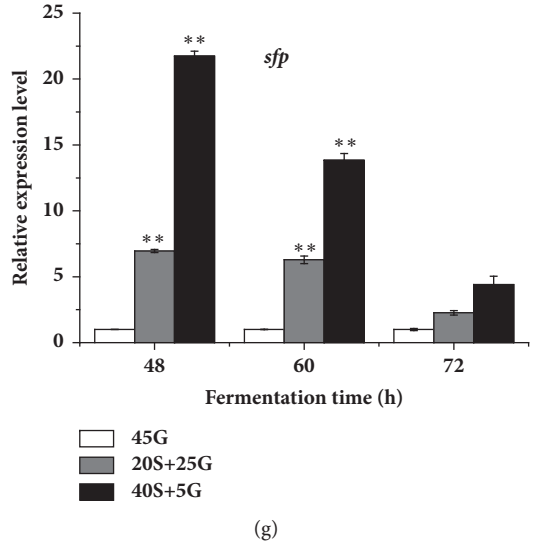

Figure 5: Effect of replacement of glucose by starch on relative expression level of genes directly involved in colistin biosynthesis and secretion. (a) *pmxA*; (b) *pmxB*; (c) *pmxC*; (d) *pmxD*; (e) *pmxE*; (f) *ectB*; (g) *sfp*. 45G: 45 g/L glucose; 20S+25G: 20 g/L starch plus 25 g/L glucose; 40S+5G: 40 g/L starch plus 5 g/L glucose. The statistically significant results are related to the condition of 45 g/L glucose.

gave overall similar patterns in relative gene expression. The higher the original concentration of glucose in fermentation medium was, the lower the relative expression of either *ccpA* or *spo0A* was. Interestingly, *abrB* had the opposite pattern in relative gene expression. The higher the original concentration of glucose in fermentation medium was, the higher the relative expression of *abrB* was. All these results indicated that the replacement of glucose by starch can stimulate the expression of both *ccpA* and *spo0A* but repress the expression of *abrB*, which in turn stimulates colistin production (Figure 1).

3.5. Effect of Replacement of Glucose by Starch on the Relative Expression of Genes Directly Involved in Colistin Biosynthesis. As shown above, the replacement of glucose by starch can promote the relative expression of genes associated with regulation of colistin production. Therefore, the relative expression levels of genes directly involved in colistin biosynthesis and secretion were examined. Figure 5 showed that the replacement of glucose by starch stimulated the relative expressions of *pmxABCDE*, *ectB*, and *sfp*. The more the glucose was replaced, the higher the relative expressions of those genes were, indicating that the replacement of glucose by starch can promote the expression of those genes and in turn increased colistin production (Figure 1).

4. Discussion

Colistin is broadly used to treat the infection of Gram-negative pathogens, particularly prevalent multidrug-resistant bacteria. It is produced by *P. polymyxa*. To date, very few reports dealt with the medium optimization for improvement of colistin production. In the present study, the effect of replacement of glucose by starch in fermentation medium on colistin production as well as transcription level of colistin biosynthesis-related genes was investigated. It was found that addition of starch could improve the production and biosynthesis rate of colistin (Figure 1). Moreover, the improvement extent was positively correlated with the amount of glucose replaced by starch (Figure 1). Our data further showed that the replacement of glucose by starch could refine two important fermentation factors, residue reducing sugar and pH (Figure 3). It seems that low concentration of residue reducing sugar and pH is better for colistin biosynthesis in *P. polymyxa* (Figure 1), but the detailed correlation mechanism needs to be further explored.

It has been found that the use of glucose represses the amylase activity and sporulation [36]. A report has shown that, relative to other carbon sources, glucose causes the strongest CCR, reducing the production of secondary metabolite [37]. It has been revealed that CCR is achieved by the global transcription regulator CcpA. The expression of *ccpA* results in the reduction of CCR [22]. Therefore, CcpA positively regulates secondary metabolism [23]. Our results showed that the replacement of glucose by starch could increase the relative expression of *ccpA* (Figure 4). As a result, the replacement of glucose by starch increased the transcription of *amyE* and amylase activity (Figure 2), which in turn is probably conducive to colistin (secondary metabolite) accumulation in *P. polymyxa* (Figure 1). Therefore, our findings are in line with the reports [22, 23, 36].

It has been demonstrated that Spo0A positively regulates secondary metabolism [38]. Our results indicated that the use of starch enhanced the relative expression level of *spo0A* (Figure 4) and subsequently increased colistin production (Figure 1), suggesting that *spo0A* also positively affects colistin biosynthesis in *P. polymyxa* at the transcriptional level. Therefore, our findings are in line with the report [38]. It has been found that the expression of *abrB* is negatively regulated

by Spo0A [15]. Our results also indicated that the relative expression level of *abrB* decreased with the increase of *spo0A* expression (Figure 4). There is evidence to show that AbrB negatively regulates colistin biosynthesis by directly binding to the upstream region of *pmxA* [11]. Thus, the decrease of *abrB* expression by adding starch (Figure 4) enhanced the relative expression of *pmxABCDE*, a gene cluster for colistin biosynthesis (Figure 5). As a result, colistin accumulation increased (Figure 1).

Data Availability

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

Conflicts of Interest

The authors have declared that no conflicts of interest exist.

Authors' Contributions

Zhiliang Yu and Juanping Qiu contributed equally to this work.

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Research Article

Inoculation with Efficient Nitrogen Fixing and Indoleacetic Acid Producing Bacterial Microsymbiont Enhance Tolerance of the Model Legume *Medicago truncatula* to Iron Deficiency

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The aim of this study was to assess the effect of symbiotic bacteria inoculation on the response of *Medicago truncatula* genotypes to iron deficiency. The present work was conducted on three *Medicago truncatula* genotypes: A17, TN8.20, and TN1.11. Three treatments were performed: control (C), direct Fe deficiency (DD), and induced Fe deficiency by bicarbonate (ID). Plants were nitrogen-fertilized (T) or inoculated with two bacterial strains: *Sinorhizobium meliloti* TII7 and *Sinorhizobium medicae* SII4. Biometric, physiological, and biochemical parameters were analyzed. Iron deficiency had a significant lowering effect on plant biomass and chlorophyll content in all *Medicago truncatula* genotypes. TN1.11 showed the highest lipid peroxidation and leakage of electrolyte under iron deficiency conditions, which suggest that TN1.11 was more affected than A17 and TN8.20 by Fe starvation. Iron deficiency affected symbiotic performance indices of all *Medicago truncatula* genotypes inoculated with both *Sinorhizobium* strains, mainly nodules number and biomass as well as nitrogen-fixing capacity. Nevertheless, inoculation with *Sinorhizobium* strains mitigates the negative effect of Fe deficiency on plant growth and oxidative stress compared to nitrogen-fertilized plants. The highest auxin producing strain, TII7, preserves relatively high growth and root biomass and length when inoculated to TN8.20 and A17. On the other hand, both TII7 and SII4 strains improve the performance of sensitive genotype TN1.11 through reduction of the negative effect of iron deficiency on chlorophyll and plant Fe content. The bacterial inoculation improved Fe-deficient plant response to oxidative stress via the induction of the activities of antioxidant enzymes.

1. Introduction

In plants, iron (Fe) plays an essential role in biochemical processes and plant metabolism such as respiration, photosynthesis, hydroxylation, nitrogen assimilation, symbiotic nitrogen fixation, and regulation of protein stability and as cofactors that carry out electron transfer functions [1, 2]. Despite the abundance of iron in calcareous soil, the bioavailability of Fe can be very low due to high pH and alkaline conditions [3, 4]. Abundance of iron-deficient calcareous soils severely affects plant growth and crop yield adversely [5, 6]. Calcareous soils cover major cultivated land of south Mediterranean lands, which decreased crop growth and yield under low Fe availability. A lot of research showed that the most obvious effect of Fe deficiency is a decrease in the amount of chlorophyll pigments [7, 8]. Therefore, there is a close relationship between plant growth and photosynthesis [7, 9]. In fact, Mann [8] and Ren [9] showed that plants responded strongly to iron deficiency in physiological traits where chlorophyll content and plant biomass were reduced [10]. Hence, in calcareous soils, cultivating Fe-efficient plants is important for maintaining yields while enhancing environmental sustainability.

The Fabaceae family is an important source of proteins [11] providing on average 33% of humans' dietary nitrogen that can reach up to 60% in developing countries [12].

Legumes establish symbiotic interactions with rhizobia leading to the formation of nitrogen-fixing nodules [13]. Nutrient deficiencies such as Fe and P are considered as a critical constraint for the nitrogen fixing and nodule [14–16]. Several studies have shown that the legume–rhizobia symbiosis is particularly sensitive to Fe deficiency [17–19]. In fact, Fe starvation limits root nodule bacterial survival and multiplication, as well as host-plant growth, nodule initiation, and development [20] since it is required for some key proteins involved in nitrogen fixation like nitrogenase, nitrogenase reductase, and leghemoglobin [21].

Indeed, Fe was implicated in cellular reactions of detoxification as a heme moiety, in several antioxidant enzymes like catalases (CAT) and peroxidases (POX), which both ensure the reduction of H_2O_2, and as metal cofactor in Fe-superoxide dismutase (Fe-SOD) which converts the superoxide anion to H_2O_2. Several research works showed that the induction and protective role of antioxidant enzymes activities under abiotic stress were widely ameliorated in legume-rhizobia by the increase of activities of antioxidant enzymes in nodules which protected biological nitrogen fixation [22–26].

Medicago truncatula represents an ideal model legume for the investigation of principal tolerance mechanisms to different environmental constraints such as drought, salt stress [14, 15, 17], and pathogenic agents [27, 28] and for understanding plant–bacteria interactions. Numerous studies of molecular, genetic, proteomic, and physiological aspects have been focused on its symbiosis with *Sinorhizobium* [29, 30]. Therefore, to cope with abiotic stress, many strategies have been developed to improve crop productivity such as microbiological approach involving use of beneficial plant growth promoting rhizobacteria (PGPR) [31]. In fact, rhizobial inoculation has been found as an economical strategy that could produce yield of legumes equal to or better than nitrogen fertilization under drought stress [32] and many studies have reported that the presence and the performance of diverse rhizobial strains were found under stress [24, 33, 34]. It has been recognized that rhizobia could modulate the growth and development of legume crops under stress via antioxidant secretion, if used as an inoculant [26, 35].

In recent years, interest is increasing in the application of plant growth promoting rhizobacteria to ameliorate plant tolerance to abiotic stress and improve plant production. The identification of Fe deficiency tolerant rhizobia has the potential to promote calcareous soils agriculture. Our hypothesis states that plant inoculation could increase plant growth and vigor under Fe deficiency conditions using bacterial strains PGPR performances such as siderophore and auxin production. To date, according to our knowledge, the implication of rhizobia inoculation on *Medicago truncatula* tolerance to iron deficiency was not explored. For that, we considered this research to study the effect of iron deficiency stress on the behavior of different *M. truncatula–Sinorhizobium* associations and to investigate the possibility of improving the tolerance of *Medicago truncatula* to iron deficiency stress by specific adapted microsymbiont inoculation.

2. Material and Methods

2.1. Bacterial Strains and Growth Conditions. Two strains belonging to the *Sinorhizobium* genus *Sinorhizobium meliloti* (TII7) and *Sinorhizobium medicae* (SII4) [36, 37] from laboratory collection were analyzed to compare their behavior and interaction with *Medicago truncatula* plants under iron deficiency conditions. Bacteria were grown in liquid yeast extract mannitol medium (YEM) [38] in an incubator with controlled growth conditions (150 rpm, 28°C) to obtain a final concentration of 10^9 cfu ml^{-1}.

2.2. Production of Indoleacetic Acid (IAA). To assess the IAA production by bacterial strains, a colorimetric method described by [39] and modified by [40] was used. The bacterial cultures were cultivated in minimal liquid medium for 48 h at 30°C [41]. Aliquots of 250 μl of the bacterial inoculum were used to inoculate 4 ml minimal liquid medium supplemented with tryptophan (0 or 250 μg ml^{-1}) and incubated at 30°C until the stationary phase was reached (48–72 h). The bacterial cells were obtained by centrifugation at 8500×g for 5 min, and then 1 ml of the supernatant was added to 200 μL orthophosphoric acid and 4 ml Salkowski reagent [42]. Following incubation at ambient temperature for 20–30 min, the optical density was measured at 535 nm. To calculate the concentration of IAA in each sample, a standard curve ranging from 0.01 to 100 μg ml^{-1} of pure IAA was used for comparison. According to the amount of IAA produced, four distinct levels of IAA production, low production (<15 μg ml^{-1}), medium production (between 15 and 30 μg ml^{-1}), high production (between 30 and 45 μg ml^{-1}), and very high production (>45 μg ml^{-1}), were considered.

2.3. Siderophore Production. The production of siderophores has been demonstrated by the chromium azurol S test in agar medium. The agar media were prepared according to Schwyn and Neilands [43]: 1/10 of a CAS indicator solution and 9/10 of yeast morphology agar medium. Inoculation was performed by central sting of the agar plates Petri dish. After incubation at 37°C, a red-orange halo appears around the fungal colony attesting to the secretion of siderophores. Measuring the halo's diameter can establish a ration for each strain reflecting the amount of siderophores secreted. The yield of siderophores production (%Ys) was determined as [(halo diameter-colony diameter)/colony diameter] ∗ 100.

2.4. Plant Materials and Growing Condition. TN8.20, A17, and TN11.11 seeds were scarified and surface-disinfected (6 min) with sulfuric acid (H_2SO_4). After imbibition with distilled H_2O, seeds were kept at 4°C overnight in darkness. Then, seeds were germinated in Petri dishes for two days at 25°C as described by [29]. After germination, seeds were transferred in autoclaved Agir perlite moistened with distilled water for six days and then to a half-strength aerated sterile nutrient solution in growth boxes for seven days. Similar sized seedlings were selected and cultured as groups of eight plants in nutrient solution (5 L) as described by [44] and modified by [29], containing macronutrients $MgSO_4$ (1 mM), KNO_3 (24 mM), K_2SO_4 (0.7 mM), and $CaCl_2$ (1.65 mM) and

micronutrients as a mixture of salts: $MnSO_4$ (6.6 μM), $CuSO_4$ (1.56 μM), $ZnSO_4$ (1.55 μM), $(Na)_2MoO_4$ (0.12 μM), $CoSO_4$ (0.12 μM) 6, and H_3BO_3 (4 μM). Plants were inoculated with two rhizobial strains, TII7 and SII4, and the control plants were nitrogen-fertilized with KNO_3 as a source of nitrogen. Two inoculations were performed: the first was in Agir perlite and the second after the transfer in the growth boxes.

Three treatments were established as follows: control (C: 50 μM Fe(III)-EDTA), direct Fe deficiency (DD: 5 μM Fe(III)-EDTA), and induced Fe deficiency (ID: 50 μM Fe + 10 mM Bic). The solution was renewed every seven days. Plants were placed in a growth chamber for 21 days under controlled conditions (16/8 h light/darkness, temperature was 18°C in the dark with a relative humidity (RH) of 60% and 24°C in the light with a RH of 80% and photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}).

2.5. Plant Biomass. After 21 days in nutrient solution, plants were separated into shoots, roots, and nodules. Plant material was rinsed with distilled water. The dry weight of each part was determined after drying at 65°C until constant weight for three days and then was used for nutrient analysis. The remaining samples were fixed at -80°C until enzyme activity assays.

2.6. Acetylene Reduction Assay. Nitrogenase activity (E.C. 1.7.9.92) was monitored "in situ" by acetylene reduction assay (ARA) using gas chromatography with Porapak T column [45]. ARA measure was performed at the flowering stage corresponding to the optimal nodule activity. Nodule-bearing roots were incubated in 10% C_2H_2 atmosphere. After 60 min of incubation, the ethylene formation rate was measured using gaseous phase chromatography. Three replicates of 0.5 ml gas samples were withdrawn from the root atmosphere of each plant, and ethylene production was determined. Pure acetylene and ethylene were used as internal standards [29].

2.7. Extraction and Determination of Plant Iron Content. Dry matter of plant parts was weighed and then crushed after drying at 60°C for 72 h. Samples of 25 mg were placed in digestion tube and extracted with 20 ml of nitric acid and perchloric acid (2.5:1, v/v) and brought to 60°C on a hot plate until total desiccation. The solution was then distilled with 20 ml of HNO_3 (N/7). Finally, the mixture was filtered with Whatman paper. The obtained filtrates are used to determine the extractable iron by means of an atomic absorption spectrophotometer [46].

2.8. Chlorophyll Content. Three plants per treatment (with three replicates for each plant) were used to determine the total chlorophyll content of young leaves according to the method of Lichtenthaler [47] with some modifications. A hundred milligrams of small discs from young leaves was incubated in 5 ml 80% acetone in darkness at 4°C for three days (until complete chlorophyll extraction). Then, the total chlorophyll content was determined by a lecture of absorbance at 649 and 665 nm.

2.9. The Leakage of Electrolyte. Fragments of 100 mg of the middle part of the freshly cut leaves (three plants/treatment) were placed in assay tubes filled with 10 ml of ultrapure water (deionized). All the tubes were incubated in a water bath for two hours at 32°C. Then, the first electrical conductivity (EC1) of the solution was measured using a type of conductivity Metrohm 712. The same tubes were autoclaved at 121°C for 20 minutes. After cooling to 25°C, the second electrical conductivity (EC2) was measured. The leakage of electrolyte was measured following the formula PE = EC1 / EC2 $*$ 100 [48].

2.10. Lipid Peroxidation Assay (MDA). Lipid peroxidation was determined as described by Cakmak and Horst [49]. 0.2 g of fresh material (three plants/treatment) was homogenized in 4 ml of a 1% (w/v) solution of trichloroacetic acid (TCA). Then, the mixture was centrifuged at 12,000 g for 15 minutes. After that, we added 3 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) (TCA) to the collected supernatant and the tubes were incubated at 95°C for two hours. The reaction was stopped by placing the reaction tubes in an ice bath. Centrifugation was subsequently done at 9000 g for 10 min. The concentration of the malondialdehyde complex (MDA-) TBA was determined by measuring the optical density of the supernatant at 532 and 600 nm and was expressed using the molar extinction coefficient of 155 mM^{-1} cm.

2.11. Antioxidant Enzymes Assays. All operations were performed at 4°C to maintain enzyme activity. Extracts (three plants/treatment) were prepared by homogenizing 200 mg of roots, shoots, and nodules in a mortar with 10% (v/v) polyvinylpolypyrrolidone and 1 ml of phosphate buffer pH 7.8 (50 mM) containing 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride as protease inhibitor. Then, the obtained extracts were centrifuged at 13,000 g for 20 minutes and the supernatant was collected for enzymatic activities. Protein content of each sample was measured according to the method of Bradford [50].

Superoxide dismutase (SOD, EC, 1.15.1.1) activity was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm [51].

Peroxidase (POX, EC 1.11.1.7) activity was determined by the measurement of the kinetic evolution of tetraguaiacol formation from guaiacol (9 mM) at 470 nm for 1 min with the extinction coefficient (e = 26.6 mM^{-1} cm^{-1}) in the presence of 19 mM H_2O_2 [52].

Catalase (CAT, EC 1.11.1.6) activity was measured by following the decline in absorbance at 240 nm caused by the catabolization of H_2O_2 (10 mM) for 3 min (e = 36 M^{-1} cm^{-1}) [53].

2.12. Statistical Analysis. Variance analysis of data (three-way ANOVA) was performed using the SPSS 18 program, and means were separated according to the HSD Tukey test at $P \leq 0.05$. Data shown are means of three replicates for each treatment.

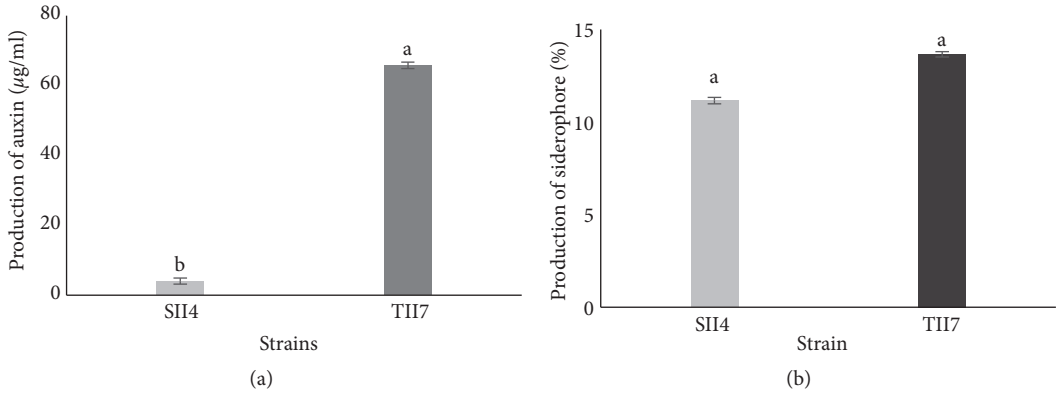

Figure 1: Production of IAA (a) and siderophore (b) of two rhizobial strains: TII7 and SII4.

3. Results

3.1. Strains: Production of Indoleacetic Acid (IAA) and Siderophore. The results of Figure 1 showed that tested bacterial strains were positive for the production of IAA and showed the ability of synthesis of this phytohormone. Our results showed a clear difference between the two strains in IAA production. In fact, higher IAA concentration was produced by TII7 (65,84 μg/ml) comparing to SII4 strain (4,04 μg/ml). In addition, both TII7 and SII4 were determined as siderophore producers.

3.2. Effect of Inoculation on Plant Responses to Iron Deficiency. The analysis of variance for plant growth, root weight and length, iron content, and chlorophyll concentration showed a significant effect for three considered factors strains, genotypes, and treatment and their interaction (Table 1). We noted a high contribution of strains effect to the variance of shoots content of iron and the effect was strongest for chlorophyll content. However, for shoots and roots weight, roots lengths, and roots content of iron, genotype showed the major effect. The treatment effect was manifested remarkably in the variance of shoots content of iron. The interaction effect was lowest for these parameters.

3.2.1. Plant Growth and Root Morphology. Results presented in Figure 2(a) showed that iron deficiency affected plant growth of *Medicago truncatula* genotypes. This fact was more detected in TN1.11 genotype under both ID and DD conditions. However, the inoculation of Fe-deficient plants with TII7 and SII4 markedly alleviated the negative effect of iron deficiency on plant growth. As shown in Figure 2(a), this improving effect is clearly identified in A17 and TN8.20 in which we have even observed an improvement in plant deficient growth inoculated with TII7 (-5,2% and -8,3, respectively) compared to the controls (-31% and -26%, respectively). On the other hand, the symbiosis TN1.11-SII4 was found effective in improving the tolerance of this sensitive genotype to iron deficiency. Based on the results of Figure 2(b), we can notice that, under Fe deficiency conditions, the inoculation improved root biomass and length in *Medicago truncatula* genotypes compared to noninoculated plants; this enhancement is more spectacular in plants inoculated with TII7 than those inoculated with SII4. In TN1.11 Fe-deficient plants, the inoculation with SII4 improved plant growth, whereas that with TII7 increased roots length (Figure 2(c)).

3.2.2. Iron Determination. Results presented in Figure 3(a) showed that shoots Fe concentrations were significantly reduced by iron deficiency conditions mainly in A17 and TN1.11 genotypes. Nevertheless, this negative effect is alleviated for the association A17-TII7 cultivated in iron deficiency conditions (DD) by 42% compared to the control. The same behavior was observed for the TN1.11 one inoculated by the two strains TII7 and SII4. Contrary, the inoculation of TN8.20 plants leads to a decrease in shoots Fe content as compared to noninoculated plants. The same responses were observed in roots (Figure 3(b)); we can notice that the inoculation ameliorates roots Fe concentrations under iron deficiency in the association A17-TII7 and in TN1.11-SII4.

3.2.3. Chlorophyll Determination. According to Figure 3(c), iron deficiency caused a significant reduction in leaves chlorophyll content of *Medicago truncatula* genotypes. Considering the genotypic variability, we noted that TN1.11 is significantly affected by this nutritional stress. Under DD treatment, the decrease in chlorophyll content can reach - 41% in TN1.11 while it does not exceed 35% in TN8.20 and 30% in A17. The inoculation moderated the observed decrease in chlorophyll content in all studied genotypes. For example, the reduction of total leaf chlorophyll content became less than -20% in A17, -30% in TN8.20, and -15% in TN1.11 inoculated with TII7 under DD treatment.

3.3. Effect of Iron Deficiency on Nodulation and Nitrogen-Fixing Capacity (ARA). As shown in Figure 4, iron deficiency affected the number and the biomass of nodules in *Medicago truncatula* genotypes inoculated with TII7 and SII4. The effect of iron deficiency on nodulation depends on genotype and strain. The analysis of our results lets us deduce that the number and the weight of nodules were higher in the associations A17-TII7 and TN8.20-TII7 which exceed 25% and 22%, respectively, compared to A17 and TN8.20 genotypes inoculated with SII4 and in TN1.11 inoculated with SII4

Table 1: Results of three-way analysis of the effect of strain (S), genotypes (G), and treatment (T) and their interaction (S∗T∗G) on shoots weight (ShW: g plant^{-1}), roots weight (RW: g plant^{-1}), roots length (RL, cm plant^{-1}), content of iron in shoots (Fe Sh: mg g^{-1} plant) and roots (Fe R: mg g^{-1} plant), chlorophyll (Chl: mg g^{-1} FW), and antioxidant enzyme activities in shoots (SOD Sh (USOD 10^3 μg^{-1} protein), CAT Sh (mM H_2O_2 min^{-1} mg^{-1} protein), and POX Sh (mM H_2O_2 min^{-1} mg^{-1} protein)) and roots (SOD R: USOD 10^3 μg^{-1} protein, CAT R (mM H_2O_2 min^{-1} mg^{-1} protein), and POX R (mM H_2O_2 min^{-1} mg^{-1} protein)) in *Medicago truncatula* genotypes.

	Sh W	RW	LR	Fe Sh	Fe R	Chl	SOD Sh	SOD R	CAT Sh	CAT R	POX Sh	POX R
S	48,4**	1588***	33,2***	247,4***	698,8***	929***	173***	133,6***	34,7***	104,4***	7,47*	19,81***
G	785**	2946,3**	715,9**	171,9**	783,3**	174**	6,25^{ns}	51,52**	89,68**	167,**	93,58**	88,78**
T	135,2**	2,68^{ns}	122,6**	194,4**	215,2**	315**	63,9**	5,58**	81,37**	5,29*	0,52^{ns}	49,26**
S∗G	108**	260**	48**	390**	515**	52**	15**	38**	72**	31**	32**	100**
S∗T	10**	17**	20**	21**	225**	52,6***	66**	12,8**	40**	29,6**	9,36**	8,6^{ns}
G∗T	14,25***	26,7***	78***	26,3***	527,4***	11***	9,25***	7,24**	30,69***	19,12***	4,59**	18,8***
S∗G∗T	3,7^{ns}	7,54**	13,4**	12,8**	314,2**	12,8***	3,69**	6,31**	7,74**	44,22**	14,65**	88,5**

Numbers represent F-value: $^{***}P<0.0001$, $^{**}P<0.001$, and $^{*}P<0.01$.
ns: nonsignificant.

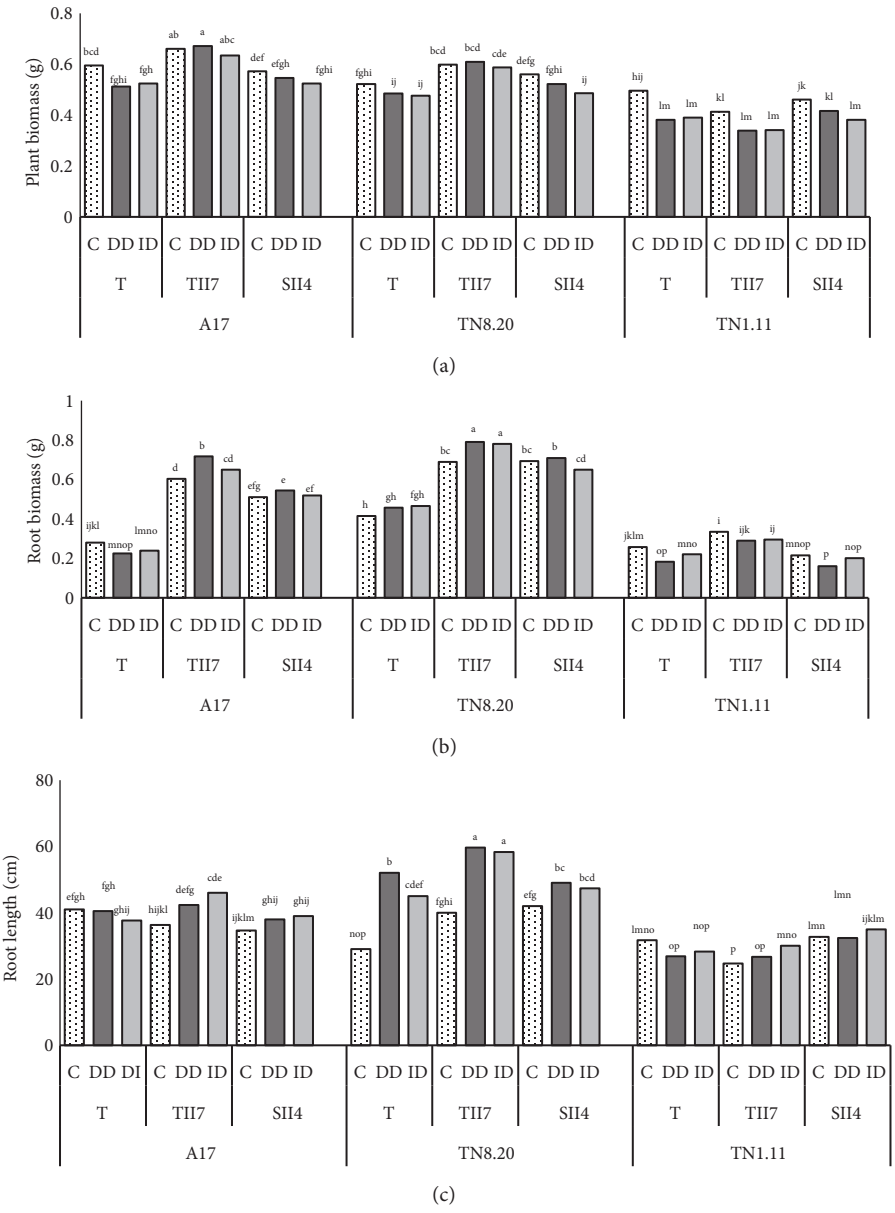

Figure 2: Plant biomass (a), root biomass (b), and root length (c) in *Medicago truncatula* genotypes. T: plants nitrogen-fertilized control, TII7: inoculated with TII7, and SII4: inoculated with SII4, growing in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Graphs denoted with different small letters are significantly different according to the Tukey test.

(42% compared to TN1.11 inoculated with TII7). Moreover, this dramatic effect of iron starvation in number and weight of nodules was concomitant with a decline in nitrogen-fixing capacity (ARA) in Fe-deficient plants. This reduction of nitrogen fixing is more important in TN8.20 inoculated with TII7 (ID) and TN1.11 inoculated with SII4 (DD).

3.4. Leakage of Electrolyte and Membrane Damage. Under iron deficiency conditions, a significant increase in leakage of electrolytes was noted in noninoculated plants of A17, TN8.20, and TN1.11 genotypes (Figure 5(a)). However, the observed increase was mitigated by plants inoculation. It is interesting to note that the best results were observed in plants inoculated with TII7. Indeed, the results showed a significant decrease of leakage of electrolytes for the association A17-TII7 (-3,2%) compared to A17-SII4 (27%) and noninoculated plants (53%) cultivated in iron deficiency conditions (DD). In the same way, Figures 5(b) and 5(c) showed similar findings concerning the beneficial effect of inoculation on shoots and roots MDA concentrations.

3.5. Antioxidant Enzyme Responses to Iron Deficiency. The analysis of variance for the antioxidant enzyme activities (SOD, CAT, and POX) showed a significant effect for the considered factors: strains, genotypes, and treatment and their interaction (Table 1). The importance of the effect varied

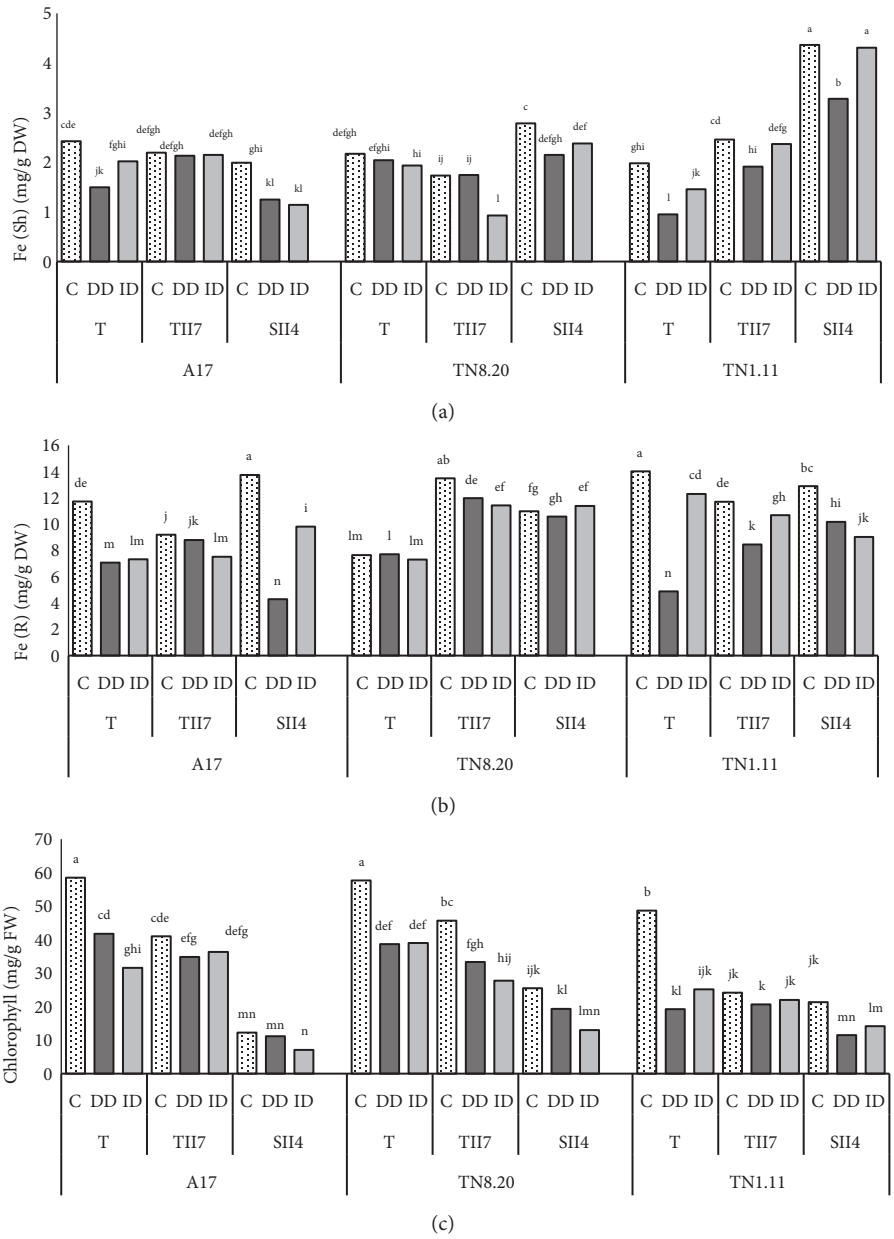

Figure 3: Iron content in shoots (a) and roots (b) and chlorophyll concentration (c) in *Medicago truncatula* genotypes. T: plants nitrogen-fertilized control, TII7: inoculated with TII7, and SII4: inoculated with SII4, growing in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Graphs denoted with different small letters are significantly different according to the Tukey test.

according to the parameter analyzed. We noted a high contribution of strains and genotypes in the variance of these activities mainly in the SOD case.

SOD activity was increased in Fe-deficient plants for all studied genotypes (Table 2). The most pronounced induction was significantly observed in the shoots of the associations A17-TII7 and TN8.20-TII7 compared to A17-SII4 and TN8.20-SII4 (Table 3). However, TN1.11 plants cultivated under DD treatment and inoculated with SII4 showed the highest values of SOD activity. In roots, the best results were found in Fe-deficient TN8.20 plants inoculated with TII7 (+ 76%) compared to those inoculated with SII4 (+ 30%). In nodules, direct iron deficiency stimulated SOD activity in the associations A17-TII7 and TN8.20-TII7. Nevertheless, in the case of TN1.11 plants, the highest increase was found in plants inoculated with SII4.

As compared to control, shoots CAT activity was significantly stimulated by inoculation. The detected increase is more pronounced in A17, TN8.20, and TN1.11 inoculated by TII7 than by SII4 (204%, 353%, and 552%, respectively) under direct iron deficiency. In roots, CAT activity was stimulated in A17 and TN8.20 inoculated with the two strains and we can

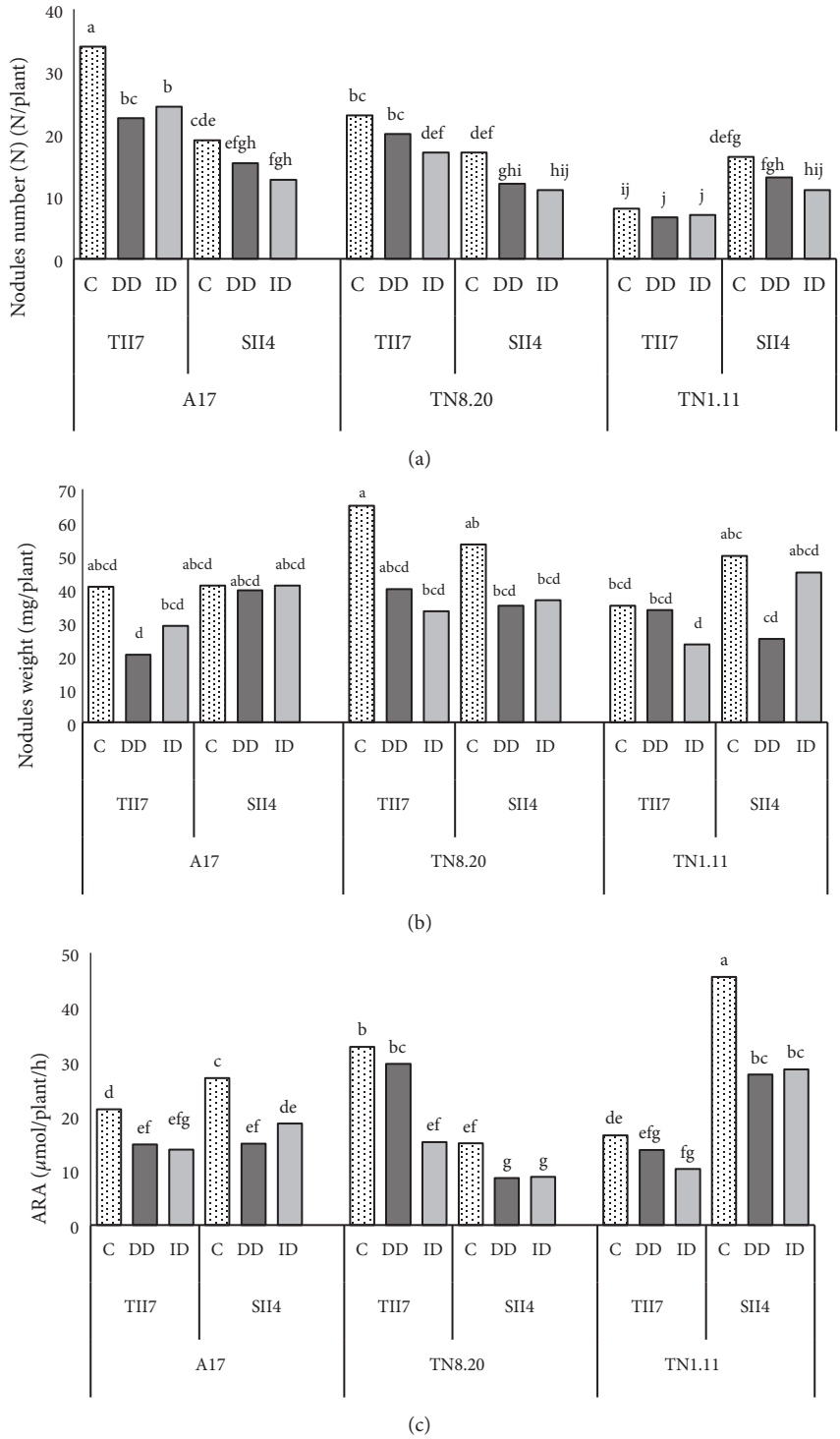

Figure 4: Nodules number (a), nodules weight (b), and acetylene reduction assay (ARA) (c) of *Medicago truncatula* genotypes grown in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Graphs denoted with different small letters are significantly different according to the Tukey test.

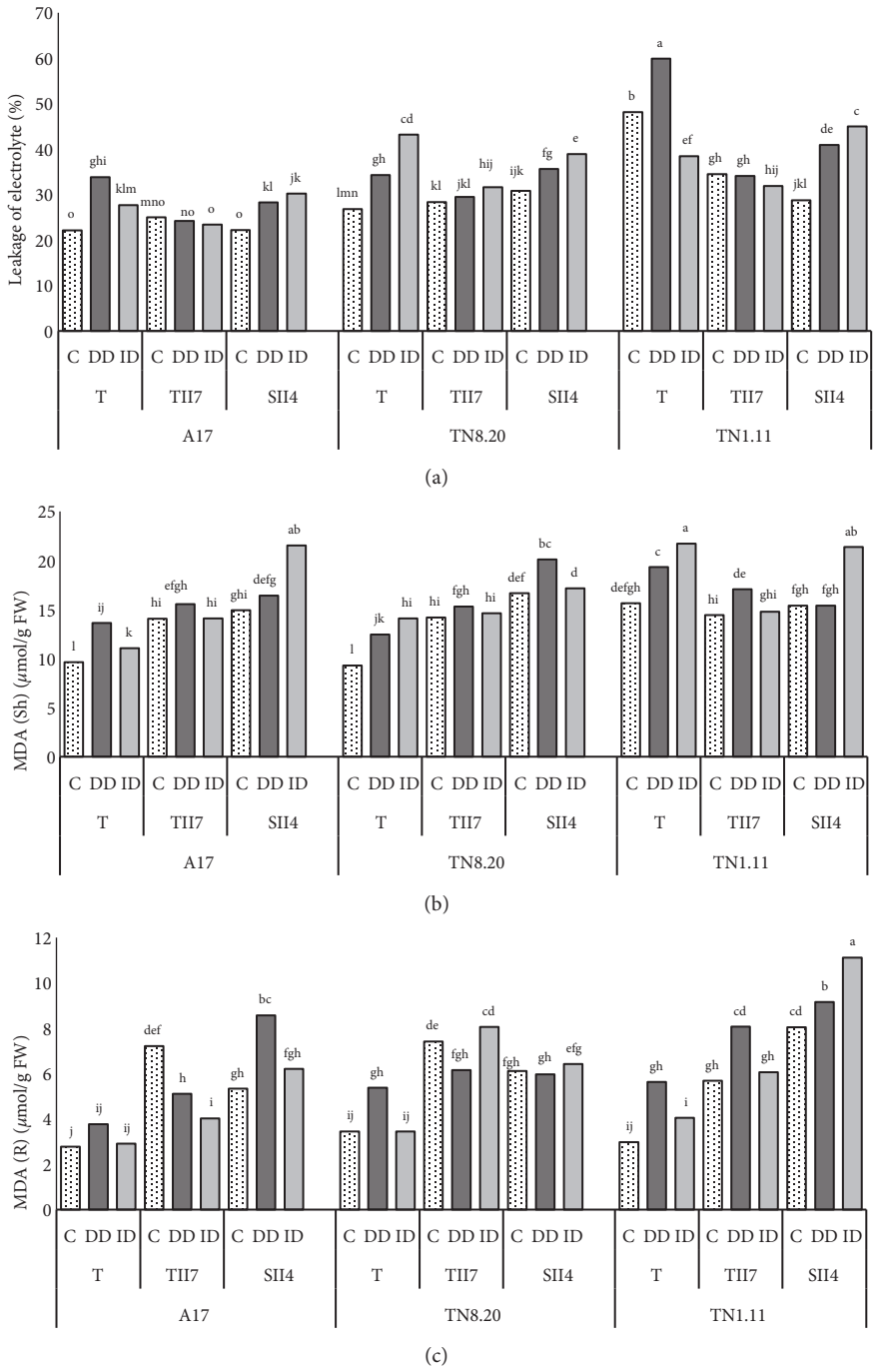

Figure 5: Electrolyte leakage (a), MDA shoots (b), and MDA roots (c) of *Medicago truncatula* genotypes. T: plants nitrogen-fertilized control, TII7: inoculated with TII7, and SII4: inoculated with SII4, growing in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Graphs denoted with different small letters are significantly different according to the Tukey test.

notice no significant change in TN1.11 genotype. In nodules, CAT activity was stimulated by iron deficiency in deficient plants inoculated with TII7.

Iron deficiency conditions stimulated POX activity in A17 and TN8.20 shoots inoculated with SII4, whereas, in the case of TN1.11, this simulation is detected in Fe-deficient plants inoculated with TII7. In roots, TN8.20 showed a similar behavior compared to leaves. However, in A17, the increase of POX was more spectacular in plants inoculated with TII7 strain (six times greater compared to control under ID

Table 2: Superoxide dismutase (SOD: USOD 10^3 μg^{-1} protein), catalase (CAT: mM H_2O_2 min^{-1} mg^{-1} protein), and guaiacol peroxidase (POX: mM H_2O_2 min^{-1} mg^{-1} protein) activities in shoots (Sh) and roots of nitrogen-fertilized plants grown in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Values denoted with different small letters are significantly different according to the Tukey test.

Genotypes	Treatment	SOD		CAT		POX	
		Sh	R	Sh	R	Sh	R
A17	C	$17{,}4^{c}$	$61{,}6^{ab}$	$2{,}15^{e}$	243^{a}	$2{,}13^{ef}$	10^{c}
	DD	$11{,}8^{c}$	93^{a}	$2{,}17^{e}$	161^{b}	$1{,}48^{ef}$	$22{,}5^{a}$
	ID	$17{,}9^{c}$	91^{a}	$7{,}23^{e}$	108^{d}	1^{ef}	$10{,}4^{c}$
TN8.20	C	$19{,}15^{c}$	$44{,}1^{bc}$	$9{,}19^{e}$	$22{,}8^{e}$	$0{,}6^{f}$	$3{,}76^{de}$
	DD	$16{,}71^{c}$	84^{ab}	$10{,}5^{e}$	$30{,}5^{e}$	$0{,}46^{f}$	$5{,}68^{d}$
	ID	$17{,}09^{c}$	89^{ab}	11^{e}	109^{cd}	$0{,}32^{f}$	15^{b}
TN1.11	C	$17{,}8^{c}$	83^{ab}	$2{,}64^{e}$	$125{,}6^{bcd}$	$0{,}38^{f}$	$13{,}02^{bc}$
	DD	$16{,}4^{c}$	91^{a}	$2{,}86^{e}$	153^{bc}	$0{,}37^{f}$	$19{,}5^{a}$
	ID	$20{,}2^{c}$	68^{ab}	$3{,}66^{e}$	$24{,}5^{e}$	$0{,}63^{ef}$	13^{bc}

treatment). POX activity in nodules increased significantly in response to iron deficiency in TN8.20 inoculated with TII7 (six times greater compared to control).

4. Discussion

In the present work, the response of three genotypes of *Medicago truncatula* to iron deficiency was investigated under inoculation conditions. The inoculation was done with two strains: TII7 and SII4. Cultivated in Fe deficiency medium, plants inoculated with TII7 produced the highest plant biomass and root biomass and length. Our results show that rhizobial inoculation of Fe-deficient plants enhanced their biomass production. Documented with several other species [54–56], the plants inoculated with rhizobia showed an increase of growth parameters compared to noninoculated plants. Moreover, it was showed that rhizobial inoculation increased the tolerance of plants under several stressful conditions [24, 57]. This improving effect was due to rhizobial partner. Our results ensure that TII7 and SII4 were able to excrete siderophore and to produce indolic compound (IAA) (Figure 1). The AIA differential production levels between strains can explain the observed improvement of Fe-deficient plant growth, root morphology, and Fe content. Many studies demonstrated that rhizobacteria produced siderophores that can affect the availability and mobility of Fe [58]. In fact, rhizobacteria are characterized by their abilities to take up siderophores de novo synthesized and released [59]. The effectiveness of this ability provides a competitive advantage and confers the efficiency of the resulting symbiosis [18]. Also, IAA is generally known as a growth phytohormone that is a principal regulator of root expansion [60], regulation of root cell elongation and development [61, 62], and functionality in terms of mineral nutrition [63, 64].

A reduction of chlorophyll content was apparent in leaves of *Medicago truncatula* genotypes submitted to DD treatment. The inoculation of Fe-deficient plants mitigated this negative effect only in A17 and TN1.11 and the best results were obtained with the TII7 strain. The same behavior was observed for Fe concentrations; the symbiosis A17/TN1.11-TII7 proved effective in overcoming the negative effect of iron deficiency of Fe content. Velázquez-Becerra and del Carmen Orozco-Mosqueda [65, 66] showed that volatile organic compounds produced by rhizobacteria may serve as signal molecules that induce plant growth by the stimulation of its iron-uptake mechanisms.

In *Medicago truncatula* genotypes, iron deficiency affected nodules number and biomass as well as the nitrogenase activity (detected by the reduction of acetylene reduction activity (ARA)). Our results are confirmed with several other studies, which showed that stressful conditions affect nodule performance. In fact, Slatni [57, 67] and Quin [68] showed that iron deficiency affected soybean nodule performance. Likewise, Mhadhbi [69] found that salt and drought stress inhibited the nitrogen-fixing activity in *Medicago truncatula* genotypes. The decrease in nodule performance observed in Fe-deficient plants can be explained by the fact that iron is an essential mineral for nodule development as it is essential for the activity of nitrogenase and leghemoglobin [70] and through nodule formation, the availability and distribution of iron within the nodule change the role of the symbiotic organ. Rodríguez-Haas [71] controlled the iron distribution in *Medicago truncatula* nodules and their results improved the process of uptake of iron from the rhizosphere and enhanced the hypothesis concerning iron movement within the nodule.

Iron deficiency generates the production of reactive oxygen species (ROS) which in turn induced oxidative stress [72]. Our results showed an increase of leakage of electrolyte and MDA in plants cultivated in Fe-deficient medium. The inoculation by TII7 and SII4 mitigated this increase. According to our results, we can suggest that the decrease in MDA and electrolyte leakage observed in inoculated plants was mainly related to the stimulation of antioxidant enzyme activities (SOD, CAT, and POX). Several works showed that, under stress conditions, the rapid elimination of excessive ROS is essential for the proper functioning of cells and survival of organisms [23]. Our results showed that the activities

Table 3: Superoxide dismutase (SOD: USOD 10^3 μg^{-1} protein), catalase (CAT: mM H_2O_2 min^{-1} mg^{-1} protein), and guaiacol peroxidase activities (POX: mM H_2O_2 min^{-1} mg^{-1} protein) in shoots (Sh), roots (R), and nodules (N) of *Medicago truncatula* genotypes(G1:A17, G2:TN8.20, and G3: TN1.11) inoculated with two strains (S1: TII7 and S2: SII4) growing in the presence of Fe (C), in the direct iron deficiency (DD), and in the induced iron deficiency (ID) during the treatment period (21 days). Values are the means of eight replicates ± SD at $P<5\%$. Graphs denoted with different small letters are significantly different according to the Tukey test.

		SOD			CAT			POX		
		Sh	R	N	Sh	R	N	Sh	R	N
G1S1	C	$18,3^{bcd}$	84^{efgh}	$38,91^{cd}$	$4,9^{efgh}$	$21,3^{fgh}$	$36,7^{def}$	$0,77^{cde}$	$2,69^{f}$	$2,8^{cde}$
	DD	$37,62^{a}$	94^{defgh}	100^{a}	$4,9^{efgh}$	$36,4^{efghi}$	182^{a}	$0,73^{cde}$	13^{bc}	$21,8^{a}$
	ID	$22,8^{b}$	148^{cdef}	110^{a}	$5,16^{efgh}$	$114,4^{b}$	$101,7^{c}$	$0,97^{bc}$	$7,9^{cdef}$	$0,33^{e}$
G1S2	C	$15,3^{bcd}$	95^{defgh}	$37,3^{cd}$	$2,48^{fgh}$	$68,9^{cd}$	$19,6^{ghi}$	$0,71^{cde}$	$3,7^{ef}$	$2,55^{cde}$
	DD	20^{bc}	75^{fgh}	$35,7^{bc}$	$7,5^{def}$	$62,2^{cdef}$	$24,4^{fg}$	$0,87^{cd}$	$9,2^{cde}$	2^{cde}
	ID	$20,5^{bc}$	125^{cdefg}	43,8	$9,2^{cde}$	236^{a}	$27,8^{efg}$	$1,59^{a}$	$27,6^{a}$	$2,6^{cde}$
G2S1	C	$12,7^{cde}$	190^{c}	$18,4^{fg}$	$2,8^{fgh}$	$23,23^{efgh}$	$10,5^{hij}$	$0,79^{cde}$	$10,8^{bcd}$	$1,2^{de}$
	DD	$16,5^{bcd}$	288^{ab}	107^{a}	$12,8^{bc}$	$12,8^{gh}$	$36,3^{def}$	$1,35^{ab}$	$16,2^{b}$	$8,8^{b}$
	ID	$5,37^{e}$	328^{a}	$81,2^{b}$	$5,84^{defg}$	$21,4^{fgh}$	41^{de}	$0,79^{cde}$	$28,1^{a}$	$2,7^{cde}$
G2S2	C	$22,3^{b}$	173^{cde}	$43,1^{bc}$	$9,9^{cd}$	$20,6^{gh}$	$28,4^{efg}$	$0,43^{e}$	$29,7^{a}$	$4,96^{c}$
	DD	$42,8^{a}$	211^{bc}	36^{bc}	$19,3^{a}$	$78,6^{bc}$	$28,7^{efg}$	$0,6^{cde}$	$33,9^{a}$	$2,9^{cde}$
	ID	21^{b}	45^{gh}	$7,4^{g}$	$4,68^{fgh}$	$13,6^{gh}$	$5,3^{j}$	$0,73^{cde}$	$9,4^{cde}$	2^{cde}
G3S1	C	$12,9^{cde}$	177^{cd}	$44,2^{bc}$	$7,65^{def}$	$9,18^{h}$	$47,8^{d}$	$0,53^{de}$	$7,4^{cdef}$	$4,07^{cd}$
	DD	$11,9^{de}$	178^{cd}	$49,7^{c}$	$18,7^{a}$	$13,2^{gh}$	$17,42^{ghij}$	$0,45^{e}$	$7,1^{cdef}$	$2,5^{cde}$
	ID	12^{de}	205^{bc}	$36,7^{bc}$	$15,2^{ab}$	$15,3^{gh}$	166^{b}	$0,54^{de}$	$5,4^{def}$	$1,27^{de}$
G3S2	C	$22,21^{b}$	25^{h}	$30,9^{def}$	$1,43^{h}$	53^{cdefg}	$5,18^{j}$	$0,6^{cde}$	$6,3^{def}$	$2,7^{cde}$
	DD	$37,3^{a}$	34^{h}	$45,8^{bc}$	$4,3^{fgh}$	$64,19^{cd}$	$23,8^{fgh}$	$0,82^{cde}$	$5,7^{def}$	$2,04^{cde}$
	ID	$12,2^{de}$	40^{gh}	21^{efg}	$2,3^{gh}$	63^{cd}	$6,5^{ij}$	$0,43^{e}$	$10,8^{bcd}$	3^{cde}

of antioxidant enzymes in nodules were influenced by iron deficiency and this influence depends on the strain and the genotype.

In conclusion, we demonstrated that iron deficiency affected plant growth parameters of all analyzed *Medicago truncatula* genotypes. Nevertheless, a variability of response was revealed; TN1.11 was more affected than A17 and TN8.20. The inoculation of plants with the two *Sinorhizobium* strains TII7 (*S. meliloti*) and SII4 (*S. medicae*) ameliorates the tolerance of Fe-deficient plants to this nutritional stress. We even observed an improvement of the tolerance of the sensitive genotype TN1.11 to iron deficiency. This enhancement could be related to the capacity of inoculated strains to fix iron via the siderophore production and mainly to produce the auxin phytohormone that was highest within the TII7 strain that showed the best enhancing effect. Both strains stimulate the antioxidant enzyme activities in Fe-deficient plants which could protect deficient plants from the deleterious effect of reactive oxygen species generated under such constraints.

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 they have no conflicts of interest.

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Research Article

Evaluation of the Efficiency of Ethanol Precipitation and Ultrafiltration on the Purification and Characteristics of Exopolysaccharides Produced by Three Lactic Acid Bacteria

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Exopolysaccharides (EPS) produced by three Lactic Acid Bacteria strains, *Lactococcus lactis* SLT10, *Lactobacillus plantarum* C7, and *Leuconostoc mesenteroides* B3, were isolated using two methods: ethanol precipitation (EPS-ETOH) and ultrafiltration (EPS-UF) through a 10 KDa cut-off membrane. EPS recovery by ultrafiltration was higher than ethanol precipitation for *Lactococcus lactis* SLT10 and *Lactobacillus plantarum* C7. However, it was similar with both methods for *Leuconostoc mesenteroides* B3. The monomer composition of the EPS fractions revealed differences in structures and molar ratios between the two studied methods. EPS isolated from *Lactococcus lactis* SLT10 are composed of glucose and mannose for EPS-ETOH against glucose, mannose, and rhamnose for EPS-UF. EPS extracted from *Lactobacillus plantarum* C7 and *Leuconostoc mesenteroides* B3 showed similar composition (glucose and mannose) but different molar ratios. The molecular weights of the different EPS fractions ranged from 11.6±1.83 to 62.4±2.94 kDa. Molecular weights of EPS-ETOH fractions were higher than those of EPS-UF fractions. Fourier transform infrared (FTIR) analysis revealed a similarity in the distribution of the functional groups (O-H, C-H, C=O, -COO, and C-O-C) between the EPS isolated from the three strains.

1. Introduction

Exopolysaccharides (EPS) are long-chain polymers, industrially used as thickeners, stabilizers, and gelling agents in food products. More recently they were used as depollution agents and there was a growing interest in their biological functions like antitumor, antioxidant, or prebiotic activities [1]. Exopolysaccharides are produced by the metabolic processes of microorganisms such as bacteria, fungi, and blue-green algae [2]. Bacterial Exopolysaccharides are widely described in the literature, offering a wide range of biological and physicochemical properties.

Lactic Acid Bacteria (LAB) represent a natural source of EPS which play an important role in the rheological behavior and texture of fermented milks [3–5]. Most LAB producing EPS belong to the genera *Streptococcus*, *Lactobacillus, Lactococcus, Leuconostoc*, and *Pediococcus* [6].

EPSs from LAB can be classified into two groups: homopolysaccharides and heteropolysaccharides. Homopolysaccharides consist of repeating units of only one type of monosaccharide (D-glucose or D-fructose) and can be divided into two major groups: glucans and fructans. By contrast, heteropolysaccharides, produced by a great variety of mesophilic and thermophilic LAB, are formed by repeating units that most often contain a combination of D-glucose, D-galactose, and L-rhamnose and, in a few cases, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), or glucuronic acid (GlcA). Sometimes, noncarbohydrate substituent such as phosphate, acetyl, and glycerol are present. The molecular mass of these polymers ranges

between 40 and 6000 kDa [4]. Heteropolysaccharides from LAB demonstrate different structures [7]. The heteropolysaccharides are constructed from multiple copies of oligosaccharides, which contain between three and eight residues. Two or more different monosaccharides are usually present in each repeating unit and show different linkage patterns [8].

EPSs produced by LAB are in great variety, depending on LAB strains, culture conditions, and medium composition [9], and often differ by monosaccharides composition, charge, linkages between units, and presence of repeated side chains. The sugar components of EPS from LAB are most commonly galactose, glucose, and rhamnose [3]. The EPSs isolated from some strains of *Lc. lactis* subsp. *cremoris* contain rhamnose, glucose, galactose, and phosphates [10–12], while others contain only glucose and galactose. Marshall et al. [13] found that *Lc. lactis* subsp. *cremoris* LC330 produced two EPSs with different sugar composition and molecular mass: a neutral EPS of 1.106 kDa and a smaller negatively charged EPS (containing phosphate groups) of about 1.104 kDa. Van Casteren et al. [14] reported that EPS from *Lc. lactis* subsp. *cremoris* B40 consists of rhamnose, galactose, and glucose in the ratio of 0.9:1.2:2.0 and that the molar ratio of carbohydrate and phosphorus is 4.7:1. *Streptococcus thermophilus* produce an EPS composed of galactose and rhamnose when grown on milk [9]. *Lactobacillus bulgaricus* grown on chemically defined medium produce an EPS composed of galactose, glucose, and rhamnose [15]. Many strains of *Leuconostoc mesenteroides* produce dextran (α-glucan). Levans are produced by several strains of *Streptococcus mutans* [3].

Wide range of exopolysaccharides extraction, purification, and analysis schemes have been developed in literature involving from simple dialysis against water of the culture medium following by freeze drying to size exclusion column for preparing of highly pure EPS extracts.

Some authors used trichloroacetic acid (TCA) for protein sedimentation, dialysis for final EPS purification from sugars, or just numerous precipitations with ethanol and/or acetone [6]. Others procedures have been used for EPS purification including microfiltration, ultrafiltration, and diafiltration which can be carried out separately or in combination with ethanol precipitation [16–18]. Different types of membranes have been used such as regenerated cellulose and polyethersulfone, as well as different molecular weight cut-off [19].

In order to obtain pure polysaccharide fractions, size exclusion chromatography is the most common method used because it allows the separation of polysaccharides according to their size and also permits the subsequent determination of their molecular weight [19].

These different methods can be compared according to two criteria: quantity and quality of extracted EPS. It was shown that different extraction procedures influence the quantity and the composition of the extracted EPS [20, 21], the quantity and the composition of the mineral fraction present in the EPS extracts [22], and EPS binding properties to protons and different metals [20, 23, 24].

More advanced technologies to obtain polysaccharides have been used recently, as ultrasonic [25] and microwave assisted extractions [26] besides the pressurized solvent extraction [27]. The latter procedure showed to be faster and more efficient in obtaining higher yield of polysaccharides, comparing to the traditional methodologies.

In the present study, we characterized the EPS fractions obtained from pure bacterial culture of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* SLT10 (*Lc. lactis* SLT10), *Lactobacillus plantarum* C7 (*Lb. plantarum* C7), and *Leuconostoc mesenteroides* B3 (*Ln. mesenteroides* B3) to evaluate the influence of purification methods on the EPS yield, composition, and molecular weight. The studied purification methods were ethanol precipitation and ultrafiltration.

2. Material and Methods

2.1. Bacterial Strains. The three studied strains were obtained from the strain collection of Laboratory of Microbial Ecology and Technology (LETMi): *Lactococcus lactis* subsp. *lactis* SLT10 strain had been previously isolated in our laboratory from Tunisian traditional fermented milk [28]. *Lactobacillus plantarum* C7 (*Lb. plantarum* C7) had been isolated from gastrointestinal tract of chickens [29] and *Leuconostoc mesenteroides* B3 (*Ln. mesenteroides* B3) had been isolated from Tunisian palm sap [30]. Stock cultures grown in MRS broth were stored at −18°C in 50% glycerol until use.

2.2. Fermentation. The MRS-sucrose medium was used for EPS production and contain: 4% (w/v) sucrose, 10 g/L tryptone, 10 g/L meat extract, 5 g/L yeast extract, 5 g/L sodium acetate, 2 g/L disodium phosphate, 2 g/L tri-ammonium citrate, 0.1 g/L $MgSO_4$, and 0.05 g/L $MnSO_4$ (pH 6.5) [31, 32]. The medium was autoclaved at 121°C for 15 min. The fermentation temperature, inoculum size, and fermentation time were 30°C, 3.0% (v/v), and 24 h, respectively. After incubation, bacterial cells were separated from the EPS preparation by centrifugation (5,000 rpm for 10 min at 4°C) of the culture broth. The supernatant, containing the EPS fraction, was filtered under vacuum through Sartorius cellulose nitrate filters of (0.45 μm pore size) to eliminate cells and large cellular. The obtained supernatant was divided into two batches for the purification step.

2.3. Purification of Exopolysaccharides. The obtained supernatant was divided in two batches. The first supernatant was treated with NaCl to a final concentration of 1 M. EPS was precipitated by the addition of v/v chilled ethanol (96%) to the supernatant (EPS-ETOH). The proportion of chilled ethanol (v/v) was chosen in order to precipitate high-molecular weight exopolysaccharides. After precipitation at 4°C (overnight) the sample was centrifuged at 6,000 rpm for 20 min at 4°C, and the pellet was washed with ethanol (96%) and recentrifuged at 6,000 rpm for 20 min at 4°C. The pellet obtained was redissolved in distilled water and lyophilized.

The second supernatant was ultrafiltrated (UF) using a 10 kDa cut-off cellulose membrane. Finally, the exopolysaccharide fraction (EPS-UF), collected from the retentate, was evaporated and lyophilized.

2.4. Exopolysaccharides Characterization

2.4.1. Monosaccharide Analysis. The monomer composition of exopolysaccharides extracted from the three studied

strains was determined after preparation of methyl glycosides trimethylsylil derivatives. Suspensions were prepared by dissolving 4 mg of lyophilized polysaccharides in 2 mL distilled water. Fifty μL of *myo*-inositol, used as internal standard, was added to 200 μL of polysaccharides suspension. The mixture was hydrolyzed for 4 h at 100°C, in screw glass tube, using 500 μL of methanol/HCl (3 N) (Supelco). After cooling to room temperature, methanolyzate fractions were neutralized with silver carbonate, centrifuged at 6,000 rpm for 5 min, and evaporated under nitrogen flow. The generated methyl glycosides were then converted to their corresponding volatile trimethylsilyl derivatives. The reaction took place by adding 70 μL pyridine and 70 μL derivatization reagent, Bis(tri-methylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) (Supelco), incubated for 30 min at 80°C. After solvent evaporation under nitrogen flow, the generated per-O-trimethylsilylmethyl glycosides were resuspended in 700 μL dichloromethane and analyzed by gas chromatography-flame ionization detector (GC-FID). An Agilent GC 6850A instrument equipped with HP-5MS capillary column (30 m length, 0.25 mm diameter, and 0.25 μm film thicknesses) was used. The GC oven temperature was set to 120°C, increased first to 180°C at 3°C/min, then increased to 200°C at 2°C/min, and held for 5 min. The helium carrier gas flow was set at 1.5 mL/min and the injection volume was 0.1 μL.

2.4.2. Molecular Mass Determination. The average molecular mass of exopolysaccharides extracted from the three studied strains was determined using high-performance size exclusion chromatography (HPSEC) equipped with Shodex OHpak SB-804 HQ and SB-805 HQ columns placed in series. The EPS was eluted with 0.1 M Sodium Nitrate ($NaNO_3$) at a flow rate of 0.5 mL.min^{-1}. Detection was performed using a refractive index detector (RI) (Waters) and a multiangle laser-light scattering detector (LS) (MiniDAWN, Wyatt Technology, Dawn).

2.4.3. Fourier Transform Infrared (FTIR) Spectroscopy of Purified EPS. The structural characterization of the purified EPS was studied using Fourier transform infrared (FTIR) spectroscopy in order to determine their functional groups distribution. The translucent pellets (5mmØ) were made by mixing and pressing the freeze-dried polysaccharides samples with KBr powder (5:100 w/w). The FTIR spectra were recorded in transmittance mode at a spectral range of 4000 and 400 cm^{-1} with an accumulation of 15 scans and a resolution was 4 cm^{-1}, using a spectrophotometer type Perkin Elmer Spectrum BX®, equipped with a He-Ne laser and a detector MCT type broadband and high sensitivity [33]. The spectra acquisition was performed via spectrum v5.3.1 software. The bands identification was accomplished according to the data cited by Wiercigroch et al. [34].

2.5. Analytical Methods

2.5.1. Total Sugar Determination. Total carbohydrate content of the exopolysaccharides obtained by ethanol precipitation and ultrafiltration was estimated by the phenol-sulfuric acid calorimetric method [35]. A 200 μl EPS sample solution (10 mg.mL^{-1}) was treated with 200 μl of a 5% phenol solution and 1 ml of pure H_2SO_4. The mixture was cooled to room temperature for 30 min. Absorbance at 492 nm of samples as well as standard sugar (10 to 100 μg.mL^{-1} ribose) was read by spectrophotometry (Hitachi U-1800®).

2.5.2. Protein Concentration Measurement. Protein content was calculated according to the Bradford's method [36]. 1 mL of reagent (Biorad) was added to 20 μL of sample (10 mg.mL^{-1}) and incubated 5 min at room temperature. OD of samples as well as the standard protein (0 to 0.75 mg.mL^{-}1 of Bovine Serum Albumin (BSA)) was measured at 595 nm (Hitachi U-1800®).

2.6. Statistical Analysis. All experiments were performed in triplicate and are reported as means ± standard deviation. Significant differences between samples were tested using a two-sample comparison analysis and a t-test. The statistical significance of the relationship was analyzed at the 95% confidence level.

3. Results and Discussion

The three studied strains were isolated from different ecological niches. *Lc. lactis* SLT10 is a starter used for the preparation of "Leben" a Tunisian fermented milk; *Lb. plantarum* C7 is a probiotic strain isolated from gastrointestinal tract of chickens. For both strains, characterization of EPS produced can provide important data concerning their uses as starters for the preparation of fermented functional foods. As regards *Ln. mesenteroides* B3, this is the first time that a strain isolated from Tunisian palm sap was investigated for EPS production.

3.1. Effect of Purification Method on Exopolysaccharides Yield. In order to isolate EPS produced by the three studied strains, two purification methods were used: ethanol precipitation and ultrafiltration (UF) through 10 kDa membrane. Molar exclusion limit of 10 kDa has been chosen based on preliminary study showing that bacterial polysaccharides fractions presented molecular weight higher than 10 kDa. Moreover, with molecular weight cut-off of 10 kDa high- and low-molecular weight, polysaccharides are retained, while oligosaccharides, polypeptides, and so forth are removed.

The evaluation of the efficiency of both methods on polysaccharides recovery after purification step was conducted using the phenol-sulfuric method described by Dubois et al. [35] for the determination of sugars and related compounds on the freeze-dried extract. This method has been widely used as an indication of the EPS yield after different purification methods. Results are summarized in Table 1.

As shown in Table 1, EPS recovery by UF was significantly higher than ethanol precipitation for *Lc. lactis* SLT10 and *Lb. plantarum* C7 ($t<0.05$). UF was more efficient than ethanol precipitation method (1.2 times more). However, EPS recovery was similar for both methods for *Ln. mesenteroides* B3. Indeed, UF is widely used method for polysaccharides

Table 1: Reducing sugars contents (%) of different exopolysaccharides fractions.

Strains	EPS-ETOH	EPS-UF
Lc. Lactis SLT10	28.06±0.041	33.63±1.38
Lb. plantarum C7	19.82±0.305	26.54±0.905
Ln. mesenteroides B3	22.07±0.019	22.044±0.366

Table 2: Proteins contents (%) of different exopolysaccharides fractions.

Strains	EPS-ETOH	EPS-UF
Lc. Lactis SLT10	1.008±0.049	1.284±0.048
Lb. plantarum C7	0.928±0.036	0.414±0.027
Ln. mesenteroides B3	0.925±0.033	0.808±0.067

purification. Similar results were found by Bergmaier et al. [16], who compared UF with a conventional method based on ethanol precipitation, dialysis, and protein removal by trichloroacetic acid; the EPS recovery by the UF was higher.

Higher EPS recovery (70.38%) was, also, found by Pan and Mei [32] when using UF for the purification of EPS obtained from *Lc. lactis* subsp. *lactis* 12. Tuinier el al. [17] used UF with a polysulfone membrane (molar exclusion limit10 kDa) after a microfiltration step for the separation of EPSB40 from *Lc. lactis* subsp. *cremoris* on whey based media; the freeze-dried extract contains 63% EPS.

Polysaccharides yields obtained in this work (Table 1) were low compared to literature. This result can be explained by the following: (i) both methods used are not appropriate to isolate EPS which affects strongly the final yield; (ii) EPS production by the three studied strains was limited in these culture conditions. Remada and Abraham [37] studied the effect of a heat treatment of the milk on EPS recovery and found that the highest recovery of EPS was obtained when samples were heated as a first step of isolation. The heat treatment allows the separation of polysaccharide attached to cells since that it has been shown that LAB express at least two distinct phenotypic forms of EPS, either ropy and/or capsular forms [38]. In the procedures without heat treatment, part of the polysaccharide attached to cells would be lost with pellet during broth culture centrifugation. Moreover, heat treatment inactivates the enzymes that could hydrolyze the polymer (glycohydrolases). Remada and Abraham [37] suggested also a method involving 1 or 2 steps of ethanol precipitation followed by dialysis with different cut-off membranes than TCA precipitation.

Proteins content of freeze-dried extracts for both methods was evaluated using Bradford's assay. Results are shown in Table 2.

The low proteins content (about 1%) of all studied samples approves the efficiency of these methods to separate proteins from polysaccharides and to provide a high purity extracts. Similar results (1% of proteins) were found by Maalej et al. [39] for EPS22 extracted from *Pseudomonas stutzeri* AS22 by applying ultrafiltration followed by dialysis. Pan and Mei [32] indicated an absence of proteins and nucleic acid in the EPS-I extracted from *Lc. lactis* subsp. *lactis* 12 by ultrafiltration. Similar results were also found after the analysis of purified EPS sample from *Lb. plantarum* YW11 using anion-exchange chromatography on DEAE-cellulose with 1.38±0.25% proteins content [40]. The proteins content of EPS from *Leuconostoc* sp. CFR 2181 extracted by Ice-cold isopropyl alcohol precipitation and washed with acetone was only 0.8% [41]. Tuinier el al. [17] obtained proteins content of 18% for *Lc. lactis* subsp. *cremoris* when grown on whey based media.

3.2. Monomer Composition and Molar Ratio of Exopolysaccharides. Different methods are available for determining the monomer composition of EPS samples. Methanolysis and per-trimethylsilylation provide samples that can be analyzed by GC. Hanko and Rohrer [42] proposed a simple method, requiring acid hydrolysis followed by monomer detection using high-pressure anion-exchange chromatography with pulsed amperometric detection.

In our work, monosaccharides composition of different polysaccharides fractions were determined according to Kamerling method [43] modified by par Montreuil [44]. Identification and quantification of monosaccharides began with a methanolysis of the polymer. The glycosidic residues are converted to their corresponding volatile trimethylsilyl derivatives and then analyzed by gas chromatography-flame ionization detector (GC-FID). The sugar composition and molar ratios of EPS-ETOH and EPS-UF extracts from the three studied strains are summarized in Table 3.

For SLT10, the main sugars were glucose (Glu) and mannose (Man) for EPS-ETOH in the molar ratio of 0.47:1, while (Glu), mannose (Man), and rhamnose (Rha) were present in EPS-UF in the molar ratio of 0.58:1:0.18. This result suggests that the strain produces heteropolysaccharides. Pan and Mei [32] showed that EPS-I extracted from *L. lactis* subsp. *lactis* 12 is mainly composed of fructose and rhamnose. According to literature, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* produce heteropolysaccharides. *Lc. lactis* subsp. *cremoris* B40 and *Lc. lactis* subsp. *cremoris* SBT 0495 produce an EPS composed of glucose, galactose, rhamnose, and phosphate at a ratio of 2:2:1:1 [11, 14]. This diversity in EPS composition produced by *Lc. lactis* can be explained by genetic studies. Indeed, genes implicated in EPS production in *Lc. lactis* are encoded by plasmid DNA. The first lactococcal eps locus identified was that of *Lc. lactis* subsp. *cremoris* NIZO B40; it comprises 14 plasmid-encoded genes [45]. Since then, partial sequences of eps gene clusters have been identified in *Lc. lactis* subsp. cremoris NIZO B891 and NIZO B35 strains [46]. Cluster consisting of 23 putative EPS biosynthetic determinants has been identified on plasmid pCI658 in *Lc. lactis* subsp. *cremoris* HO2 [47].

GC-MS analysis of the monosaccharide composition of the EPS produced by *Lb. plantarum* C7 showed that the EPS was composed of glucose and mannose in a molar ratio of 0.607:1 for EPS-ETOH and 4.07:1 for EPS-UF, which suggested that the strain produces heteropolysaccharides. Different monomers composition and proportion of *Lb. plantarum* strains are available in the literature. The EPS purified from *Lb. plantarum* YW11 using anion-exchange chromatography is composed of glucose and galactose in a

Table 3: Sugar composition and molar ratio of exopolysaccharides isolated from the three studied strains (SLT10, C7, and B3).

Strains	Monomers	Molar ratio		Mol (%)	
		EPS-ETOH	*EPS-UF*	*EPS-ETOH*	*EPS-UF*
Lc. Lactococcus SLT10	Glucose	0.47±0.033	0.58±0.0001	1.97±0.008	8.46±0.35
	Mannose	1	1	4.15±0.27	14.59±0.6
	Rhamnose	-	0.18±0.002	-	2.48±0.13
	Total			6.13±0.264	25.55±1.09
Lb. plantarum C7	Glucose	0.607±0.162	4.07±0.82	3.34±1.31	13.36±8.11
	Mannose	1	1	6.01 ± 3.77	8.98±7.73
	Total			9.35 ± 5.09	22.34±2.73
Ln. mesenteroides B3	Glucose	0.61± 0.02	3.48± 0.02	1.97± 0.167	13.35± .09
	Mannose	1	1	3.21 ±0.38	3.85 ±0.62
	Total			5.19 ±0.54	17.20 ±2.72

molar ratio of 2.71:1 [40]. EPS extracted from *Lb. plantarum* KF5 presented a monomer composition of mannose, glucose, and galactose in a molar ratio of 1:4.99:6.90 [48]. Li et al. [49] showed that the strain *Lb. plantarum* 70810 isolated from Chinese paocai produced two types of EPS with a monomer composition of glucose, mannose, and galactose in a molar ratio of 18.21:78.76:3.03 and 12.92:30.89:56.19, respectively. Indeed, the monosaccharide composition of EPS produced by LAB can be affected by the type of strains; Laws et al. [8] revealed that the genes coding for EPS synthesis are of plasmid origin in the mesophilic LAB strains (e.g., *Lactococcus*), but they are chromosomally based in the thermophilic strains (*Streptococcus* and *Lactobacilli*). In general, the EPS-producing ability of LAB is regarded as being unstable. For mesophilic LAB strains, the unstable nature of EPS synthesis is consistent with the genes for EPS synthesis being plasmid bound. For the thermophilic LAB strains, it has been proposed that the loss of EPS-producing character is due to deletions and rearrangement resulting from genetic instability.

For *Ln. mesenteroides* B3, GC analysis showed a monomeric composition of glucose and mannose in a molar ratio of 0.61:1 for EPS-ETOH and 3.48:1 for EPS-UF. Even though, Leuconostocs are well known for the production of homopolysaccharides such as alternan, dextran, and levan from sucrose metabolism [50], the strain *Leuconostoc mesenteroides* B3 produces a heteropolysaccharide composed of glucose and mannose. Indeed, Welman and Maddox [51] revealed that themesophilic LAB such as *Leuconostoc mesenteroides* also produces heteropolysaccharides. Previous study showed the ability of Leuconostocs to produce heteropolysaccharides; the EPS isolated from *Leuconostoc* sp. CFR 2181 consisted mainly of glucose (91%) with minor quantities of rhamnose and arabinose [41].

Based on Table 3, the main observation was the difference in monomer composition of EPS extracted by ultrafiltration and ethanol precipitation for *Lc. lactis* SLT10. Moreover, for the strains *Lb. plantarum* C7 and *Ln. mesenteroides* B3 when comparing both purification methods and in spite of similarity in monomer composition, the molar ratios were different.

This result can be explained by the hypothesis that when sucrose was used as carbon source, the studied strains synthesize mixtures of EPSs. The EPSs can have different structures (different monomeric composition) in the case of the strain SLT10 or different molar ratios with the same monomers for *Lb. plantarum* C7 and *Ln. mesenteroides* B3. Thus, we can suggest that the strain SLT10 produces at least two types of exopolysaccharides with different molecular weight and different monomeric composition: the first one with the higher molecular weight is composed of glucose and mannose and the second one with lower molecular weight probably contains rhamnose. When ultrafiltration is used as purification method, with a 10 kDa cut-off membrane that could retain either high- and low-molecular weight polysaccharides, both EPSs were retained. However for ethanol precipitation only one EPS was precipitated; probably the one with the higher molecular weight composed of glucose and mannose.

For the strains *Lb. plantarum* C7 and *Ln. mesenteroides* B3, there is also the possibility of recovering EPS samples that have identical structure (glucose and mannose) but different molecular masses. In previous studies Comte et al. [20] have showed that the EPS characteristics present qualitative and quantitative differences depending on the method used. They found that the extraction methods using chemical reagents strongly affected the HPSEC (high-pressure size exclusion chromatography) fingerprints of EPS, whereas the physical methods influenced only molecular weight distribution but not HPSEC fingerprints. It had been shown that *Lactobacillus* spp. G-77 produces two homopolysaccharides with different structures [52] (Duenas-Chasco et al., 1998). Degeest and de Vuyst [53] reported the production of a high-molecular mass and a low-molecular mass EPS by *Streptococcus thermophilus* LY03. The production of two polysaccharides by *Lb. rhamnosus* has been reported [54]. A strain of *Lb. reuteri* LB 121 is able to produce two types of homopolysaccharides mainly composed of D-glucose or D-fructose [55].

3.3. Molecular Mass Estimation. The molecular mass of different EPSs isolated from the three studied strains with both methods was estimated using high-performance size

Table 4: Molecular weight (Mw), molecular number (Mn), and polydispersity index of EPSs extracted from the three studied stains.

Purification method	Strains	Mw (kDa)	Mn (kDa)	Polydispersity Index (Mw/Mn)	Mass recovery (%)
EPS-ETOH	*Lc. lactis* SLT10	62.4±2.94	19.3±2.07	3.231±0.47	37.3
	Lb. plantarum C7	33.7±2.89	8.5±3.44	3.968±1.64	46.0
	Ln. mesenteroides B3	-	-	-	-
EPS-UF	*Lc. lactis* SLT10	51.5±1.75	12.3±1.38	4.178±0.49	54.6
	Lb. plantarum C7	11.6±1.83	2.1±0.95	5.586±2.66	53.5
	Ln. mesenteroides B3	18.6±2.21	8.4±1.52	2.204±0.407	46.1

exclusion chromatography (HPSEC). The molecular weight distribution of EPS-UF and EPS-ETOH fractions of *Lb. plantarum* C7, *Lc. lactis* SLT10, and *Ln. mesenteroides* B3 is presented in Figures 1 and 2.

Molecular weight (Mw), molecular number (Mn), and polydispersity index of EPSs isolated from the three studied stains are summarized in Table 4.

The result showed that the three strains present different EPS molecular masses. Indeed, the molar mass of the EPS produced by LAB varied according to strains and polymer type [6]. Generally, the EPSs produced by the bacterial strains in MRS-sucrose media are lower-molecular-mass fractions, whose molecular masses do not exceed 62.4±2.94 kDa. The MW of EPS extracted from *Lc. lactis* SLT 10 was the highest with both purification methods (62.4±2.94 kDa and 51.5 kDa±1.75). Heteropolysaccharides ranged from 40 to 9000 kDa for *Streptococcus thermophilus* strains and 100 to 2000 kDa for *Lc. lactis* spp. *cremoris* strains [6].

The molecular mass of the EPS produced by *Lb. plantarum C7* was determined to be 33.5±2.89 kDa (EPS-ETOH) and 11.6±1.83 kDa (EPS-UF) which was similar to that (44 kDa) of the EPS of *L. plantarum* EP56 [56] but lower than that (110 kDa) of the EPS of *Lactobacillus plantarum* YW11 [40] and (1150 kDa) of the EPS of *Lb. plantarum* C88 [57].

The molecular mass of EPS purified from *Ln. mesenteroides* B3 was only available for EPS-UF and was 18.6±2.21 kDa which in the same range of magnitude of those reported for the EPS fractions of *Leuconostoc* sp. CFR 2181 with molecular weights ranging from 10 kDa to 1500 kDa [41].

The polydispersity index Mw/Mn (Mw, weight-average; Mn, number-average), which reflects the degree of heterogeneity of the polymer's chain lengths, is ranging from 2.204±0.407 to 5.586±2.66 (>1) which represents a heterogeneous populations in terms of polysaccharide chains size. The lower polydispersity index value was obtained for the EPS fraction purified from *Ln. mesenteroides* B3 (2.204±0.407) which can be considered as moderately polydisperse distribution type even when the EPS fractions purified from *Lc. lactis* SLT10 and *Lb. plantarum* C7 were considered as broadly polydisperse distribution type.

The results from Table 4 show two mains observations: (i) EPS-ETOH fractions molecular weights were higher than those of EPS-UF fractions and (ii) the heterogeneity of EPS fractions is more pronounced when UF was used as purification method (polydispersity index ranged from 4.178±0.49 to 5.586±2.66).

These observations can be explained by the hypothesis given above that the studied strains grown in MRS-sucrose produce more than one polymer with various molecular masses causing the heterogeneity of the freeze-dried extracts (high polydispersity index). This hypothesis could explain variations in monomer composition and molecular ratios of EPS-ETOH and EPS-UF. When using UF as purification methods, polymers produced by the lactic strains remain in the retentate. However, when using ethanol for purification, only one polymer precipitates (probably the one with the highest molecular weight). Grobben et al. [15] found that *Lb. bulgaricus* strain NCFB 2772, grown in chemically defined medium, produced two EPS fractions with molecular masses of 40 and 1700 kDa. Similar results were reported with *Lc. Lactis* subsp. *cremoris* LC 330 [13].

3.4. The Structural Characterization of the Purified EPS. FTIR spectroscopy has been a powerful and valuable analytical method to investigate the nature of the functional groups of EPS in terms of monomeric units and their linkages. Figure 3 shows the FTIR spectra of EPS fractions from *Lb. plantarum* C7, *Lc. lactis* SLT10, and *Ln. mesenteroides* B3 obtained by precipitation in a final ethanol concentration 96%.

The spectrum of purified EPS was studied in the region between 400 cm^{-1} and 4000 cm^{-1} and showed numerous peaks from 3434 cm^{-1} to 534 cm^{-1}. In comparison with the IR spectra of polysaccharides listed in the literature, all the peaks obtained were in agreement with the typical absorption peaks of polysaccharides.

As can be seen, no significant difference in the main absorption intensity was observed among the three fractions. However, upon close comparison of the spectra, small differences are observed.

The broad absorption peak observed at around 3434-3420 cm^{-1} indicated the presence of intensive hydroxyl groups (O-H) stretching frequency confirming the polysaccharide nature of the material [58]. The C–H stretching vibration gives signals between 2928 and 2850 cm^{-1} [59]. The peak observed around 2366 cm^{-1} is attributed to O-H bond groups, which could be explained by their carbohydrate nature.

The absorption peaks at 1634 cm^{-1}, 1628 cm^{-1}, and 1626 cm^{-1} were due to the stretch vibration of carboxyl group (C = O) [60]. The absorption at 1404 and 1406 cm^{-1} was due to the symmetric stretching of –COO.

There were peaks near 1000-1200 cm^{-1}, indicating that the polysaccharide contained α-pyranose. Indeed, the

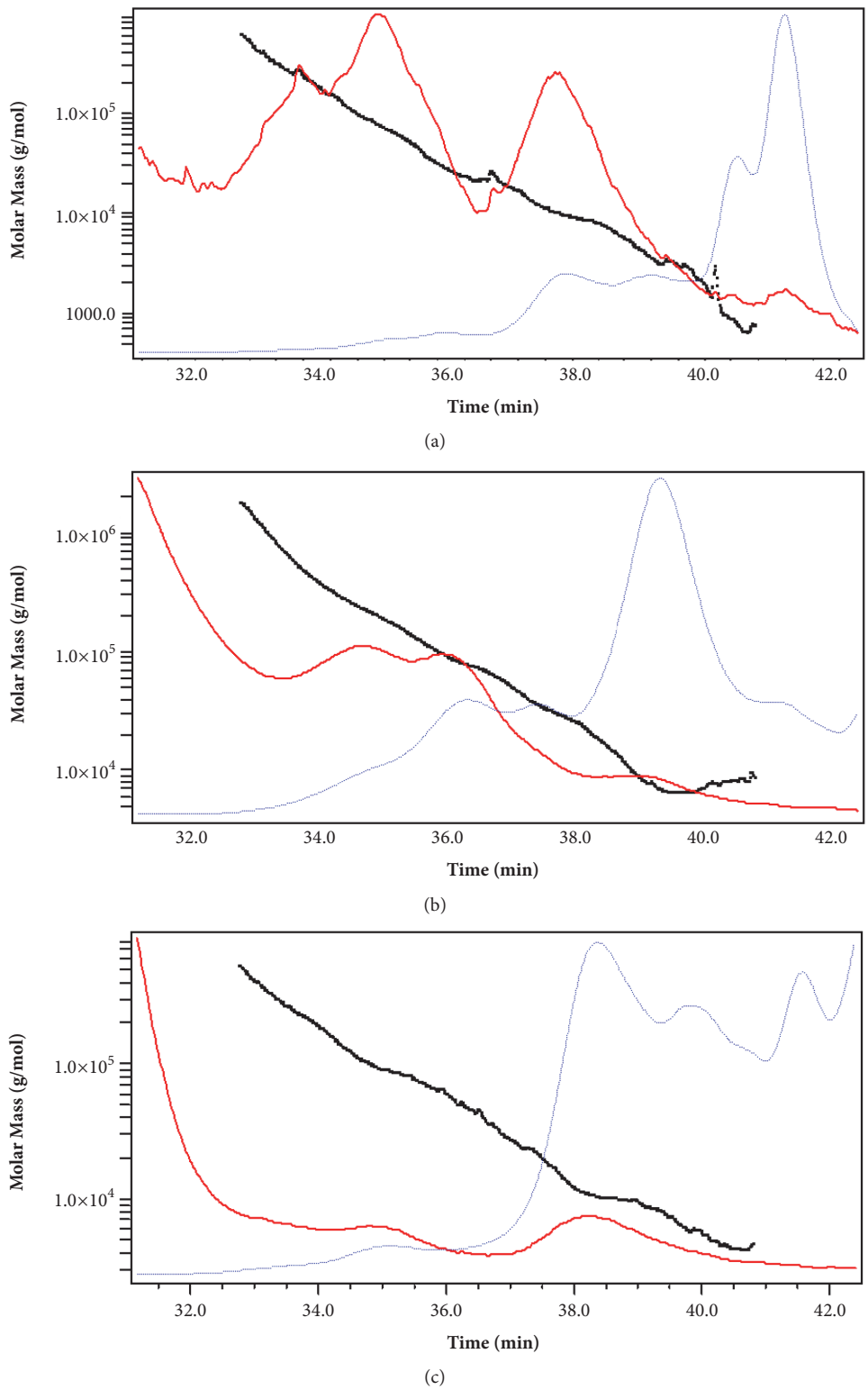

Figure 1: The molecular weight distribution of EPS-UF fractions of *Lb. plantarum* C7 (**a**), *Lc. lactis* SLT10 (**b**), and *Ln. mesenteroides* B3 (**c**) (black squares: molar mass; red dashed line: LS (laser-light scattering); blue dotted line: RI (refractive index)).

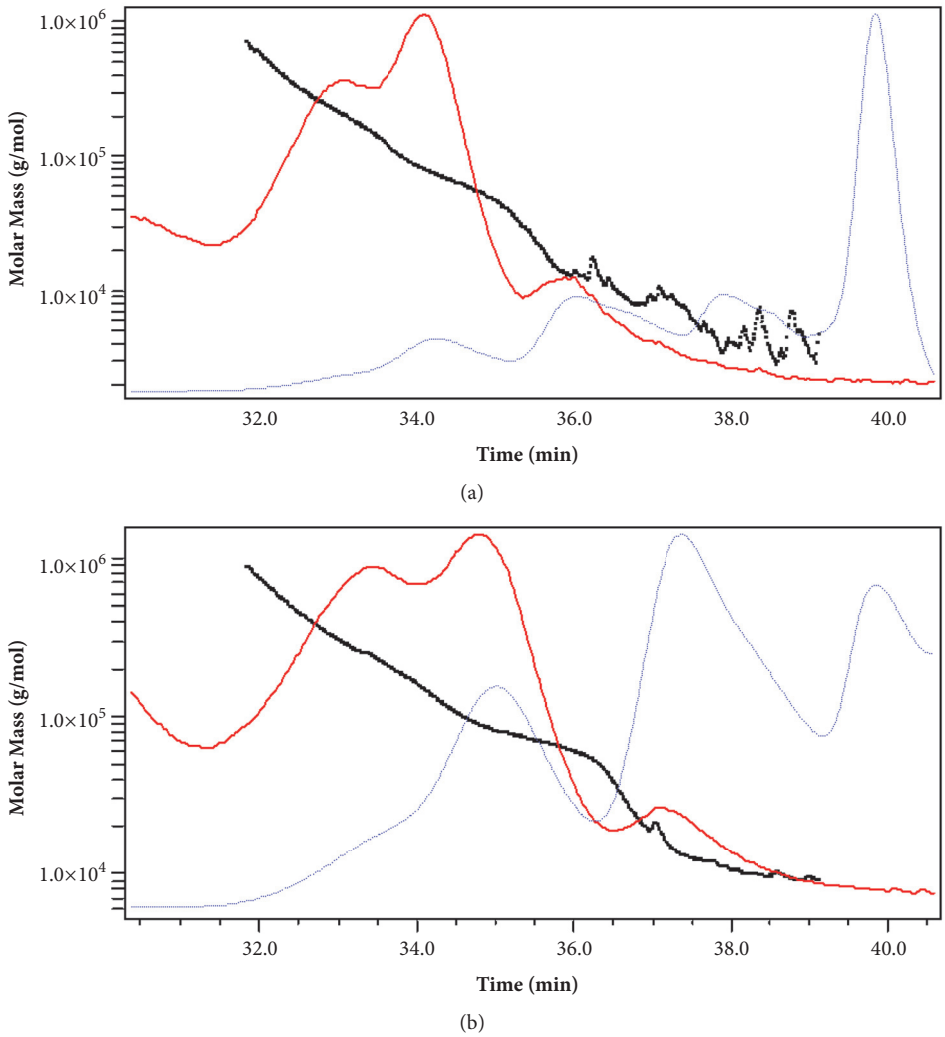

Figure 2: The molecular weight distribution of EPS-ETOH fractions of *Lb. plantarum* C7 (**a**) and *Lc. lactis* SLT10 (**b**) (black squares: molar mass; red dashed line: LS (laser-light scattering); and blue dotted line: RI (refractive index)).

carbohydrates show high absorbencies in this region, which is within the so-called fingerprint region, where the position and the intensity of the bands are specific for every polysaccharide, allowing its possible identification [61].

The intense peaks at 1094 cm^{-1}, 1074 cm^{-1}, and 1048 cm^{-1} were attributed to the vibration of the glycosidic linkage C-O-C of glucose [60].

Along with these peaks, more characteristic peaks at 870 and 804 cm^{-1} region were also detected indicating that the EPS contained both α and β-type glycosidic linkages between sugar monomers [62]. These peaks are absent in the EPS fraction extracted from *Ln. mesenteroides* B3.

The weak adoption band at 534-538 cm^{-1}, absent in the EPS fraction purified from *Ln. mesenteroides* B3, was indicative of glycosidic linkage peak for polysaccharide.

4. Conclusion

In this work, ultrafiltration and ethanol precipitation were used for the purification of EPSs produced by three Lactic Acid Bacteria strains isolated from different Tunisian biotopes. Results confirmed that EPS recovery by ultrafiltration was significantly higher than ethanol precipitation for *Lc. lactis* SLT10 and *Lb. plantarum* C7. GC-MS and HPSEC analysis of EPSs showed that the three studied strains produce a heteropolysaccharides with low-molecular masses. Depending on purification method, the monomeric composition and molar ratios of the different EPS fractions are affected.

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 they have no conflicts of interest.

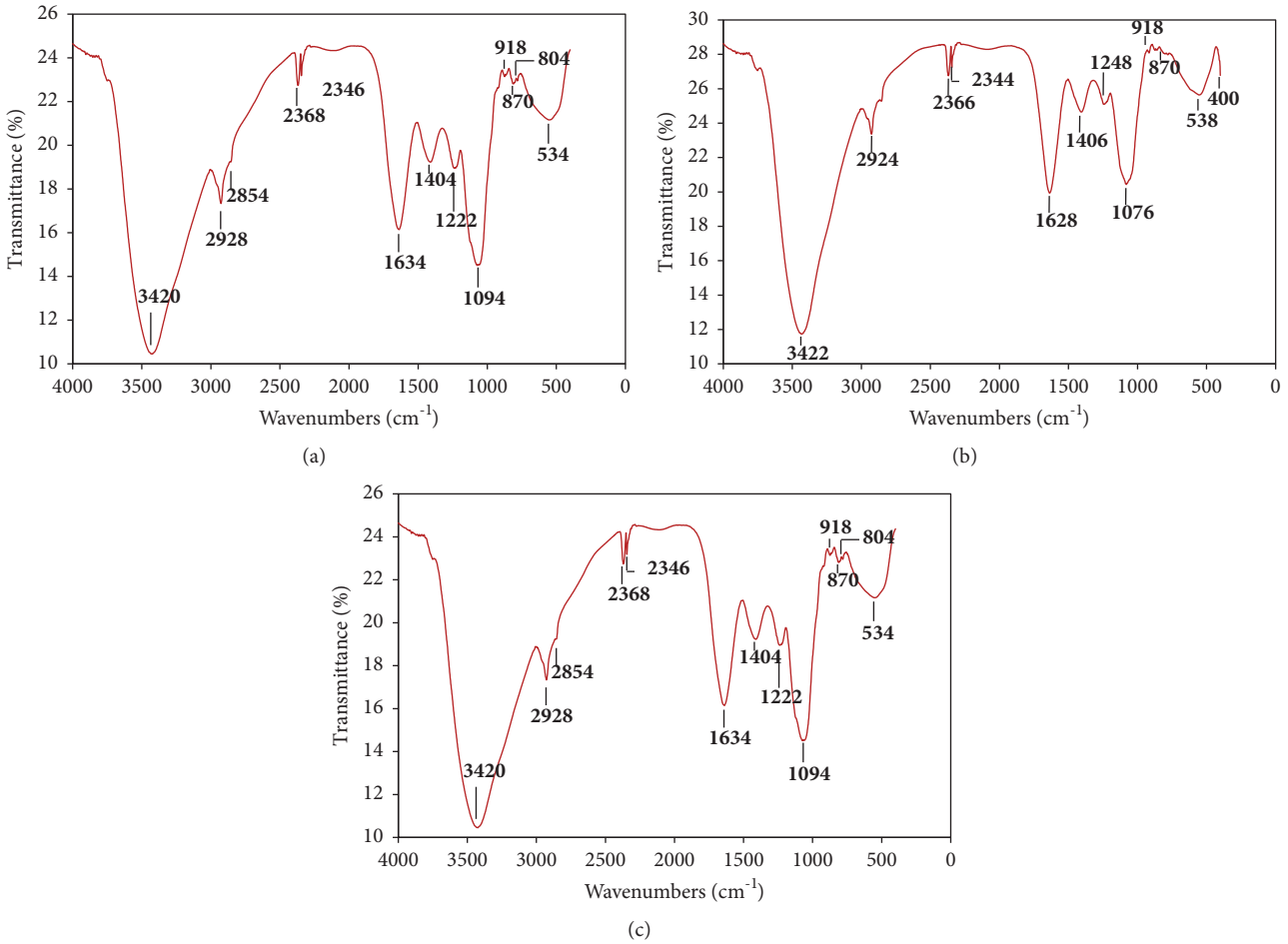

Figure 3: FTIR spectra of EPS-ETOH fractions of *Lb. plantarum* C7 (**a**), *Lc. lactis* SLT10, (**b**) and *Ln. mesenteroides* B3 (**c**).

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Research Article

In Vitro Antibacterial Activity of Propyl-Propane-Thiosulfinate and Propyl-Propane-Thiosulfonate Derived from *Allium* spp. against Gram-Negative and Gram-Positive Multidrug-Resistant Bacteria Isolated from Human Samples

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Background. The aim of this study was to compare the *in vitro* antibacterial activity of two compounds derived from *Alliaceae*, PTS (propyl-propane-thiosulfinate), and PTSO (propyl-propane-thiosulfonate), with that of other antibiotics commonly used against bacteria isolated from humans. *Materials and Methods*. A total of 212 gram-negative bacilli and 267 gram-positive cocci isolated from human clinical samples and resistant to at least one group of antibiotics were selected. In order to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) to various antibiotics as well as PTS and PTSO, all isolates underwent broth microdilution assay. *Results*. PTS showed moderate activity against *Enterobacteriaceae* with MIC_{50} (and MBC_{50}) and MIC_{90} (and MBC_{90}) values of 256-512 mg/L, while PTSO showed greater activity with MIC_{50} and MIC_{90} values of 64-128 mg/L and MBC_{50} and MBC_{90} values of 128-512 mg/L. These data show the bactericidal activity of both compounds and indicate that PTSO was more active than PTS against this group of bacteria. Both compounds showed lower activity against *P. aeruginosa* (MIC_{50} = 1024 mg/L, MIC_{90} = 2048 mg/L, MBC_{50} = 2048 mg/L, and MBC_{90} = 2048 mg/L, for PTS; MIC_{50} = 512 mg/L, MIC_{90} = 1024 mg/L, MBC_{50} = 512 mg/L, and MBC_{90} = 2048 mg/L, for PTSO) compared to those obtained in others nonfermenting gram-negative bacilli (MIC_{50} = 128 mg/L, MIC_{90} = 512 mg/L, MBC_{50} = 128 mg/L, and MBC_{90} = 512 mg/L, for PTS; MIC_{50} = 64 mg/L, MIC_{90} = 256 mg/L, MBC_{50} = 64 mg/L, and MBC_{90} = 256 mg/L, for PTSO) and also indicate the bactericidal activity of both compounds against these groups of bacteria. Finally, the activity against *S. aureus*, *E. faecalis*, and *S. agalactiae* was higher than that observed against enterobacteria, especially in the case of PTSO (MIC_{50} = 8 mg/L, MIC_{90} = 8 mg/L, MBC_{50} = 32 mg/L, and MBC_{90} = 64 mg/L, in *S. aureus*; MIC_{50} = 4 mg/L, MIC_{90} = 8 mg/L, MBC_{50} = 8 mg/L, and MBC_{90} = 16 mg/L, in *E. faecalis* and *S. agalactiae*). *Conclusion*. PTS and PTSO have a significant broad spectrum antibacterial activity against multiresistant bacteria isolated from human clinical samples. Preliminary results in present work provide basic and useful information for development and potential use of these compounds in the treatment of human infections.

1. Introduction

The use of conventional antibiotics for the prevention of infectious diseases and as growth promoters in animal production has fostered the appearance of resistant bacteria and the transmission of these pathogens to humans [1]. In addition, the use and sometimes misuse of antibiotics in humans has increased the occurrence of infections (urinary

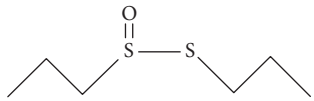

Figure 1: Chemical structure of propyl-propane-thiosulfinate (PTS).

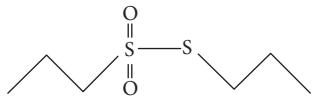

Figure 2: Chemical structure of propyl-propane-thiosulfonate (PTSO).

tract infections, respiratory tract infections, skin and soft tissue infections, etc.) caused by multiresistant bacteria, which has reduced the therapeutic options and has made necessary the selection of new molecules with antibacterial properties [2]. Natural compounds obtained from vegetables with antibacterial properties could be considered an alternative to conventional antibiotics [3].

In recent years, the antibacterial properties of some compounds obtained from *Allium* plants such as garlic (*Allium sativum*) and onion (*Allium cepa*) have been described. These can inhibit the growth of a range of gram-positive and gram-negative bacteria, including both pathogenic and commensal bacteria in humans and animals [4, 5]. *Allium*-derived products have been reported to be effective even against those strains that have become resistant to antibiotics [6].

Two of these *Allium*-derived compounds, propyl-propane-thiosulfinate (PTS) (Figure 1) and propyl-propane-thiosulfonate (PTSO) (Figure 2), are organosulphurate products obtained by decomposition of initial compounds naturally present in garlic bulbs as alliin and allicin. In several *in vitro* and *in vivo* studies against pathogenic bacteria from animals, both compounds have showed an antibiotic activity [3, 7, 8]. While the precise mechanism of action is not yet known, the main antibacterial effect of thiosulfinates (as allicin) has been reported to be due to (i) its accessibility resulting from high permeability through phospholipid membranes [9]; (ii) its chemical reaction with thiol groups of various enzymes such as the bacterial acetyl-CoA-forming system, consisting of acetate kinase and phosphotransacetyl-CoA synthetase, blocking acetate incorporation into fatty acids and inhibiting the formation of lipids [10]; and (iii) the inhibition of RNA polymerase and RNA synthesis [11].

Therefore, the aim of this study was to compare the *in vitro* antibacterial activity of the compounds derived from garlic PTS and PTSO with that of other antibiotics commonly used against gram-negative and gram-positive multidrug-resistant bacteria isolated from human clinical samples.

2. Material and Methods

2.1. Antibiotics, PTS and PTSO. All antibiotics were purchased from Sigma-Aldrich (Madrid, Spain) and each antibiotic was dissolved according to the manufacturer's recommendations.

PTS and PTSO (95% purity) were supplied by DMC Research (Alhendín, Granada, Spain) and dissolved in polysorbate-80 to a final concentration of 50%. The biosynthesis of propyl-propane-thiosulfinate (PTS) and propyl-propane-thiosulfonate (PTSO) is made from propiin, an amino acid derived from L-cysteine found in *Allium* species. The first step of the biosynthesis is the formation of a sulfenic acid, which is highly reactive and immediately produces PTS by a condensation reaction. In the last step, oxidation of PTS induces its dismutation in PTSO and propyl disulfide that can be oxidized and transformed to PTSO and that way the oxidation of PTS to PTSO is completed.

2.2. Bacterial Isolates. A total of 212 gram-negative bacilli and 267 gram-positive cocci isolated from clinical samples obtained from 479 different patients were selected. Identification and susceptibility studies were performed using WIDER system (Francisco Soria Melguizo, Madrid, Spain) or MicroScan system (Siemens Healthcare Diagnostics, Madrid, Spain). The susceptibility results obtained through these systems allowed the selection of isolates, based on the resistance presence to at least one group of antibiotics commonly used in the treatment of infections caused by these bacteria.

The presence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL) was confirmed by the diffusion method with disks containing cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazidime (30μg), and ceftazidime/clavulanic acid (30/10μg). The resistance to methicillin was confirmed using the Mueller–Hinton agar diffusion procedure with 30 μg cefoxitin disks. Both procedures were performed as recommended by the Clinical and Laboratory Standards Institute [12].

A total of 151 clinical isolates of *Enterobacteriaceae* (68 *Escherichia coli*, 33 *Klebsiella pneumoniae*, 6 *Klebsiella oxytoca*, 15 *Salmonella* spp., 17 *Yersinia enterocolitica*, 7 *Enterobacter cloacae*, 2 *Providencia stuartii*, 1 *Citrobacter amalonaticus*, 1 *Kluyvera cryocrescens*, and 1 *Proteus vulgaris*), 61 of non-fermenting gram-negative bacilli (40 *Pseudomonas aeruginosa*, 9 *Acinetobacter baumannii*, 7 *Aeromonas hydrophila*, 3 *Stenotrophomonas maltophilia*, 1 *Achromobacter xylosoxidans*, and 1 *Comamonas acidovorans*), 112 *Staphylococcus aureus* (all of them methicillin-resistant), 54 *Enterococcus faecalis* (all of them fluoroquinolone-resistant), and 101 *Streptococcus agalactiae* were selected. All isolates were stored at -40°C until the susceptibility study by microdilution.

2.3. In Vitro Antibacterial Assay. In order to determine the antibacterial susceptibilities, all 479 isolates underwent broth microdilution assay in Cation-Adjusted Mueller–Hinton Broth (CAMHB) following the guidelines of the CLSI [12]. Broth microdilution testing was performed with 96-well, round-bottom microtiter plates with a final concentration of the bacterial cell suspension equal to 1 x 10^5 colony forming units per milliliter (CFU/ml) in each well.

Each plate included negative controls (medium only) and 11 serial twofold dilutions of each antibiotic, PTS, or PTSO. The positive controls (only bacterial suspension without

antibiotics) were added per well in a separate round-bottom plate.

The concentration ranges (in mg/L) assayed for *Enterobacteriaceae* for each antibiotic were the following: amoxicillin/clavulanate (0.25/0.125-256/128), piperacillin/tazobactam (0.5/4-512/4), cefuroxime (0.5-512), cefoxitin (0.5-512), cefotaxime (0.125-128), ceftazidime (0.5-512), cefepime (0.25-256), imipenem (0.016-16), meropenem (0.016-16), gentamicin (0.125-128), tobramycin (0.125-128), amikacin (0.5-512), ciprofloxacin (0.125-128), trimethoprim/sulfamethoxazole (0.06/1.1875-64/1216), and nitrofurantoin (1-1024). The concentration ranges assayed for nonfermenting gram-negative bacilli for each antibiotic were piperacillin/tazobactam (0.5/4-512/4), ceftazidime (0.5-512), cefepime (0.25-256), imipenem (0.125-128), meropenem (0.125-128), gentamicin (0.125-128), tobramycin (0.125-128), amikacin (0.5-512), and ciprofloxacin (0.125-128). The concentration ranges for staphylococci were gentamicin (0.25-256), tobramycin (0.25-256), erythromycin (0.06-64), clindamycin (0.06-64), levofloxacin (0.06-64), linezolid (0.03-32), vancomycin (0.015-16), teicoplanin (0.03-32), daptomycin (0.008-8), rifampicin (0.03-32), and trimethoprim/sulfamethoxazole (0.06/1.1875-64/1216). The concentration ranges for enterococci were ampicillin (0.03-32), levofloxacin (0.06-64), linezolid (0.008-8), vancomycin (0.06-64), teicoplanin (0.03-32), and daptomycin (0.008-8). Finally, the concentration ranges assayed for *S. agalactiae* for each antibiotic were ampicillin (0.004-4), erythromycin (0.06-64), clindamycin (0.06-64), levofloxacin (0.06-64), linezolid (0.008-8), vancomycin (0.008-8), and daptomycin (0.008-8).

The concentration ranges of PTS were 2-2048 mg/L in *Enterobacteriaceae*, nonfermenting gram-negative bacilli and *S. aureus*, and 4-4096 mg/L in *E. faecalis* and *S. agalactiae*. For PTSO, they were 2-2048 mg/L in *Enterobacteriaceae* and nonfermenting gram-negative bacilli and 0.125-128 mg/L in gram-positive cocci. Thus, the final concentration of polysorbate-80 in the wells was less than 1%.

The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration to completely inhibit the visible growth of a microorganism after overnight incubation and the isolates were considered to be susceptible, intermediate, or resistant, according to the recommendations of the CLSI [12]. A "susceptible" result indicates that the patient's organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species. Conversely, a microorganism with a MIC interpreted as "resistant" should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally used with that drug. An "intermediate" result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. MIC_{50} and MIC_{90} values were defined as the lowest concentration of the antibiotic at which 50 and 90% of the isolates were inhibited, respectively.

For minimum bactericidal concentration (MBC) testing, 100 μl of broth from 1 to 5 wells containing no growth (which showed no visible turbidity) was plated onto antibiotic-free Columbia agar and incubated overnight at 37°C. The highest dilution that yielded no single bacterial colony on the agar plates was taken as MBC. Allium extracts were then considered as bacteriostatic or bactericidal depending on the MBC/MIC ratio which were, respectively, greater than 2 or between 2 and 1. MBC_{50} and MBC_{90} values were defined as the concentration of the antibiotic which kills 50 and 90% of the isolates, respectively.

Following the CLSI guidelines, we used the following strains as quality control in the procedures: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212.

2.4. Statistical Analysis. Data analysis was performed using the software IBM SPSS Statistics v19. The Mann–Whitney U test was used to compare the distribution of MIC and MBC values of PTS and PTSO in the different groups of bacteria studied. A level of significance was considered with a $p< 0.05$.

3. Results

Tables 1 and 2 show the values (in mg/L) of the MIC_{50}, MIC_{90}, MBC_{50}, and MBC_{90} and percentages of resistance to the antibacterial agents tested of the 479 clinical isolates.

There was 59 ESBL-producing *Enterobacteriaceae* (42 *E. coli*, 12 *K. pneumoniae*, and 5 *K. oxytoca*). The presence of this resistance phenotype in 39.1% of *Enterobacteriaceae* was the main determinant of the high rates of resistance to beta-lactam antibiotics, whose range oscillated from 1.3% to meropenem (MIC_{50} = 0.125 mg/L, MIC_{90} = 1 mg/L) to 81.5% to cefuroxime (MIC_{50} > 512 mg/L, MIC_{90} > 512 mg/L).

ESBL-producing strains were more resistant to second to fourth-generation cephalosporins, such as cefuroxime (MIC_{50} > 512 mg/L, MIC_{90} > 512 mg/L, 100% resistant), cefotaxime (MIC_{50} = 128 mg/L, MIC_{90} > 128 mg/L, and 100% resistant), ceftazidime (MIC_{50} = 64 mg/L, MIC_{90} = 256 mg/L, and 78.0% resistant), and cefepime (MIC_{50} = 32 mg/L, MIC_{90} = 128 mg/L, and 93.2% resistant) that combinations of beta-lactams with beta-lactamase inhibitors such as piperacillin-tazobactam (MIC_{50} = 8/4 mg/L, MIC_{90} = 256/4 mg/L, and 30.5% resistant) and amoxicillin/clavulanate (MIC_{50} = 16/8 mg/L, MIC_{90} > 256/128 mg/L, and 52.5% resistant) or to carbapenems such as imipenem (MIC_{50} = 0.5 mg/L, MIC_{90} = 1 mg/L, and 100% susceptible) or meropenem (MIC_{50} = 0.125 mg/L, MIC_{90} = 1 mg/L, and 100% susceptible). Nevertheless, the absence of ESBL in *Salmonella* spp. and *Yersinia* spp. explains the lower number of isolates resistant to beta-lactam antibiotics in this group of enterobacteria (range 0-28.1%). Finally, in case of bacteria such as *Enterobacter* spp., *Proteus* spp., or *Providencia* spp., among others (*remaining enterobacteria* group in Table 1), high rates of resistance to beta-lactams were observed: 16.7% to meropenem (MIC_{50} = 0.06 mg/L, MIC_{90} = 1 mg/L) and 100% to cefuroxime (MIC_{50} > 512 mg/L, MIC_{90} > 512 mg/L).

Among the aminoglycosides, amikacin was the antibiotic with a higher rate of activity against enterobacteria (MIC_{50} = 16 mg/L, MIC_{90} > 512 mg/L, and 29.1% resistant), against 35.8% resistant to gentamicin (MIC_{50} = 4 mg/L, MIC_{90} = 128

Table 1: Activity *in vitro* of PTS, PTSO, and others antibacterial agents against gram-negative organisms.

Organisms (number of isolates)	MIC_{50} (in mg/L)	MIC_{90} (in mg/L)	MBC_{50} (in mg/L)	MBC_{90} (in mg/L)	% of resistant isolates
***Enterobacteriaceae* (n=151)**					
Amoxicillin/clavulanate	32/16	256/128	64/32	256/128	59.6
Piperacillin/tazobactam	8/4	256/4	32/4	512/4	31.8
Cefuroxime	>512	>512	>512	>512	81.5
Cefoxitin	8	256	64	512	37.1
Cefotaxime	64	>128	128	>128	77.5
Ceftazidime	16	256	64	512	58.3
Cefepime	8	128	32	256	65.6
Imipenem	1	1	2	16	2.0
Meropenem	0.125	1	0.25	4	1.3
Gentamicin	4	128	32	>128	35.8
Tobramycin	4	128	32	>128	42.4
Amikacin	16	>512	64	>512	29.1
Ciprofloxacin	64	>128	128	>128	67.6
Trimethoprim/sulfamethoxazole	2/38	>64/1216	64/1216	>64/1216	53.0
Nitrofurantoín	32	256	128	512	43.0
PTS	256	512	256	512	-
PTSO	64	128	128	512	-
***Escherichia coli* (n=68)**					
Amoxicillin/clavulanate	16/8	256/128	64/32	>256/128	55.9
Piperacillin/tazobactam	8/4	128/4	32/4	256/4	26.5
Cefuroxime	>512	>512	>512	>512	95.6
Cefoxitin	8	128	32	256	33.8
Cefotaxime	128	>128	>128	>128	94.1
Ceftazidime	32	256	64	>512	75.0
Cefepime	16	128	64	256	80.9
Imipenem	0.5	1	2	4	0.0
Meropenem	0.06	1	0.125	4	0.0
Gentamicin	4	64	16	128	30.9
Tobramycin	4	64	16	128	33.8
Amikacin	8	32	32	128	14.7
Ciprofloxacin	64	128	128	>128	73.5
Trimethoprim/sulfamethoxazole	>64/1216	>64/1216	>64/1216	>64/1216	61.8
Nitrofurantoin	32	64	64	256	14.7
PTS	128	256	256	512	-
PTSO	64	128	128	512	-
***Klebsiella* spp. (n=39)**					
Amoxicillin/clavulanate	32/16	64/32	64/32	256/128	82.1
Piperacillin/tazobactam	32/4	512/4	64/4	>512/4	59.0
Cefuroxime	>512	>512	>512	>512	97.4
Cefoxitin	32	512	64	>512	53.8
Cefotaxime	64	>128	128	>128	97.4
Ceftazidime	64	256	128	512	76.9
Cefepime	16	64	32	256	79.5
Imipenem	1	1	2	4	0.0
Meropenem	0.06	0.25	0.125	1	0.0
Gentamicin	64	>128	64	>128	66.7
Tobramycin	32	>128	32	>128	76.9

Table 1: Continued.

Organisms (number of isolates)	MIC_{50} (in mg/L)	MIC_{90} (in mg/L)	MBC_{50} (in mg/L)	MBC_{90} (in mg/L)	% of resistant isolates
Amikacin	64	>512	128	>512	53.8
Ciprofloxacin	128	>128	>128	>128	87.2
Trimethoprim/sulfamethoxazole	>64/1216	>64/1216	>64/1216	>64/1216	79.5
Nitrofurantoin	64	128	128	256	61.5
PTS	256	512	256	512	-
PTSO	128	256	128	512	-
ESBL-producers (n=59)					
Amoxicillin/clavulanate	16/8	>256/128	64/32	>256/128	52.5
Piperacillin/tazobactam	8/4	256/4	32/4	512/4	30.5
Cefuroxime	>512	>512	>512	>512	100
Cefoxitin	8	64	32	128	25.4
Cefotaxime	128	>128	>128	>128	100
Ceftazidime	64	256	256	>512	78.0
Cefepime	32	128	64	>256	93.2
Imipenem	0.5	1	2	4	0.0
Meropenem	0.125	1	0.25	4	0.0
Gentamicin	4	128	16	>128	37.3
Tobramycin	4	128	32	>128	47.5
Amikacin	16	128	64	128	25.4
Ciprofloxacin	64	>128	128	>128	74.6
Trimethoprim/sulfamethoxazole	>64/1216	>64/1216	>64/1216	>64/1216	62.7
Nitrofurantoin	32	128	64	256	32.2
PTS	128	256	256	512	-
PTSO	64	128	128	512	-
***Salmonella* spp. and *Yersinia* spp. (n=32)**					
Amoxicillin/clavulanate	8/4	256/128	64/32	256/128	28.1
Piperacillin/tazobactam	2/4	128/4	16/4	128/4	12.5
Cefuroxime	4	>512	32	>512	25.0
Cefoxitin	8	64	64	128	12.5
Cefotaxime	1	1	8	64	12.5
Ceftazidime	4	4	16	64	0.0
Cefepime	2	32	16	64	0.0
Imipenem	1	1	16	16	0.0
Meropenem	1	1	4	8	0.0
Gentamicin	4	4	32	32	3.1
Tobramycin	4	4	32	32	6.3
Amikacin	16	128	128	256	34.4
Ciprofloxacin	1	128	8	128	37.5
Trimethoprim/sulfamethoxazole	2/38	2/38	16/304	>64/1216	3.1
Nitrofurantoin	256	512	512	1024	65.6
PTS	256	256	256	512	-
PTSO	64	128	64	128	-
Remaining enterobacteria (n=12)					
Amoxicillin/clavulanate	64/32	128/64	256/128	256/128	91.7
Piperacillin/tazobactam	4/4	64/4	8/4	256/4	25.0
Cefuroxime	>512	>512	>512	>512	100
Cefoxitin	256	>512	256	>512	66.7
Cefotaxime	64	>128	128	>128	91.7
Ceftazidime	8	128	32	512	58.3

Table 1: Continued.

Organisms (number of isolates)	MIC_{50} (in mg/L)	MIC_{90} (in mg/L)	MBC_{50} (in mg/L)	MBC_{90} (in mg/L)	% of resistant isolates
Cefepime	8	64	8	256	66.7
Imipenem	1	4	2	16	25.0
Meropenem	0.06	1	0.125	4	16.7
Gentamicin	4	32	32	>128	50.0
Tobramycin	8	32	16	>128	58.3
Amikacin	4	256	8	>512	16.7
Ciprofloxacin	1	>128	8	>128	50.0
Trimethoprim/sulfamethoxazole	2/38	>64/1216	16/304	>64/1216	50.0
Nitrofurantoin	64	>1024	128	>1024	83.3
PTS	128	256	256	256	-
PTSO	64	128	128	256	-
Nonfermenting gram-negative bacilli (n=61)					
Piperacillin/tazobactam	16/4	512/4	128/4	512/4	34.4
Ceftazidime	8	128	64	512	32.8
Cefepime	8	32	64	256	42.6
Imipenem	16	128	32	>128	52.5
Meropenem	4	64	16	128	52.5
Gentamicin	4	>128	32	>128	39.3
Tobramycin	4	>128	32	>128	27.9
Amikacin	8	128	32	256	19.7
Ciprofloxacin	32	>128	64	>128	59.0
PTS	1024	2048	1024	2048	-
PTSO	256	1024	512	2048	-
***Pseudomonas aeruginosa* (n=40)**					
Piperacillin/tazobactam	16/4	256/4	128/4	256/4	25.0
Ceftazidime	8	64	64	256	27.5
Cefepime	8	32	64	128	32.5
Imipenem	16	128	32	128	57.5
Meropenem	4	64	32	128	57.5
Gentamicin	4	>128	16	>128	37.5
Tobramycin	4	128	16	>128	17.5
Amikacin	8	32	32	128	12.5
Ciprofloxacin	32	>128	64	>128	62.5
PTS	1024	2048	2048	2048	-
PTSO	512	1024	512	2048	-
Remaining nonfermenting gram-negative bacilli (n=21)					
Piperacillin/tazobactam	128/4	512/4	512/4	>512/4	52.4
Ceftazidime	8	128	64	512	42.9
Cefepime	16	64	128	256	61.9
Imipenem	2	>128	16	>128	42.9
Meropenem	2	16	8	>128	42.9
Gentamicin	4	>128	32	>128	42.9
Tobramycin	4	>128	32	>128	47.6
Amikacin	16	256	64	256	33.3
Ciprofloxacin	4	>128	32	>128	52.4
PTS	128	512	128	512	-
PTSO	64	256	64	256	-

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; % of resistant isolates: percentages of isolates intermediate or resistant according to the criteria published by the CLSI (2016).

Table 2: Activity *in vitro* of PTS, PTSO, and others antibacterial agents against gram-positive organisms.

Organisms (number of isolates)	MIC_{50} (in mg/L)	MIC_{90} (in mg/L)	MBC_{50} (in mg/L)	MBC_{90} (in mg/L)	% of resistant isolates
***Staphylococcus aureus* methicillin-resistant (n=112)**					
Gentamicin	4	256	16	>256	48.2
Tobramycin	64	>256	>256	>256	79.5
Erythromycin	>64	>64	>64	>64	69.6
Clindamycin	>64	>64	>64	>64	49.1
Levofloxacin	8	32	32	>64	89.3
Linezolid	2	4	4	8	0.0
Vancomycin	0.5	1	1	4	0.0
Teicoplanin	0.25	1	0.5	4	0.0
Daptomycin	0.25	0.5	0.5	2	0.0
Rifampicin	≤0.03	0.5	0.125	1	3.6
Trimethoprim/sulfamethoxazole	≤0.06	0.5	0.5	2	3.6
PTS	64	128	512	1024	-
PTSO	8	8	32	64	-
***Enterococcus faecalis* (n=54)**					
Ampicillin	1	2	2	8	0.0
Levofloxacin	32	64	>64	>64	100
Linezolid	2	2	4	8	0.0
Vancomycin	0.5	1	2	4	0.0
Teicoplanin	≤0.03	0.125	0.25	1	0.0
Daptomycin	2	4	4	8	0.0
PTS	128	128	2048	4096	-
PTSO	4	8	8	16	-
***Streptococcus agalactiae* (n=101)**					
Ampicillin	0.06	0.125	0.125	0.5	0.0
Erythromycin	>64	>64	>64	>64	94.1
Clindamycin	>64	>64	>64	>64	85.1
Levofloxacin	0.5	1	2	8	6.9
Linezolid	1	2	2	4	0.0
Vancomycin	1	1	2	4	0.0
Daptomycin	0.125	0.5	0.5	2	0.0
PTS	64	128	512	2048	-
PTSO	4	8	8	16	-

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; % of resistant isolates: percentages of isolates intermediate or resistant according to the criteria published by the CLSI (2016).

mg/L) or 42.4% to tobramycin (MIC_{50} = 4 mg/L, MIC_{90} = 128 mg/L). The resistance to aminoglycosides was higher among *Klebsiella* spp. and the group "remaining enterobacteria" than *E. coli*, *Salmonella* spp., or *Yersinia* spp. In general, enterobacteria showed high resistance to fluoroquinolones (MIC_{50} = 64 mg/L, MIC_{90} > 128 mg/L, and 67.6% of resistant isolates to ciprofloxacin) and to trimethoprim-sulfamethoxazole (MIC_{50} = 2/38 mg/L, MIC_{90} > 64/1216 mg/L, and 53.0% resistant), except for *Salmonella* spp. and *Yersinia* spp., which showed the lowest rates (37.5% and 3.1% of resistant isolates to ciprofloxacin and trimethoprim-sulfamethoxazole, respectively). *E. coli* was the bacteria with a lower resistance to nitrofurantoin (MIC_{50} = 32 mg/L, MIC_{90} = 64 mg/L, and 14.7% resistant).

As previously mentioned, bacteria were selected for their detection of resistance to, at least, a group of antibiotics. However, a relevant characteristic of the 151 enterobacteria included in the study was the high frequency to coresistance to two or more of these groups (multidrug-resistant bacteria), as described in Table 3. Therefore, 74.0% of the isolates resistant to some beta-lactams antibiotics were also resistant to ciprofloxacin, 61.8% to trimethoprim-sulfamethoxazole, and 48.8% to some aminoglycoside. It should be noted that 22.8% of that resistant to beta-lactams was also resistant to all the other groups of antibiotics assayed.

The behaviour of PTS and PTSO against multidrug-resistant enterobacteria was quite homogeneous, regardless the group analyzed (Table 1). The values of MIC_{50} and MIC_{90}

Table 3: Analysis of coresistance to different groups of antibiotics.

	Enterobacteria resistant to some beta-lactams antibiotics (n=123; 81.5%)	ESBL-producers enterobacteria (n=59; 39.1%)	Non ESBL-producers enterobacteria resistant to some beta-lactams antibiotics (n=64; 42.4%)	Non-fermenting gram-negative bacilli resistant to fluoroquinolones (n=36; 59.0%)	Methicillin-resistant *Staphylococcus aureus* (n=112)
Resistance to beta-lactams	-	-	-	75.0%	-
Resistance to aminoglycosides	48.8%	47.5%	50%	63.9%	79.5%
Resistance to fluoroquinolones	74.0%	74.6%	73.4%	-	89.3%
Resistance to TMX	61.8%	62.7%	60.9%	-	3.6%
Resistance to nitrofurantoin	41.5%	32.2%	50.0%	-	-
Resistance to macrolides (erythromycin)	-	-	-	-	69.6%
Resistance to lincosamides (clindamycin)	-	-	-	-	49.1%
Resistance to rifampicin	-	-	-	-	3.6%
Resistance to aminoglycosides & fluoroquinolones	46.3%	44.1%	50.0%	-	75.9%
Resistance to aminoglycosides & fluoroquinolones & TMX	41.5%	39.0%	45.3%	-	2.7%
Resistance to aminoglycosides & fluoroquinolones & TMX & nitrofurantoin	22.8%	22.0%	23.4%	-	-
Resistance to beta-lactams & aminoglycosides	-	-	-	55.6%	-
Resistance to aminoglycosides & fluoroquinolones & macrolides	-	-	-	-	60.7%
Resistance to aminoglycosides & fluoroquinolones & macrolides & lincosamides	-	-	-	-	45.5%

TMX: Trimethoprim/sulfamethoxazole.

of PTS ranged from 128 to 256 mg/L and from 256 to 512 mg/L, while the MBC_{50} and MBC_{90} ranged from 256 mg/L and 256 to 512 mg/L, respectively. On the other hand, the values of MIC_{50} and MIC_{90} of PTSO ranged from 64 to 128 mg/L and 128 to 256 mg/L, while MBC_{50} y MBC_{90} ranged from 64 to 128 mg/L and from 128 to 512 mg/L, respectively. These data show the bactericidal activity of both compounds (MIC and MBC values were equal or differed in only one dilution) and indicate that PTSO was significantly more active than PTS against this group of bacteria ($p<0.001$).

Among the 61 nonfermenting gram-negative bacilli, the resistance to beta-lactams antibiotics ranged from 32.8% to ceftazidime (MIC_{50} = 8 mg/L, MIC_{90} = 128 mg/L) and 52.5% to imipenem (MIC_{50} = 16 mg/L, MIC_{90} = 128 mg/L) and meropenem (MIC_{50} = 4 mg/L, MIC_{90} = 64 mg/L). Carbapenems showed more activity against bacteria such as *Acinetobacter* spp., *Aeromonas* spp., and *Stenotrophomonas* spp. (MIC_{50} = 2 mg/L, MIC_{90} > 128 mg/L, and 42.9% of isolates resistant to imipenem and MIC_{50} = 2 mg/L, MIC_{90} = 16 mg/L, and 42.9% of isolates resistant to meropenem), than against *Pseudomonas* spp. (MIC_{50} = 16 mg/L, MIC_{90} = 128 mg/L, and 57.5% of isolates resistant to imipenem and MIC_{50} = 4 mg/L, MIC_{90} = 64 mg/L, and 57.5% of isolates resistant to meropenem). Among the aminoglycosides assayed, amikacin was the most active against both groups (MIC_{50} = 8 mg/L, MIC_{90} = 128 mg/L, and 19.7% resistant). Finally, 59.0% of isolates were resistant to ciprofloxacin (MIC_{50} = 32 mg/L, MIC_{90} > 128 mg/L), which resulted in less active against *P. aeruginosa* isolates than against other bacteria of this group. As shown in Table 3, 75.0% of the isolates resistant to fluoroquinolones (ciprofloxacin) were also resistant to some beta-lactam antibiotic; 63.9% to some aminoglycoside and 55.6% showed resistance to these three groups of antibiotics.

Just as with the rest of antibiotics, when comparing the results obtained in *P. aeruginosa* with those obtained in others nonfermenting gram-negative bacilli, the behaviour, both of PTS and PTSO, was significantly different (Table 1). In the case of PTS, the results shown in *P. aeruginosa* were MIC_{50} = 1024 mg/L, MIC_{90} = 2048 mg/L, MBC_{50} = 2048 mg/L, and MBC_{90} = 2048 mg/L, while in the rest of bacteria they showed more activity (MIC_{50} = 128 mg/L, MIC_{90} = 512 mg/L, MBC_{50} = 128 mg/L, and MBC_{90} = 512 mg/L) ($p < 0.001$). Likewise, the results for PTSO indicated less activity against *Pseudomonas* spp. (MIC_{50} = 512 mg/L, MIC_{90} = 1024 mg/L, MBC_{50} = 512 mg/L, and MBC_{90} = 2048 mg/L) than against the rest of isolates (MIC_{50} = 64 mg/L, MIC_{90} = 256 mg/L, MBC_{50} = 64 mg/L, and MBC_{90} = 256 mg/L) ($p < 0.001$). In any case, these data also indicate the bactericidal activity of both compounds, especially PTSO that showed significantly more activity than PTS ($p < 0.001$).

Concerning the gram-positive cocci, all the isolates were susceptible to vancomycin, teicoplanin (*S. agalactiae* was not tested), daptomycin, and linezolid. Besides, all the isolates of *E. faecalis* and *S. agalactiae* were also susceptible to ampicillin (Table 2).

All the isolates of *S. aureus* were resistant to methicillin (this was the selection criteria in this bacteria) and therefore to all beta-lactams antibiotics. High rates of resistance to fluoroquinolones (MIC_{50} = 8 mg/L, MIC_{90} = 32 mg/L, 89.3% resistant to levofloxacin), to aminoglycosides (MIC_{50} = 64 mg/L, MIC_{90} > 256 mg/L, 79.5% resistant to tobramycin), to macrolides (MIC_{50} > 64 mg/L, MIC_{90} > 64 mg/L, 69.6% resistant to erythromycin), or to lincosamides (MIC_{50} > 64 mg/L, MIC_{90} > 64 mg/L, 49.1% resistant to clindamycin) were observed. In contrast, trimethoprim-sulfamethoxazole (MIC_{50} < 0.06 mg/L, MIC_{90} = 0.5 mg/L, 3.6% resistant) and rifampicin (MIC_{50} < 0.03 mg/L, MIC_{90} = 0.5 mg/L, and 3.6% resistant) showed the lowest rates of resistance. The 75.9% of these bacteria were resistant, both to aminoglycosides and fluoroquinolones, and 60.7% also showed resistance to macrolides and 45.5% also to clindamycin (Table 3). Finally, 100% of isolates of *E. faecalis* were resistant to levofloxacin (MIC_{50} = 32 mg/L, MIC_{90} = 64 mg/L) and resistance to any other antibiotic was not associated, whereas 86 out of 101 isolates of *S. agalactiae* were resistant to erythromycin and clindamycin.

PTSO showed significantly more activity than PTS in the three groups of gram-positive bacteria tested ($p < 0.001$, in all cases) and the values for MIC_{50}, MIC_{90}, MBC_{50}, and MBC_{90} were, for both compounds, lower than those obtained against gram-negative bacteria (Table 2). However, MIC and MBC values in gram-positive bacteria differed significantly, especially for PTS (more than 2 dilutions), which indicates that these compounds could have a bacteriostatic but not a bactericidal effect against these bacteria at least at low concentrations.

4. Discussion

Organosulfur compounds obtained from *Allium* spp. such as PTS and PTSO have been proposed as an effective alternative to antibiotics to improve animal performance and prevent gastrointestinal disorders. This is due on the one hand to their greater stability in comparison to other natural compounds [13] and on the other hand to their activity against bacterial groups, such as *Enterobacteriaceae*, *Staphylococcus* spp., *Enterococcus* spp., *Clostridium* spp., *Bacteroides* spp., *Lactobacillus* spp., *Bifidobacterium* spp., or *Campylobacter* spp., among others [3, 4, 7]. Furthermore, it has been shown that feed supplementation with these compounds improves the digestion and absorption of nutrients in the gastrointestinal tract by modulating the intestinal microbiota and increases the villus height and mucosal thickness [7, 8]. Beyond its use in animals, it is possible that these molecules may as well be useful in the human clinical practice, due to the fact that alliaceous plants have been traditionally used for their antibacterial, antioxidant, and cardiovascular properties, as has been known for centuries [6].

To our knowledge, this is the first study to evaluate the activity of PTS and PTSO against a selection of gram-negative and gram-positive multiresistant bacteria isolated from human clinical samples. Antibiotic susceptibility tests were performed in accordance with the procedure outlined by CLSI in order to determine if a bacterium is susceptible or resistant to each of the antibiotic assayed. Although the cut-off points for PTS or PTSO are unknown, perform the assay under the same conditions as the other antibiotics allow us to make comparisons with them.

Our results revealed that PTS showed moderate activity against *Enterobacteriaceae* with MIC_{50} (and MBC_{50}) and MIC_{90} (and MBC_{90}) values of 256-512 mg/L, while PTSO showed greater activity with MIC_{50} and MIC_{90} values of 64-128 mg/L and MBC_{50} and MBC_{90} values of 128-512 mg/L. These homogeneous results among the different groups of enterobacteria selected, regardless of the resistance shown to different antibiotics commonly used in clinical practice, reveal the bactericidal action of these compounds. According to these results, Ruiz et al. also proved a bactericidal effect against enterobacteria, such as *E. coli* and *Salmonella typhimurium* [3].

The activity against methicillin-resistant *S. aureus*, *E. faecalis*, and *S. agalactiae* was higher than that observed against enterobacteria, especially in the case of PTSO (MIC_{50} = 8 mg/L, MIC_{90} = 8 mg/L, MBC_{50} = 32 mg/L, MBC_{90} = 64 mg/L, in *S. aureus*; MIC_{50} = 4 mg/L, MIC_{90} = 8 mg/L, MBC_{50} = 8 mg/L, and MBC_{90} = 16 mg/L, in *E. faecalis* and *S. agalactiae*). The PTS activity against this group of bacteria was significantly lower, especially in the case of enterococci. Some authors have evaluated the potential of garlic allicin, a molecule structurally similar to PTS, to control oral pathogens, reporting inhibitory concentrations of 600 mg/L against *Streptococcus* spp. [14]. Other studies have reported a bacteriostatic effect of allicin against vancomycin resistant enterococci [15].

However, in contrast to the relatively good results obtained previously, both compounds showed lower activity against *P. aeruginosa* (MIC_{50} = 1024 mg/L, MIC_{90} = 2048 mg/L, MBC_{50} = 2048 mg/L, MBC_{90} = 2048 mg/L, for PTS; MIC_{50} = 512 mg/L, MIC_{90} = 1024 mg/L, MBC_{50} = 512 mg/L, and MBC_{90} = 2048 mg/L, for PTSO). It is possible that PTS and PTSO may be affected by active removal mechanisms when they come in contact with these bacteria. Further research is needed to determine with certainty the mechanisms involved in this increased resistance.

All these results are in agreement with the antibacterial effects of garlic previously described in the literature against bacterial isolates from animals and reference strains [3–6]. However, MBC determined in our experiment were much higher compared to Llana-Ruiz-Cabello et al. who demonstrated MBC lower than 5 mg/L in all cases [16]. The differences may be caused by different methodology.

In the present study, the values obtained for MIC and MBC in PTS and PTSO were very similar to those obtained in antibiotics such as nitrofurantoin, aminoglycosides, fluoroquinolones, and some beta-lactams. Based on the data obtained from MIC, the CLSI determines that a very large percentage of enterobacteria should be resistant to these antibiotics (as shown in Tables 1 and 2). It should therefore not be considered for clinical use. Likewise, we may think that the activity shown by PTS and PTSO should also not be considered for clinical use in humans considering the results obtained. However, due to the lack of susceptibility cut-off points for the compounds derived from garlic, no final conclusion can be drawn.

In correspondence with the need of discovering new potentially antibacterial natural products, the activity of these organosulfur compounds described in this study may be considered as promising. Furthermore, the use of naturally and potentially innocuous compounds that can be administered without high restrictions provided us with the possibility to discuss the viability of their application for the treatment of specific infectious pathologies, provided that adequate formulations are developed.

In our opinion, several therapeutic possibilities may be considered, i.e., superficial skin infections, such as acne, folliculitis or impetigo by topical use, the treatment of oral and gastrointestinal infections by oral administration, or even the treatment of urinary tract infections caused by multidrug-resistant bacteria applied by intravesical instillation (in the same way that colistin is used). The concentration of the substance in the source of the infection should always be high enough to guarantee that it exceeds the values of MIC against the bacteria causing these processes.

It is clear that, in order to evaluate the real effectiveness of these substances, either in this or another situation, further testing would be necessary with a more diverse and larger group of bacteria. Furthermore, it would be necessary to establish suitable administration routes for the compounds and its efficacy *in vivo*. Finally, the concentrations that they achieve in the different tissues and fluids would also need to be known.

Lastly, PTS and PTSO are perceived as harmless since these compounds occur naturally in foods such as garlic or onion. Nevertheless, further studies on pharmacokinetic and toxicological characteristics are required before safe clinical use is considered. Some recent studies on cell lines and experimental animals reported low acute and subchronic oral toxicity in PTSO and a lack of genotoxicity, both *in vitro* and *in vivo* models [16–19].

5. Conclusion

Our results demonstrate that PTS, but mainly PTSO, have a significant broad spectrum antibacterial activity against a selection of gram-negative and gram-positive multiresistant bacteria isolated from human clinical samples. Further work is needed to demonstrate the effectiveness of these compounds *in vivo* models, although preliminary results in present work provide basic and useful information for development and its potential use in the treatment of human infections.

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 wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Research Article

Effect of Ionizing Radiation on the Microbiological Safety and Phytochemical Properties of Cooked *Malva sylvestris* L.

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Nowadays, recent studies have demonstrated that plant-derived foods were characterized by their richness in bioactive phytochemicals and their consumption has a protective effect for human health. The effects of ionizing radiation on phytochemical properties of cooked *Malva sylvestris* L. (Mallow) were investigated. Irradiation increased significantly *($P<0.05$)* the total polyphenols and flavonoids content of cooked Mallow. Irradiation at 2 and 4 kGy doses resulted in a significant increase in the DPPH and ABTS radical-scavenging ability of cooked Mallow extracts. There was no significant change on carbohydrate, lipid, ash, and protein content. While the mineral composition of K and Na was affected slightly after irradiation, the amounts of Mg, P, Ca, Fe, Z, and Cu remain unaffected at 2 kGy and reduced slightly at 4 kGy. The antimicrobial activity was unaffected after irradiation. Postirradiation storage studies showed that the cooked irradiated Mallow was microbiologically safe even after 20 days of storage period. Sensory properties of cooked irradiated Mallow were unaffected by the treatment. This study supports that cooking process followed by gamma irradiation did not compromise the chemical composition and sensory characteristics of Mallow.

1. Introduction

Aromatic and medicinal plants have been reported to contain a higher content of bioactive phytochemicals such as substantial amount of vitamins, phenolic compounds, and essential oils and thus can be used as important sources of natural antioxidants for food application and pharmaceuticals [1]. Currently, scientists are interested in developing value added products from wild and cultivated plants [2]. In fact, it is very important to increase the antioxidant intake in our nutrition, for that, there is a considerable attention to enriching food products with aromatic and medicinal plants which are considered rich in natural antioxidants [3]. *Malva sylvestris* L. (Malvaceae family) known as common Mallow, is one of the most well-known medicinal herbs. Native to Europe, North Africa, and Asia, it is largely cultivated in the Mediterranean countries including Tunisia. In particular, flowers and leaves are used as a remedy for dermal infected wounds [4]. The therapeutic guide of herbal medicine in German, France, and Switzerland approved Mallow for cough [5], bronchitis, and inflammation of the mouth and pharynx [6]. Young leaves are eaten raw in salads, and leaves and shoots are consumed in soups and as boiled vegetables. Immature fruits are sucked or chewed by children, shepherds, and hunters. In Tunisia, Mallow leaves can be cooked traditionally and served as a dish. According the literature, there are many phytochemicals studies of these plants [7, 8] and to the best of our knowledge, no studies focused on the effect of ionizing radiation effect on cooked Mallow have been reported. With today's demand for high-quality convenience foods, irradiation in combination with other processes holds a promise for enhancing the safety of many minimally processed foods. Ionizing radiation

has been demonstrated to be very effective for pathogen inactivation in both raw and cooked foods [9]. A 10 kGy dose is permitted by the World Health Organization for irradiation sterilization of food where toxicity testing is not necessarily involved [10]. Thus, the aims of the present report were to study the effect of ionizing radiation of cooked Mallow leaves on their microbiological safety, phytochemical, sensory, and antioxidant properties.

2. Materials and Methods

2.1. Plant Material and Sample Preparation. Samples of Mallow were collected in February 2017 from the region of Jdaida (Manouba). The collected plant material consisted of total aerial parts. The pretreatment and cooking procedures were adapted from [11]. The vegetables were washed in water and all inedible parts were removed manually. 100 g of leaves was added to 500 ml of water and cooked for 2-3 minutes at 50°C in pressure cooker thermostatically controlled. Samples were drained and rapidly cooled with cold water. Then, the samples were stored in sealed boxes (Length: 14 cm, width: 9 cm, depth: 6 cm) at 4°C. The use of only leaves is mainly due to the follow-up of the traditional cooking process of Mallow.

2.2. Irradiation of Cooked Mallow. The Tunisian gamma irradiation facility (at Sidi Thabet) is designed for the preservation of food stuff and sterilization of medical devices. The source consists of eight encapsulated 60Co pencils with a diameter of 9.7 mm and an overall length of 452 mm. The starting activity of the source was 99.162 kCi. The installation is equipped with a stainless steel telescopic source rack that allows obtaining a linear source of approximately 900 mm height. The source pencils are distributed circularly on a diameter of 140 mm for the upper source rack and of 80 mm for a lower one. The source rack comprises 20 housings allowing sources loading for several years. These sources are stored in dry condition in a cylindrical shield container in which they were transported. Mallow samples were exposed to gamma radiation dose of 2 and 4 kGy at a dose rate of 22.21 Gy/min and at room temperature (27±2°C). Nonirradiated (0kGy) samples were kept at 4°C and used as a control for comparative analysis. The irradiation time was 1.5 and 3 hours, respectively, for 2 and 4 kGy. Each experiment was done in triplicate.

2.3. Polyphenols Extraction. Irradiated Mallow cooked and control samples were dried at room temperature and ground to a fine powder. Thirteen to fourteen grams of Mallow powder from nonirradiated and irradiated samples is macerated in the presence of 140 ml of aqueous methanol solvent (80 % v/v). After filtration, the methanol solvent was evaporated at 40°C on a rotary evaporator. To prevent oxidation of the polyphenols, extraction was achieved rapidly and extracts were immediately used or conserved in darkness at -20°C until further use [12].

2.4. Total Polyphenols Content (TPC). The TPC of Mallow extracts was estimated spectrometrically by the Folin–Ciocalteu method, as described by Lin and Tang [13]. Briefly, 100 μL of diluted sample was added to 400 μL of 1:10 diluted Folin–Ciocalteu reagent. After 5 min, 500 μL of 10 % (w/v) sodium carbonate solution was added. Following 1 h of incubation at room temperature, the absorbance at 765 nm was measured in triplicate. TPC was calculated from the equation determined from linear regression after plotting known solutions of Gallic acid (10–100 ppm). Results are expressed in mg of Gallic acid equivalent (GAE) per gram of dry weight (dw) of plant material.

2.5. Total Flavonoids Content (TFC). The TFC in the extracts was determined by a spectrophotometric method based on the formation of complex flavonoid-aluminium with an absorptivity maximum between 420 and 430 nm [14]. Briefly, 500 μL of each extract was separately mixed with 1500 μL methanol (95 %), 100 μL of AlCl310 % (m/v), 100 μl of sodium acetate 1M, and 2.8 mL of distilled water. The experiments were run in triplicate, and after incubation at room temperature for 30 minutes, the absorbance of the reaction mixtures was measured at 420 nm. The TFC values were determined from a standard curve prepared with quercetin (ranging from 10 to 50 μg/mL final volumes) and expressed as mg quercetin equivalents (QE) / g dw.

2.6. Assessment of Antioxidant Capacity

2.6.1. DPPH Scavenging Activity. The antioxidant activity of the polyphenolic extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical [2]. A DPPH methanolic solution was prepared at a concentration of 4 x10-5 M. Then, 1 mL of the stock DPPH solution was added in each test tube, followed by the addition of 25 μL of each polyphenolic extract. In parallel, the control was prepared containing all reagents except the polyphenolic extract and methanol was used as a blank solution. The mixture was shaken vigorously and left in the dark at room temperature. After 60 min, readings were taken using a spectrophotometer at a wavelength of 517 nm. Percent inhibition of the DPPH radical by the samples was calculated according to the formula Yen and Duh (1994):% inhibition = ((AC(o) – AS(t)) / AC(o) * 100, where AC(o) is the absorbance of the control at t = 0 min and AS(t) is the absorbance of the sample at t = 60 min.

2.6.2. ABTS Radical-Scavenging Assay. The radical-scavenging capacity of antioxidant for the ABTS (2,2′-azinobis-3-ethylbenzothiazoline- 6-sulphonate) radical action was determined as described by Belkhir et al. [15]. The absorbance of the reaction mixture was measured at 734 nm and compared to the antioxidant potency of Trolox used as a reference. The results were expressed in terms of Trolox. The estimate of the antiradical activity is expressed by the value of the inhibition percent (% I) calculated using the following formula: %I = [(Abs0 – Abs1)/Abs0)] x100.

2.7. Vitamins. The contents of individual vitamin C and vitamin E (alpha-tocopherol, beta-tocopherol, and gamma-tocopherol) were quantified by High Performance Liquid

Chromatography (HPLC), based on the normalized methods EN 14130, 2003 and EN 12822, 2014, respectively [16, 17].

2.8. Lipids. A Soxtec System Extraction Unit Tecator was used. The crude fat was determined by extracting 0.5 g of freeze-dried sample with petroleum ether. Containers were removed and dried at 105°C, cooled, weighted, and expressed as mg/100 mg.

2.9. Total Proteins, Carbohydrates, Ash, and Moisture Content. Total proteins were determined as the nitrogen content by the Kjeldahl method according to the AOAC method AOAC 1995 [18]. The carbohydrate content was determined by titration in the presence of methylene blue: the Lane–Eynon method AOAC 2005 [19]. The AOAC method 942.05 was used for the determination of ash content [20]. The moisture was determined according to the AOAC 1996 [21].

2.10. Mineral Analysis by Atomic Absorption Spectrophotometer. Different mineral constituents (potassium [K], sodium [Na], calcium [Ca], magnesium [Mg], iron [Fe], zinc [Zn], copper [Cu], and phosphor [P]) were analyzed separately using an atomic absorption spectrophotometer.

2.11. Antimicrobial Effect. Whatman filter paper is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish Mueller-Hinton solidified with agar and sterilized in a hot air oven. The loop used for delivering the antibiotics is made of 20 gauge wire and has a diameter of 2 mm. This delivers 15 μL of treated and untreated cooked Mallow extract (200 mg/mL) to each disc. The bacterial concentration of *Salmonella typhimurium ATCC 14028, Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecium* ATCC 19434, and *Streptococcus agalactiae* ATCC 13813 is 108 CFU/mL.

2.12. Microbial Decontamination of Cooked Irradiated M. sylvestris. 10 g of each sample (irradiated and nonirradiated) was homogenized in sterile Stomachers bags containing 90 ml of sterile buffered peptone water for 2 minutes using a Blender stomacher (Model 400). This mixture corresponds to the diluted 1/10 of stock suspension. A dilution series is thus prepared from 10-1 to 10-7. The fraction of surviving microorganisms in sublethally irradiated samples and unirradiated was determined according to the standards ISO 4833-1 2013 [22], for total aerobic mesophilic flora, the standard NF V 08 – 060, 2009 for fecal coliforms [23], the standard XP V 08 057 – 1, 2004 for *Staphylococcus aureus* [23], the standard NF ISO 6579, 2002 for *Salmonella* [24], and the standard ISO 7954, 2002 for molds and yeast [25].

The total mesophilic flora counting, 1 mL of each dilution, is spread on the solidified PCA medium and then incubated 72 hours at 30°C. For fecal coliforms, 1 ml of each dilution is seeded in double layer in VRBG agar medium. The inoculum is thoroughly mixed with the culture medium until solidification and incubated 24 hours at 44°C. For the counting of *Staphylococcus aureus*, 0.1 ml of the Mallow homogenate is deposited on the surface of Baird-Parker agar medium with egg yolk and potassium tellurite. Then, the inoculum is spread as fast as possible on the surface of the BP agar medium. The dishes are incubated at 37°C for 48 h.

For Salmonella enumeration, 1 ml of the preenrichment medium is transferred into 10 ml of the selenite cysteine broth medium. The medium is incubated 24 hours at 44°C.

After 24 h incubation, inoculate with a platinum loop in parallel striations on the four-side surface of the Petri dishes containing the Hektoen selective isolation medium, and the dishes are then incubated at 37°C for 24 h. The typical colonies of Salmonella are green with black centers or green or bluish colonies

For molds and yeast, the medium used for the counting of yeasts and molds is Sabouraud agar and incubation was maintained at 30°C for 3-4 days.

2.13. Sensory Analysis. Sensory acceptances of the cooked Mallow and irradiated cooked Mallow at 2 and 4 kGy were evaluated with 10 experimented panelists. Samples were presented in an anonymous way with a simple three-digit code. Three samples of Mallow were analyzed by panelists in individual cabins sensory evaluation. Panelists were instructed to evaluate each attribute using a ten-point hedonic scale ranging from "dislike extremely" to "like extremely". Six different parameters were used to grade the overall quality in terms of color intensity, herbaceous smelling, flavor, melting texture, cooking taste, and overall acceptance. The proposed question was: How much do you like this product on a scale of 1 to 10, where 1 = dislike extremely, and 10 = like extremely?

2.14. Statistical Analysis. The results of this work were analyzed using SPSS software, version 20, by an analysis of variance test (ANOVA) to compare different means between the control samples (nonirradiated) and irradiated at 2 and 4 kGy.

3. Results and Discussion

3.1. Effect of Ionizing Radiation on TPC and TFC. It is well known that phenolic and flavonoids substances contribute directly to the antioxidant activity of plant materials. In fact, phenolic compounds exhibit considerable free radical-scavenging activities (through their reactivity as hydrogen-donating or electron-donating agents) and metal ion-chelating properties. The herein obtained TPC and TFC of the unirradiated cooked Mallow were, respectively, 186.59±14,55 mg GAE/g dw and 12,17 ± 3,88 (mg QE/g dw) (Table 1). The obtained results are in agreement with those reported by [7, 25]. The TPC of the extracts obtained from irradiated cooked Mallow at 2 kGy increases significantly ($P<0.05$) by 13.8% (212.4 ± 11.7g /g dw) compared to the unirradiated ones (Table 1). This increase is more pronounced for the samples treated at 4 kGy reaching the rate of 104 % (382.25 ± 19.35 /g dw). The same trend was observed for TFC and a pronounced increase, reaching 5 and 7 times, respectively, for 2 and 4 kGy in irradiated cooked Mallow compared to the unirradiated samples suggesting that flavonoids are less radioresistant than the other phenolic classes. Harrison and Were [26] studied the irradiation effects of almond skin extracts and

Table 1: Polyphenols and flavonoids content of untreated and irradiated Mallow at 2 and 4 kGy (n=3).

Doses (kGy)	0	2	4
TPC (mg EAG/g dw)	$186.59^{a} \pm 14.55$	$212.4^{b} \pm 11.7$	$382.25^{c} \pm 19.35$
TFC (mg QE/g dw)	$12.17^{a} \pm 3.88$	$65.80^{b} \pm 16.75$	$92.53^{c} \pm 10.45$
DDPH EC_{50} (μg/ml)	$158.81^{a} \pm 3.62$	$150.63^{b} \pm 066$	$101.8^{c} \pm 0.2$
ABTS EC_{50} (μg/ml)	$65.96^{a} \pm 0.56$	$61.1^{b} \pm 0.9$	$54.81^{c} \pm 0.43$
Vitamin C (mg/100 g)	$24.32^{a} \pm 0.67$	$25.53^{b} \pm 0.89$	$26.67^{c} \pm 0.02$
α-tocopherol (mg/100 g)	$87.94^{a} \pm 1.32$	$86.54^{a} \pm 1.02$	$87.53^{a} \pm 0.54$
β-tocopherol (mg/100 g)	$5.56^{a} \pm 0.86$	$5.34^{a} \pm 0.81$	$5.32^{a} \pm 0.84$
γ-tocopherol (mg/100 g)	$15.54^{a} \pm 0.32$	$14.89^{a} \pm 1.25$	$14.68^{a} \pm 1.53$
Carbohydrates (g/100g)	$80.65^{a} \pm 0.37$	$81.05^{a} \pm 0.57$	$79.76^{a} \pm 0.83$
Lipids (g/100g)	$2.34^{a} \pm 0.03$	$2.28^{a} \pm 0.12$	$2.31^{a} \pm 0.04$
Proteins (g/100g)	$14.21^{a} \pm 1.01$	$15.5^{b} \pm 1.5$	$16.4^{c} \pm 1.42$
ash (g/100g)	$12.98^{a} \pm 0.02$	$12.56^{a} \pm 0.76$	$12.78^{a} \pm 0.83$
Moisture (%)	$75.76^{a} \pm 0.53$	$75.21^{a} \pm 0.32$	$75.01^{a} \pm 0.51$

Values followed by the same letter along the row are not significantly different ($P<0.05$).

found that TPC increased at 4 and 12.7 kGy. A similar increase of TPC and TFC was reported in the literature for irradiated Purslane (Portulaca oleracea) Plant [27] and Dill herb irradiated at 2, 4, and 8 kGy [28]. This increase of the TPC is due to the release of phenolic compounds from the glycosidic components and degradation of the larger phenolic molecules into smaller ones by gamma irradiation [26]. In addition, this increase is probably related to the effect of the irradiation, which breaks down the polyphenol chemical bonds and consequently induces the release of low molecular weight and soluble phenols. Similar observations have been reported for different plant material treated with different doses of ionizing radiation [29].

3.2. Effect of Ionizing Radiation on the Antioxidant Capacity. Table 1 showed a significant increase *($P<0.05$)* of the antioxidant activity value in the irradiated cooked Mallow at 2 and 4 kGy. The EC50 calculated from the calibration curve DPPH = f (Trolox) of irradiated cooked Mallow at 2 and 4 kGy were, respectively, 150.63 ± 0.65μg/mL and 101.79 ± 0.17μg/mL, significantly lower *($P<0.05$)* than that of unirradiated sample (158.81 ± 3,62μg/mL) (Table 1). The same trend was observed in the ABTS radical-scavenging activity. Indeed, the EC50 calculated from the calibration curve ABTS= f (Trolox) were 61.1± 0.9μg/mL and 54.81 ± 0.28μg/mL, respectively, for 2 and 4 kGy (Table 1), significantly lower *($P<0.05$)* than that of unirradiated sample (65.96±0.56μg/mL). The significant increase in TPC was thus suggestive of their enhanced antioxidant properties. Similar report funded by Mohammad Akbari et al. [30] showed that ionizing radiation leads to an increase of TPC followed by an increase of antioxidant property in three different Persian pistachio nuts.

3.3. Effect of Ionizing Radiation on Vitamin C, E, Carbohydrates, Lipids, and Proteins. The nutrient composition is depicted in Table 1. The vitamin C content (24.32 mg/100 g) was higher than those reported by **Barros et al.** [7]. The carbohydrates, lipids, proteins, and ash content were similar to those described by the same authors. Therefore, the cooked Mallow is an excellent source of antioxidant phenols and flavonoids, being vitamin E (α-tocopherol) the most abundant component.

Studies on the effects of ionizing radiation in macronutrients revealed no significant difference in total carbohydrates and lipids between irradiated (2 and 4 kGy) and nonirradiated samples (Table 1). Data obtained by other authors also showed that gamma irradiation, using a dose up to 10 kGy, did not induce significant loss on lipid and carbohydrates content [10, 31]. The ash and moisture content were stable after irradiation. Significant increase in quantity of proteins was observed after irradiation at 2 and 4 KGy (Table 1). This increase could be attributed to the fact that the gamma irradiation can lead to the degradation or polymerization of protein enhancing the solubility of nitrogen [32]. Vitamins E and C appeared to be stable after irradiation since no loss was observed in an irradiated cooked Mallow.

3.4. Effect of Ionizing Radiation on Mineral Composition. Mineral element's contents of the irradiated and unirradiated cooked Mallow are shown in Table 2. The elements K, P, Na, Mg, and Ca were the major inorganic constituents, while Fe, Zn, and Cu were also present as minor constituents. The values reported for these elements in this study were in agreement with the findings of Hiçsönmez et al. [33]. The Na and K concentration in the control were, respectively, 460.558±20.149 and 952.934±36.413 mg/ 100 g which was significantly reduced at 2 and 4 kGy (Table 2).

While the finding reported by Sanni et al. [3] indicating that Na and K in irradiated Sorrel Seeds were dramatically reduced after 2.5 and 5 kGy, our results showed a slight decrease in these elements and the negative effect of irradiation on sodium and potassium may not be sufficient reason

Table 2: Mineral composition of untreated and irradiated cooked Mallow at 2 and 4 kGy on dry weight basis mg/100 g (n=3).

	Dose (kGy)		
Minerals	0	2	4
Potassium	952.934[a] ±36.413	749.153[b] ±27.085	631.293[c] ±4.328
Sodium	460.558[a] ±20.149	336.712[b] ±36.828	235.782[c] ±10.733
Phosphor	379.967[a] ±7.611	369.832[a] ±9.545	330.963[b] ±3.155
Copper	2.416[a] ± 0.198	2.425[a] ±0.076	1.955[b] ±0.091
Zinc	6.646[a] ± 0.078	6.564[a] ±1.234	5.014[b] ±0.029
Magnesium	189.160[a] ±3.925	188.712[a] ±1.560	145.419[b] ±2.592
Iron	16.137[a] ±0.639	16.384[a] ±0.735	12.791[b] ±0.997
Calcium	199.889[a] ±2.541	197.97[a] ±1.93	161.138[b] ±0.637

Values followed by the same letter along the row are not significantly different (P<0.05).

to foreclose the use of ionizing radiation on cooked Mallow. However, the concentrations of Mg, P, Ca, Fe, Z, and Cu were not affected at 2kGy and reduced slightly at 4 kGy (Table 2).

3.5. Microbial Decontamination. Table 3 shows microbial counts measured by plate method in control and irradiated cooked Mallow. The initial mean populations of the total aerobic mesophilic flora and total coliforms were 3×102 CFU/g and 103 CFU/g, respectively (Table 3). These concentrations are low due to the cooking process used before irradiation. The pathogenic *Salmonella*, *Staphylococcus aureus* bacteria, and fecal coliforms were absent in cooked Mallow samples. Samples that were irradiated at 2 and 4 kGy did not show any molds and yeast count after 20 storage days (Table 3). This result was in agreement with findings reported by Farkas indicating that molds, fungi, and coliforms are eliminated by doses lower than those required for bacteria [9]. Previous studies indicated the minimum dose as low as 4-5 kGy will destroy these organisms. The ionizing radiation at 2 and 4 kGy, compared to the control sample, reduced considerably the amount of total mesophilic bacteria and total coliforms in 10 and 20 days of storage to the permissible level recommended by the World Health Organization [9]. Thus, the cooking process followed by ionizing radiation at low doses 2 and 4 kGy improves the microbiological safety of cooked irradiated Mallow.

3.6. Effect of Ionizing Radiation on Antimicrobial Activity. The analysis of cooked Mallow antimicrobial activity was investigated immediately after irradiation at 2 and 4 kGy. The mean of zone inhibiting growth (ZIG) for irradiated cooked Mallow extract was particularly unchanged ($P<0.05$) at 2 and 4 kGy (Table 4) and confirms that the irradiation dose of 2 and 4 kGy has no significant effect on the antimicrobial activities of an irradiated cooked sample against gram positive bacteria, i.e., *Staphylococcus aureus, Enterococcus faecium,* and *Streptococcus agalactiae* ATCC, and gram negative bacteria like *Salmonella typhimurium* and *Escherichia coli*. Previous works in concordance with our results demonstrated that pharmacological activity of medicinal herbs has been found satisfactory after microbiological decontamination by irradiation [34]. In addition, pharmacological tests of Brazil medicinal herbs concluded identical therapeutic action as unirradiated preparations after exposure to a dose of 10, 20, and 30 kGy of ionizing radiation [35]. Thus, the antimicrobial activity of bio-actives substances in Mallow did not change significantly after cooking process followed by ionizing radiation.

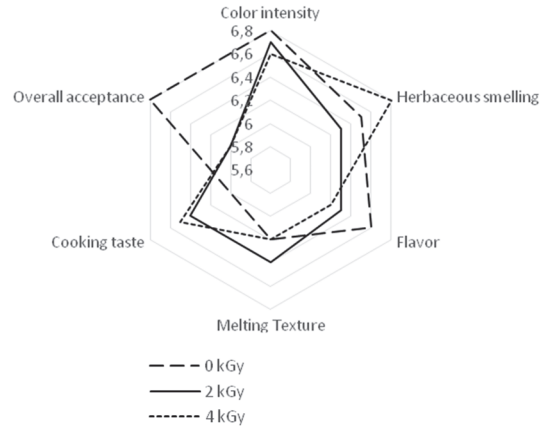

Figure 1: Mean sensorial ratings of untreated (0kGy) and cooked irradiated Mallow at 2 and 4 kGy.

3.7. Effect of Ionizing Radiation on Sensory Characteristics. Testers were invited to express a judgment of pleasantness ranging from "1 Dislike Very Much" to "10 like Very Much" to indicate their preferences. Mean sensorial ratings for all samples irradiated at 2 and 4 kGy received good overall acceptance scores, not significantly *($P<0.05$)* different from unirradiated samples. The lowest mean score was 6 on a scale of 1 to 10 (Figure 1). Given these results, it can be asserted that irradiation at doses 2 and 4 kGy might well apply for decontamination of cooked Mallow without adversely affecting their sensory attributes.

4. Conclusions

In this work, the effect of cooking process followed by ionizing radiation at low doses of *M. sylvestris* was investigated. As summarized in supplementary materials (available here) the results obtained from this study confirm the significant increase of TPC and TFC after ionizing radiation.

Table 3: Effect of postirradiation on microbial load (CFU/g) of cooked Mallow during 20 days of storage at 3°C ± 1°C (n=3).

Dose (kGy)	**Total aerobic mesophilic flora (CFU/g)**			**Fecal coliforms (CFU/g)**			**Molds and yeast (CFU/g)**			***Staphylococcus aureus* (CFU/g)**			***Salmonella* (CFU/g)**			**Total coliforms (CFU/g)**		
Days	0	10	20	0	10	20	0	10	20	0	10	20	0	10	20	0	10	20
0	$3\times10^2\pm30$	$4\times10^3\pm52$	$2\times10^5\pm34$	Abs∗	Abs	Abs	1	4	36 ± 5	Abs	Abs	Abs	Abs	Abs	Abs	$10^3\pm22$	$4\times10^3\pm28$	$4\times10^4\pm 62$
2	<10	19	$2\times10^2\pm38$	Abs	Abs	Abs	0	0	0	Abs	Abs	Abs	Abs	Abs	Abs	<10	<10	$10^2\pm 51$
4	<10	13	$10^2\pm25$	Abs	Abs	Abs	0	0	0	Abs	Abs	Abs	Abs	Abs	Abs	<10	<10	10

Table 4: Antimicrobial activities of untreated and cooked irradiated Mallow under 2 and 4 kGy dose. The zone inhibition growth (ZIG) was measured in triplicate (n=3), SD= standard deviation.

	Irradiation dose (kGy)		
Strains	**0**	**2**	**4**
		ZIG (mm)	
Staphylococcus aureus ATCC 6538 G(+)	7±2[a]	8±3[a]	8±1[a]
Salmonella typhimurium ATCC 14028 G(-)	11.5±1[a]	10±2[a]	10±3[a]
Enterococcus faecium ATCC 19434 G(+)	12±2.5[a]	11±3[a]	9.5±4[a]
***Streptococus B G(+)*ATCC 13813**	7±2[a]	8±1.5[a]	7±2[a]
***Escherichia coli G(-) ATCC* 8739**	**7**±0.5[a]	**7**±1[a]	**7**±2[a]

Values followed by the same letter along the row are not significantly different (P<0.05).

Antioxidant response was also manifested in the increase of DPPH and ABTS scavenger ability at 2 and 4 kGy applied doses. The antimicrobial activity of irradiated cooked Mallow was significantly unaffected at 2 and 4 kGy. The mineral composition was slightly affected in K and Na amount after irradiation; however, the amount of Mg, P, Ca, Fe, Z, and Cu was unaffected at 2kGy and reduced slightly at 4 kGy. The nutraceutical properties of irradiated cooked Mallow were not affected after irradiation. Therefore, cooking process followed by ionizing radiation improves the microbiological safety and maintaining sensory characteristics or even enhancing the antioxidant activity. It may emerge as one of the important techniques for preserving or improving the nutritional effect of the edible medicinal plant.

Abbreviations

KGy: KilloGray
DPPH: 2,2-diphenyl-1-picrylhydrazyl
ABTS: 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Trolox: 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid
TPC: Total polyphenols content
TFC: Total flavonoids content
GAE: Gallic acid equivalent
QE: Quercetin equivalent
HPLC: High Performance Liquid Chromatography
AOAC: Official Method of Analysis Chemistry
CFU: Colony Forming Unit.

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 paper.

Supplementary Materials

Graphic summary description. The cooked Mallow stored in sealed boxes was irradiated at 2 and 4 kGy in Tunisian 60Co gamma irradiation facility. The starting activity of the source was 99.162 kCi. The results obtained from this study confirm the significant increase of TPC after ionizing radiation at 2 and 4 kGy. Global acceptance ratings for all samples irradiated at 2 and 4 kGy received good overall acceptance scores, not significantly different from unirradiated samples. *(Supplementary Materials)*

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Research Article

Synthesis and Evaluation of Biological Activity of New Arylphosphoramidates

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The synthesis of new substituted arylphosphoramidates is performed in two steps through phosphorylation of the corresponding alcohols followed by aminolysis. The formation of the desired phosphoramidates depends on the subsequent addition of the two alcohols with the amine being added at the last step. The products were obtained in 58–95% yields. They were characterized mainly by multinuclear (^{1}H, ^{13}C, ^{31}P, and ^{19}F) NMR and IR spectroscopy. In addition, the antimicrobial and antiacetylcholinesterase activities were evaluated. The results showed acetylcholinesterase activity by some compounds, whilst no significant inhibitory effect against the tested bacterial strains has been recorded.

1. Introduction

Organophosphorus compounds are widely used as pesticides and chemical weapon agents because of their inhibitory effect on acetylcholinesterase [1]. The development in the field of medicinal chemistry of these compounds is currently characterized by a more marked orientation towards the synthesis of their derivatives as prodrugs for pharmaceutical purposes [2, 3]. Recent studies have shown that phosphoramidates and phosphates can be used as anticancer agents [4, 5], anti-HIV [6], and against Alzheimer's disease [7]. It was shown [8] that some phosphoramidates are active against strains of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*. It was also shown [9] that they are bacterial enzyme inhibitors, aspartate semialdehyde dehydrogenase (ASA-DH), which is involved in the biosynthesis of the aspartate family of amino acids. The biological activity of these compounds was also shown to depend significantly on the phosphorus atom substituents [10]. Thus, p-nitrophenylphosphoramidate derivatives were proven to be considerably stronger [11] than the methamidophos which is known for its acetylcholinesterase (AChE) inhibition and insecticidal property [12]. Furthermore, it was shown that phosphoramidates could be very useful for studying the mechanism of prophylaxis against poisoning by organophosphates and also reported that the p-nitrophenylphosphoramidates protect the guinea pigs against poisoning by Soman neurotoxic gas [13].

Inspired by these results and in continuation of our research on the complexing properties of phosphorylated compounds [14–17], we have already studied in a previous paper the complexes $SnCl_4$.2L by multinuclear NMR at variable temperature of a series of new arylphosphoramidates with the formula $(ArO)P(O)(NR_2)(OR')$ [18]. We have found that tuning substituents nondirectly bounded at phosphorus atom as R, R', and Ar groups have affected the donor character of the phosphoryl group towards tin atom. In this paper we describe the synthesis of these arylphosphoramidates and their biological activity tests against bacterial strains and acetylcholinesterase enzyme.

2. Materials and Methods

2.1. Chemistry

2.1.1. Synthesis of Phosphoramidates. All reactions were performed under nitrogen using anhydrous solvents. The Et_3N products, $POCl_3$, Me_2NH, Et_2NH, piperidine, morpholine, p-cresol, nitrophenol, and 2,2,2-trifluoroethanol, are commercial. The liquids are distilled before use and the solvents were dried by conventional methods. The synthesis of $CF_3CH_2OP(O)Cl_2$ was performed according to the literature [19] and the synthesis of 4-nitrophenyldichlophosphate was also performed according to literature [20]. The proton NMR spectra (TMS) at 300 MHz, ^{31}P (H_3PO_4 85%) at 121 MHz, and ^{19}F ($CFCl_3$) at 282 MHz were recorded on a Bruker AVANCE III-300. HRMS were recorded on Q-Tof 6500 Series.

2.1.2. Synthesis of 4-methylphenyl-2,2,2-trifluoroethylchlorophosphate. In an Erlenmeyer flask surmounted by a funnel under nitrogen, a solution of 2,2,2-trifluoroethyldichlorophosphate (16 mmol) was introduced into 120 mL of anhydrous ether. The mixture of p-cresol (16 mmol) with triethylamine (16 mmol) in 50 mL of anhydrous ether was added dropwise at room temperature. After 12 hours of stirring, the precipitate was removed by filtration and the filtrate concentrated distilled. A pale yellow liquid was obtained, with yield = 93%, $E_{b0,3mmHg}$ = 80°C. RMN δ: ^{31}P: 0.52; ^{1}H: 2.35 (s, CH_3-Ph); 4.5 (m, OCH_2); 7.5 (m, -Ph-, 4H).

2.1.3. Synthesis of 4-methylphenyl-2,2,2-trifluoroethyl phosphoramidates. A solution of 2,2,2-trifluoroethyldichlorophosphate (15 mmol) was placed in 100 mL of anhydrous ether in a flask equipped with a funnel under a flew of nitrogen, then HNR_2 (30 mmol) in 20 mL of anhydrous ether is added dropwise. Viscous liquid is obtained without no further purification. The ^{31}P NMR spectra show that crude compounds are pure.

4-Methylphenyl-2,2,2-trifluoroethyl dimethylamidophosphate 3a is as follows: It is a colorless viscous liquid, yielding 92%; ^{1}H NMR δ: 2.32 (CH_3-Ph); 2.76 (d, CH_3N, 6H, $^{3}J_{H-P}$ = 12 Hz); 4.32 (m, OCH_2); 7.1 (m, -Ph-, 4H). ^{19}F NMR δ: −75.27 (t, $^{3}J_{H-F}$ = 8.5 Hz). ^{13}C NMR δ: 20.6 (CH_3Ph); 36.5 (CH_3N); 62.7 (q, CH_2CF_3; J = 37 Hz); 122 (q, CF_3, J = 276 Hz); (117; 130; 134; 148; C_{arom}). ^{31}P NMR δ: 6.15 (9 peaks, $^{3}J_{P-H}$ = 9.7Hz).

4-Methylphenyl-2,2,2-trifluoroethyl diethylamidophosphate 3b is as follows: It is a colorless liquid, yielding 78%, $E_{b0.01mmHg}$ = 100°C. ^{1}H NMR δ: 1.0 (t, 3H, CH_3); 2.3 (s, 3H, CH_3-Ph); 3.2 (m, 4H, CH_2N); 4.32 (m, 2H, OCH_2-); 7.1 (m, -Ph-, 4H). ^{31}P NMR δ: 5,53 (11 peaks, $^{3}J_{P-H}$ = 7.3Hz). ^{19}F NMRδ: −75,4 (t, $^{3}J_{H-F}$ = 8,5 Hz).^{13}C NMRδ: 13.8 ($\underline{C}H_3CH_2$); 20.7 (CH_3Ph); 39.9 ($2N\underline{C}H_2CH_3$); 62.7 (q, CH_2CF_3; ^{3}J = 33 Hz); 122 (q, CF_3, ^{1}J = 267 Hz); (117; 130; 134; 148; C_{arom}). ESI MS m/z 348 $[M+Na]^+$; 673 $[2M + Na^+]$; $C_{13}H_{19}F_3NO_3P$: calc. 325.1055; found 325.1056

4-Methylphenyl-2,2,2-trifluoroethylpiperidin-1-yl-phosphonate 3c is as follows: It is a colorless liquid, yielding 95%, NMR δ: ^{31}P: 3.95 (7 raies, $^{3}J_{P-H}$ = 7.9 Hz); ^{19}F: −75.4 (t, $^{3}J_{H-F}$ = 8.5 Hz); ^{13}C: 19.6 (CH_3Ph); 23.2 (CH_2); 24.7 ($2CH_2$); 44.5 ($2CH_2N$); 62.7 (q, CH_2CF_3; ^{2}J =37 Hz); 122 (q, CF_3, ^{1}J = 276 Hz) (117; 130; 134; 148; C_{arom}); ^{1}H: 2.3 (s, 3H, CH_3-Ph); 7.1 (m, -Ph-, 4H).

4-Methylphenyl-2,2,2-trifluoroethylmorpholin-4-yl-phosphonate 3d is as follows: It is a colorless viscous liquid, with yield 95%, NMR δ: ^{31}P: 2.92 (7 raies, $^{3}J_{P-H}$ = 7.3 Hz); ^{19}F: −75.4 (t, $^{3}J_{H-F}$ = 7.3 Hz); ^{13}C: 20.5 (CH_3Ph); 44.5 ($2CH_2N$); 62.9 (q, CH_2CF_3; ^{2}J = 33 Hz); 66.6 ($2OCH_2$); 122.0 (q, CF_3, ^{1}J = 267 Hz) (119; 130; 134; 148; H_{arom}); ^{1}H: 2.3 (s, 3H, CH_3-Ph); 3.2 (m, 4H, CH_2N); 3.6 (m, 4H, OCH_2); 4.32 (m, 2H, OCH_2CF_3); 7.1 (m, -Ph-, 4H).

2.1.4. Synthesis of 2,2,2-trifluoroethyl(4-nitrophenyl)phosphonochloridate. A solution of trifluoroethanol (18 mmol) and triethylamine (18 mmol) in 20 mL was added at 0°C to a solution of p-$O_2NPhP(O)Cl_2$ (15 mmol) in 120 mL of anhydrous ether under a flew of nitrogen. After 48 h of stirring at room temperature, the precipitate is filtered and the filtrate is concentrated and then distilled. It is a yellow viscous liquid, with yield = 61%, $E_{b0.5mmHg}$ = 138°C, NMR δ: ^{31}P: −0.43 (t, J = 9.7 Hz); ^{19}F: −75.0 (t, J = 8.5 Hz); ^{13}C: 65. 0 (q, OCH_2CF_3, J = 32 Hz); 122 (q, CF_3, J = 274 Hz); (154, 146, 127, 121 C_{arom}); ^{1}H: 8.3 et 7.4 (2m, $4H_{arom}$); 4.6 (m, 2H, OCH_2CF_3).

2.1.5. Synthesis of 4-nitrophenyl-2,2,2-trifluoroethylphosphoramidates. 6 mmol of 2,2,2-trifluoroethyl (4-nitrophenyl) phosphonochloridate in 50 mL of anhydrous ether was added to 13.2 mmol of triethylamine in 10 mL of anhydrous ether under nitrogen. Stirring is continued for 5 hours. The precipitate is filtered and the filtrate was concentrated to give an oil, which unless otherwise is not further purified.

2,2,2-Trifluoroethyl-4-nitrophenyl-*N*,*N*-dimethyl-phosphoramidate 6a is as follows: It is colorless viscous liquid, with yield (94%), NMR δ: ^{31}P: 4,85; ^{13}C: 36,6 (CH_3N); 63,5 (q, CH_2CF_3, J = 33 Hz); 122 (q, CF_3, J =275 Hz); (164, 157,156, 145, 126, 121, C_{arom}); ^{1}H: 2,8 (d, $2CH_3$); 4,4 (m, CH_2); 7.4 et 8.2 (2m, $4H_{arom}$).

2,2,2-Trifluoroethyl-4-nitrophenyl-*N*,*N*-diethylphosphoramidate 6b is as follows: It is a yellow viscous liquid, with yield (58%), $E_{b0.01mmHg}$ = 152°C, NMR δ: ^{31}P: 5.0 (hept. J = 7.4 Hz); ^{13}C: 36.6 (CH_3N); 63.5 (q, CH_2CF_3, J = 33 Hz); 122 (q, CF_3, J = 275 Hz); (154, 146, 126, 121, C_{arom}); ^{19}F: −73.6 (t, J = 8.5 Hz), ^{1}H: 1.0 (t, CH_3); 3.2 (m, CH_2CH_3); 4.4 (m, CH_2); 7.4 et 8.2 (2m, $4H_{arom}$).

2,2,2-Trifluoroethylmorpholin-4-yl(4-nitrophenyl) phosphoramidate 6c is as follows: It is a yellow solid, with yield (95%), RMN δ: ^{31}P: 2.4 (hept. J = 7.9 Hz); ^{13}C: 45.0 ($2CH_2N$); 62.7 (q, CH_2CF_3; ^{2}J = 35 Hz); 67,0 ($2OCH_2$); 122 (q, CF_3, J = 275 Hz); (163, 156, 144, 126, 121, C_{arom}),^{19}F: −75.6 (t, J = 8.5 Hz); ^{1}H: 3,1 (m, 4H, CH_2N); 3.6 (m, 4H, OCH_2); 4.32 (m, 2H, OCH_2CF_3); 7.4 et8.2 (2m, $4H_{arom}$).

2.2. Biological Activity

2.2.1. Antimicrobial Activity. Different bacterial strains are maintained by subculture on BHI agar (Brain Heart Infusion,

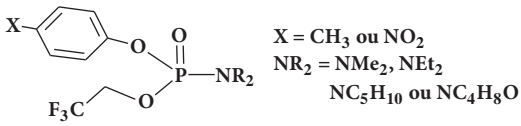


Figure 1: Designed phosphoramidates.

agar and brain-heart infusion) favorable to their growth for 24 hours *C. B. cereus* at 37°C with the exception of *L. monocytogenes* and incubated at a temperature of 30° grown on nutrient agar at 30°C. The agar diffusion method (method of disc). Filter paper disc was impregnated by different tested compounds and deposited on the surface of agar petri dishes. Minimal inhibitory concentrations were determined by the dilution method in solid medium.

2.2.2. Anticholinesterase Activity. Chemicals : Acetylcholinesterase (AChE) type VI-S, from electric eel 137 U/mg solid, 217 U/mg protein, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (AChI), tris[hydroxymethyl] aminomethane (tris buffer), and dimethylsulfoxide (DMSO) were supplied from Sigma-Aldrich. Acetylcholinesterase enzymatic activity was measured by the Ellman test [21]: 98 μL (50mM/L) tris-HCl buffer (pH 8), 30 μL of the sample, and 7.5 μL of the acetylcholinesterase solution containing 0.26 U/mL were well mixed in 96-well microplates and incubated for 15 min. Subsequently, 22 μL of (3mmol/L) DTNB was added. The absorbance at 405 nm was read when the reaction reached the equilibrium. A control reaction using DMSO instead the sample and a blank with Tris-HCl buffer instead of enzyme solution were used. Tests were carried out in duplicate.

Inhibition, in %, was calculated in the following way: I (%) = 100 - (A sample/A control)∗100,

where A sample is the absorbance of the sample containing reaction and A control the absorbance of the reaction control.

3. Results and Discussion

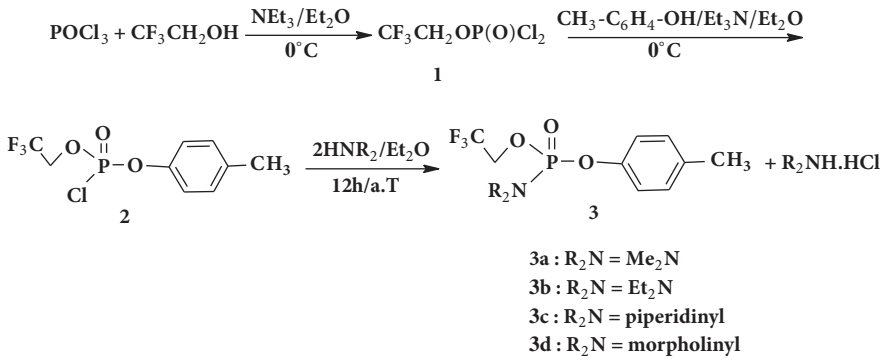
3.1. Synthesis. The design of arylphosphoramidates in this work (Figure 1) is based on phosphoramidate structures already used as prodrugs. (NR_2) is the masking group which hydrolyzes first. (Ar-X) is the leaving group and (OR') is the active group that should be supplied to the cell to be treated, avoiding hydrolysis thereof to the surface of the cell by the NR_2 group in Figure 1.

For the synthesis of the designed arylphosphoramidates, several attempts have been carried out. On the basis of the reported literature by [2], we have attempted the synthesis of the phosphoramidates $(R_2N)P(O)(OCH_2CF_3)(OphCH_3)$ in a one pot by mixing phosphorus oxychloride, alcohols ($HOPhCH_3$, $HOCH_2CF_3$), and the amine (R_2NH) as shown in Scheme 1. However, in addition to the expected phosphoramidates, the ^{31}P NMR spectrum showed signals relating to the formation of several byproducts such as $CF_3CH_2OP(O)Cl$, $(CF_3CH_2O)_2P(O)(OPhCH_3)$, and $P(O)(OPhCH_3)_3$. These byproducts could not be separated by distillation. The reaction was then undertaken in multisteps with several assays: first, the p-cresol and triethylamine are added to phosphorus oxychloride in anhydrous ether at −10°C and kept at room temperature for 12 hours. The corresponding ^{31}P NMR spectrum showed the corresponding dichlorophosphate in addition to other unknown phosphorus compounds. Then, the addition of alkylamine on phosphorus oxychloride followed by the addition of $HOCH_2CF_3$ gave the desired dialkylphosphoramidic dichloride. However the addition of CF_3CH_2OH in the presence of DMAP as catalyst afforded the expected phosphoramidate in low proportion with the appearance of a new compound due to the substitution of the-NR_2 group by –OR group located at -6 ppm. Finally, we have reacted $POCl_3$ with CF_3CH_2OH in presence of triethylamine in anhydrous ether for 12 hours at room temperature and subsequent addition of p-cresol and amine afforded the desired compounds **3** with good yields and satisfactory purity. In these optimized conditions, the other amines were used and gave the corresponding phosphoramidates (Scheme 1).

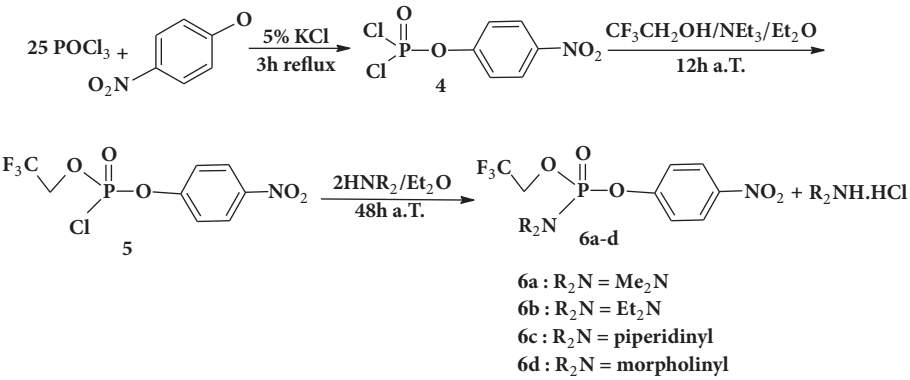
The ^{31}P NMR coupled to ^{1}H spectrum of the compound **2** showed a triplet at 0.5 ppm with a coupling constant value $^{3}J_{H\text{-}P}$ = 8 Hz with the two protons of the methylene group. The reaction of two equivalents of amine in anhydrous ether with compound **2** for 12 hours at room temperature gave the pure arylphosphoramidates **3**. The ^{1}H NMR spectrum of compound **3a** shows a doublet at 2.8 ppm resulting from the coupling with the phosphorus atom. The methylene group shows a multiplet at 4.4 ppm due to the coupling with both fluorine and phosphorus atoms. The corresponding ^{31}P NMR spectrum (Figure 1) shows a multiplet of 9 peaks resulting from the coupling between the phosphorus atom and 8 protons (CH_2O and $2CH_3$). ^{19}F NMR spectrum shows a triplet due to coupling of the fluorine atom with the methylene group (Figure 2).

We have also used nitrophenol instead of p-cresol following the same sequence described for the synthesis of compounds **3**. However the purification of the reaction products was tedious and gave a mixture of products together with the desired phosphoramidates. We have therefore attempted to do the synthesis using a different sequence where the addition of the amine with phosphorus oxychloride was followed by nitrophenol and then by trifluoroethanol allowing the desired phosphoramidate but the reaction took 5 days. Finally using the starting compound **4** described in the literature [20], the reaction with trifluoroethanol gave the corresponding chlorophosphoramidate in a good yield. The reaction of the compound **5** with two equivalents of amine in anhydrous ether at room temperature for 48 hours led to the desired phosphoramidates with yields ranging from 58 to 95% (Scheme 2).

The ^{1}H NMR spectrum of the compound **6a** shows the coupling with methyl protons with the phosphorus atom at 2.8 ppm (Figure 3). On the other hand, the ^{31}P NMR spectrum of **6d** shows a multiplet of seven peaks reflecting the coupling between phosphorus atom and methylene protons at 2.4 ppm, at lower field compared to



Scheme 1: Synthesis of p-tolylphosphoramidates **3**.



Scheme 2: Synthesis of p-nitrophenol phosphoramidates **6**.

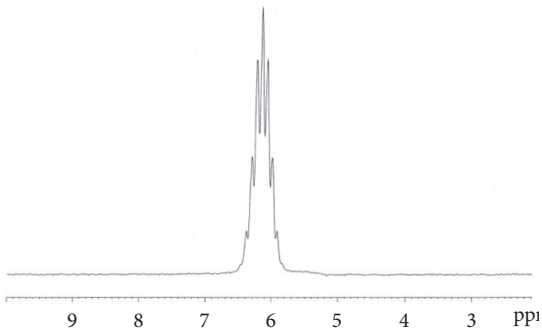


Figure 2: ^{31}P NMR of **3a** in $CDCl_3$ at 298 K.

p-tolylphosphoramidate. The spectroscopic data of these arylphosphoramidates are shown in Table 1.

The above results in Schemes 1 and 2 show that the formation of phosphoramidates is sensitive to the nature of the alcohols used. Thus preparing phosphoramidates**3** is in the order CF_3CH_2OH followed by p-cresol and the amine, whilst for phosphoramidates **6**, the addition of the aromatic alcohol, nitrophenol, should be the first step then the addition of the second alcohol CF_3CH_2OH and amine as the final step.

3.2. Biological Activity

3.2.1. Antimicrobial Activity. The phosphoramidates **3a**, **3c,** and **3d** have been tested towards different Gram negative and Gram positive bacteria. Chloramphenicol was taken as reference to study the effect of different substituents on biological activity. The compound 2,2,2-trifluoroethyl N,N,N′,N′-tetramethylphosphorodiamidate (**TMP**) [22] has also been tested in order to evaluate the effect of electrodonating effect on the phosphorus atom. The reactivity of each compound was evaluated towards the different bacterial strains by the agar diffusion method. The inhibition diameters of bacterial growth area are summarized in Figure 4.

The results show that all the tested compounds in a pure state have diameters of inhibition zone of bacterial growth ranging between 6 and 10 mm for all strains of Gram negative and Gram positive bacteria. The compounds **3a**, **3c, 3d,** and **TMP** do not exhibit a particular antimicrobial activity. To better assess the sensitivity of the strains towards the activity of these compounds, their minimum inhibitory concentration (MIC) was determined by the dilution method on solid medium. The results show that these values (i.e., 1000 to 2000 μg/mL) are high compared to those of usual therapeutic agents. The lack of antimicrobial activity may be due to the low solubility in water [23, 24], as well as instability in alkaline hydrolysis [25].

3.2.2. Antiacetylcholinesterase Activity. The determination of the inhibitor activity of acetylcholinesterase (AChE) of compounds **3a, c, d,** and **TMP** with galantamine, taken as a reference, was performed according to the method of Ellman

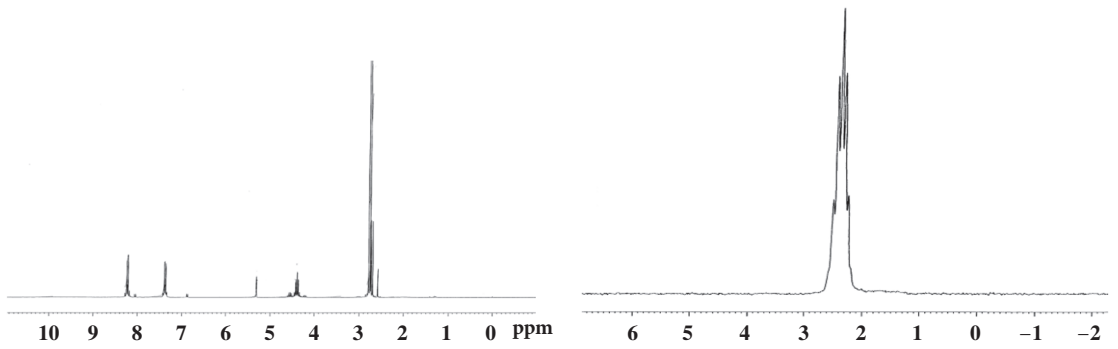


Figure 3: (a) ^{1}H NMR of **6a** in $CDCl_3$ and (b) ^{31}P NMR coupled to ^{1}H of **6d** in $CDCl_3$.

Table 1: Spectroscopic data of arylphosphoramidates δ^{31}P (ppm),^{3}J (Hz), and ν_{PO} (cm^{-1}).

Phosphoramidates	yield%	δ^{31}P (ppm)	^{3}J (Hz)	ν_{PO} (cm^{-1})
3a	91	6.15	9,7	1167
3b	80	5.53	7,3	1165
3c	95	3.95	7,9	1165
3d	90	2.92	7,3	1168
6a	60	4.85	9,7	1180
6b	58	5.0	7,4	1181
6c	90	3.1	7,5	1180
6d	95	2.4	7,9	1178

[21]. The results of optical density measurements of all the tested compounds are shown in Figure 5.

As can be seen from Figure 3, the negative values of the phosphoramidates **3a** and **3d** indicate that the compounds have no inhibitory activity against AChE. The compounds **3c** and **TMP** exhibit some AChE activity. The compound **3c** is more active than the compound **3d** probably due to the hydrophobicity and the more electrodonating character of **3d.** However, the difference in activity found between phosphoramidates **3a** and **3c** both bearing electrodonating groups could be mainly due to steric hindrance which would enhance AChE activity in **3c**. On the other hand, the direct substitution of phosphorus atom by amine group in **TMP** can enhance sensitively of the AChE inhibitory effect. This electrodonating group enhances nucleophilic character which facilitates the nucleophilic attack on the phosphorous atom and the elimination of the leaving group. This is consistent with the literature [26, 27] which showed that the AChE inhibition increases when the polarity of the amine group increases related to the electrostatic attraction between this group and the enzyme which becomes stronger.

Therefore the inhibitor-enzyme interaction would be influenced mainly by the reactivity of the phosphorus atom, which determines the rate of the phosphorylation reaction and the ease of bonding between the inhibitor and the enzyme to form a complex before the phosphorylation step and the electronic and steric effects of hydrophobic moieties directly bounded to the phosphorus atom. The binding affinity is determined by the structural features in particular the instability of the P=O bond as reported in the literature [28, 29] which may also influence the cholinesterase activity.

4. Conclusions

In this paper, we have synthesized new phosphoramidates $R_2N(pX\text{-}ArO)P(O)OR'$ using convenient steps. All synthesized phosphoramidates were characterized by ^{31}P NMR, ^{1}H, and ^{13}C NMR, IR spectroscopy. The biological study of some of arylphosphoramidates did not show particular antibacterial activity even when the phosphorus atom was directly substituted by an electrodonating group ($-N(Me)_2$). However the AChE activity has shown that the directly substituted electrodonating group on the phosphorus atom has some AChE inhibitory effect. Therefore the substituents nondirectly bounded to the phosphorus atom did not affect sensitively the reactive sites of the arylphosphoramidates towards AChE enzyme.

Data Availability

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

Conflicts of Interest

There are no conflicts of interest in this manuscript.

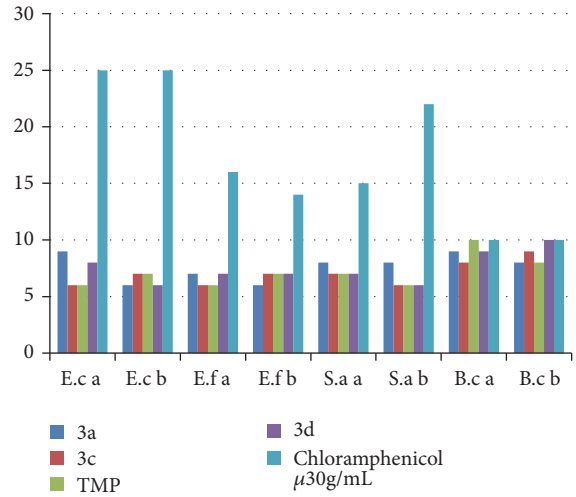


Figure 4: Inhibition diameters zones of bacterial growth (mm) of phosphoramidates **3a, c, d,** and **TMP**. *E.c a: Escherichia coli* ATCC 8739; E.c b: *Escherichia coli* DH5α; E.f a: *Enterococcus faecalis* ATCC 29212; E.f b: *Enterococcus faecium* ATCC19436; S.a a: *Staphylococcus aureus* PIC 4.83; S.a a: *Staphylococcus aureus* ATCC 25923; B.c 49: *Bacillus cereus* 49; B.c: *Bacillus circulans* (ATCC: American Type Culture Collection; PIC: Pasteur Institute Collection).

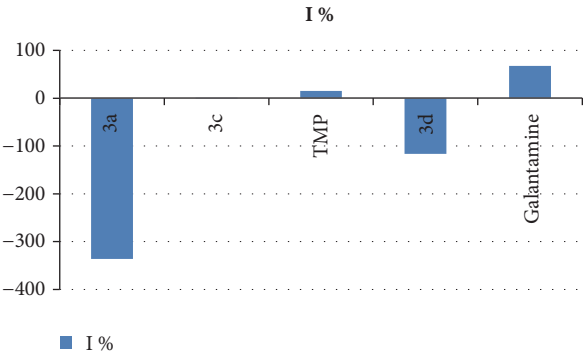


Figure 5: Measurements of optical density DO406.

Acknowledgments

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Research Article

Congo Red Decolorization and Detoxification by *Aspergillus niger*: Removal Mechanisms and Dye Degradation Pathway

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Congo red is one of the best known and used azo dyes which has two azo bonds (-N=N-) chromophore in its molecular structure. Its structural stability makes it highly toxic and resistant to biodegradation. The objective of this study was to assess the congo red biodegradation and detoxification by *Aspergillus niger*. The effects of pH, initial dye concentration, temperature, and shaking speed on the decolorization rate and enzymes production were studied. The maximum decolorization was correlated with lignin peroxidase and manganese peroxidase production. Above 97% were obtained when 2 g mycelia were incubated at pH 5, in presence of 200 mg/L of dye during 6 days at 28°C and under 120 to 150 rpm shaking speed. The degraded metabolites were characterized by using LC-MS/MS analyses and the biodegradation mechanism was also studied. Congo red bioconversion formed degradation metabolites mainly by peroxidases activities, i.e., the sodium naphthalene sulfonate (m/z = 227) and the cycloheptadienylium (m/z = 91). Phytotoxicity and microtoxicity tests confirmed that degradation metabolites were less toxic than original dye.

1. Introduction

Water is necessary to sustaining life on earth. However, available water represents less than 1% of the total volume of fresh water on earth [1]. However, pollution reduces its availability for human use. The water pollutants are usually generated by industries and can be divided into various classes. Every class has its own specific dangers.

The textile industry is one of the most polluting industries of clean water. In fact, during the manufacturing processes, a large percentage of the synthetic dye does not bind and is lost in wastewaters, which are usually discharged untreated. Congo red is the most common dyes that can be found in textile industry. It is a benzidine based anionic diazo dye [2]. This dye is known to be metabolized into benzidine, which is a human carcinogen and mutagen; that is why it is banned in many countries.

Various chemical and physical methods of these colored waste waters have been proposed in the last few decades such as coagulation–flocculation, oxidation, and electrochemical methods [3]. Nowadays scientists work on the implementation of innovative processes to treat these recalcitrant compounds. Among the most recent treatments is the advanced oxidation process (AOP), which allows mineralization of toxic organic molecules through formation of extremely reactive and nonselective radicals such as hydroxyl radicals. However, AOP have many disadvantages, such as high-energy costs and sometimes formation of toxic by-products [4].

However, bioprocessing can be considered as a preferred option to overcome these disadvantages because it is cost saving and environmentally friendly. Biological treatments can be used to degrade and/or to adsorb azo dyes contaminants [5]. The most efficient microorganisms to break down colored pollutants so far reported are white-rot fungi. These comprise mostly basidiomycetous fungi, which are capable of extensive aerobic lignin degradation and mineralization. This is possible through several extracellular lignin-degrading enzymes [6], such as lignin peroxidase, manganese-dependent peroxidase, and laccase. A previous report showed dye degradation potential of *Aspergillus niger* [7]. A total decolorization of Procion Red MX-5B by *Aspergillus niger* was obtained after 336 h of treatment. On the other hand, the enzymatic cleavage of azo dyes leads to the formation of toxic products, mainly amines. Therefore, it is important to identify and evaluate toxicity of degradation products [8].

The aim of the present work was to study the *Aspergillus niger* potential for detoxification and decolorization of CR dye. The enzymes involved in the decolorization process were identified and the effects of various parameters (pH, temperature, initial dye concentration, and shaking speed) on dye decolorization and enzymes production were investigated. The degraded metabolites were characterized by using LC-MS/MS analyses. The study also aimed to assess the toxicity of the metabolites formed after the degradation of CR dye by this fungus.

2. Materials and Methods

2.1. Fungal Strain and Biomass Generation. *Aspergillus niger* was isolated from processing wastewater [9]. The fungal strain was maintained on Potato Dextrose Agar (Merck) at room at 28 ± 2°C. To generate biomass, three mycelia plugs (0.7 cm diameter) taken from the edge of the colony were transferred into 100 ml synthetic nutrient broth medium containing (g. L^{-1}) glucose 10 g, yeast extracts 1 g, and peptone 2 g (Scharlau). The pH was adjusted to 6.0 ± 0.2 and the culture was incubated for 8 days at 30°C. The fungal biomass was homogenized, filtered through Whatman filter paper No. 1, and washed with sterile distilled water. This freshly prepared biomass was used for dye biodegradation experiments.

2.2. Condition Optimization for CR Decolorization. The fungus ability to decolorize the CR dye (Sigma Aldrich) under different conditions was investigated using decolorization rate as the index. The influence of pH and temperature on decolorization was studied in presence of 200 mg. L^{-1} CR under pH values ranging from 3 to 10 and temperature ranging from 15 to 45°C. The agitation effect was studied under different speed shacking conditions (0, 50, 100, 150, and 200 rpm). The influence of initial dye concentration was tested using 100, 250, 500, and 1000 mg. L^{-1}. In all cultures, 2g of fungal fresh biomass was used to inoculate 100 mL synthetic nutrient broth. Cultures were incubated at 30°C, for 6 days. The supernatant was used for color reduction measurements and peroxidases activities.

2.3. Biodegradation and Biosorption Treatments. The CR biodegradation treatment was performed with an aqueous solution inoculated with 20g L^{-1} of fresh fungal biomass. In 250 mL Erlenmeyer containing 100 mL of synthetic broth medium was supplemented with 200 mg. L^{-1} CR dye at pH 6.0 ± 0.2. After inoculation, cultures were placed in a rotary shaker at 150 rpm and 30°C for 10 days. Noninoculated dye solution was designated as negative control. At regular intervals, a sample from the broth medium was collected and centrifuged at 5000 rpm for 15 min to remove the fungal mycelium. The supernatant was used for pH, color reduction measurements, and enzyme activities assay. The fungal biomass was determined by measuring the dry weight of the pellets suspensions washed twice with distilled water. All cultures were performed in triplicate, and the results are the average.

CR biosorption treatment was conducted with dye aqueous solution in 250 mL Erlenmeyer containing 100 mL broth medium and 200 mg. L^{-1} CR dye inoculated by 2g of autoclaved fresh fungal biomass of *A. niger* (inactivated biomass). Flasks were incubated at 30°C for 48 h under stirred conditions (120 rpm). Noninoculated dye solution was designated as negative control.

2.4. Enzymatic Assay. Lignin peroxidase (LiP) activity was determined using veratryl alcohol as a substrate [10]. The assay mixture contained 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the increase in absorbance at 310 nm because of the formation of veratraldehyde from ε_{310} = 9300 M^{-1} cm^{-1}.

Manganese peroxidase (MnP) activity was determined using MnSO4 as a substrate [11]. The assay mixture contained 0.5 mM MnSO4 and 0.5 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} was followed by measuring the increase in absorbance at 270 nm due to the formation of Mn^{3+}-malonate from ε_{270}=11590 M^{-1} cm^{-1}.

Laccase activity was measured spectrophotometrically with a Genesys 5 spectrophotometer using 1 μmol 2, 2-azino–bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as a substrate. One unit of enzyme activity was defined as of ABTS oxidized per minute at 25°C (ε_{420}= 29 300 M^{-1} cm^{-1}) [12]. All enzyme activity was expressed as international units (IU).

2.5. UV-Visible and FTIR Analyses. Color reduction was assayed by absorbance measurement at λ_{max} =495nm nm using a Jenway 3540 UV/VIS spectrophotometer. After culture centrifugation at 5000 rpm for 15 min, the supernatants were analyzed by measuring the absorbance differences. Decolorization rate was determined according to the following formulation:

$$\text{Decolorization rate}\,(\%) = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad (1)$$

A_0 is the dye absorbance before decolorization and A_1 is the dye absorbance after decolorization.

The decolorization analysis of the biodegraded and crude CR solution was performed by the change in the absorption spectrum in the wavelength range of 200-800 nm region using a quartz cuvette with an optical path of 5nn.

FTIR analysis of lyophilized fungal biomass was monitored on a Thermo Scientific IR 200 FT-IR spectrophotometer. The FTIR spectra were then recorded between 4000 and 400 cm^{-1}, at a rate of 16 nm/s.

2.6. LC-MS/MS Analyses of Transformed Metabolites. CR metabolites were analyzed on a LC-MS/MS with an electrospray ionization- (ESI-) interface (Agilent Technologies, USA) equipped with C18 waters column (4.6 mm-250 mm; particle size 5um). Isocratic elution was performed with mobile phase of methanol: water (90:10 v/v) at a flow rate of 0.7 mL/min.

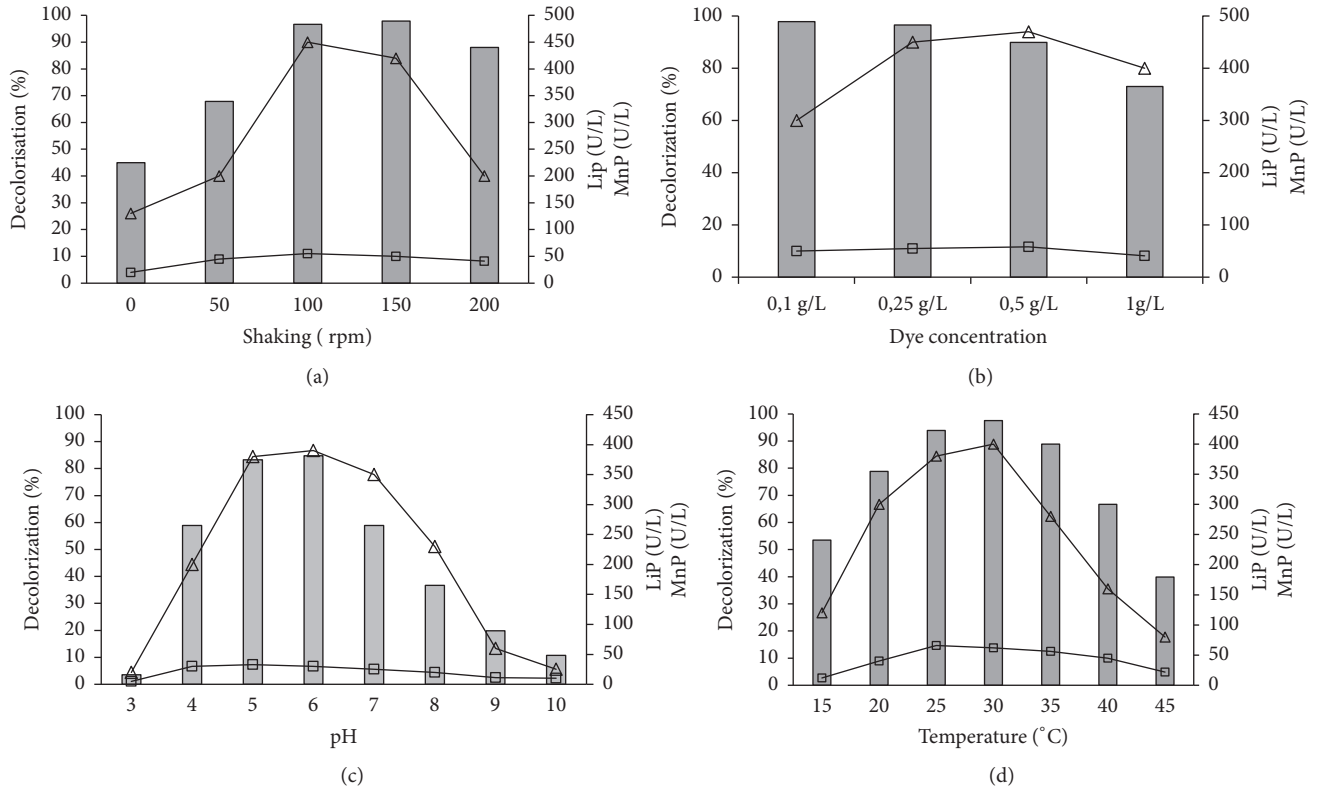

Figure 1: Effect of initial dye shaking (a), dye concentration (b), pH (c), and temperature (d) on CR decolorization efficiency (Gray colored bars), LiP (□), and MnP activities (△).

2.7. Toxicity Assay

2.7.1. Phytotoxicity Assay. The phytotoxicity assays were performed to assess the toxicity of dye before and after degradation. The experiment was conducted using *Zea mais* and *Solanum lycopersicum* seeds. 10 seeds were wetted (3mL per day) with dye solution (200 mg. L^{-1}) or treated CR solution in separated Petri dishes. The control groups were treated with distilled water. All samples were incubated at the same environmental conditions and repeated three times. Percent of germination and length of shoot and root were recorded after 7 days.

2.7.2. Microtoxicity Assay. *Bacillus cereus* ATCC 11778 and *Escherichia coli* ATCC 10536 strains were used for toxicity evaluation of the untreated and treated RC solution by *Aspergillus niger*. The two strains growths were studied on nutrient broth (NB) medium as control and on NB medium supplemented with 200 mg L^{-1} of CR before and after biodegradation. Incubation temperature was 30°C and 37°C for *B. cereus* and *E. coli*, respectively. The bacterial growth was assessed by OD at 600 nm recorded at 1 h interval during 8 h [13].

2.8. Statistical Analysis. An analysis of variance (a one-way ANOVA) was conducted by employing performed (SPSS) version 16.0 software. SAS 9.0 software was used for all statistical analysis with multiple comparison tests. Effects were considered significant when the P value was < 0.05.

3. Results and Discussion

3.1. Process Parameters' Optimization for Congo Red Decolorization by A. niger. CR decolorization efficiency and enzyme production (LiP and MnP) by *A. niger* were investigated by different process parameters. Effects of speed shaking, pH, temperature, and initial dye concentration are shown in Figure 1. The effect of shaking speed was found to be highly significant ($p < 0.01$) on CR decolorization. When the speed increases from 0 to 150 rpm, the decolorization efficiency increases from 45% to 98% after six days of culture, indicating that shaking increases the mixing of the oxygen present in the medium, thus helping *Aspergillus niger* growth (Figure 1(a)). The same results were obtained by Kumar et al. [14], who found that shaking was beneficial for achieving maximum dye decolorization of brilliant green by *Aspergillus sp*. as a result of better oxygen transfer and nutrient distribution through the medium. However, a decline was observed beyond a speed of 150 rpm, which can be explained by pellet formation decrease in spite of the high biomass produced. The maximum LiP and MnP activities were obtained at a speed of 100 rpm (54 and 452 $U.L^{-1}$, respectively). The differences between enzymatic activities recorded between 100 and 150 rpm are not significant ($P > 0.05$). When the speed increased from

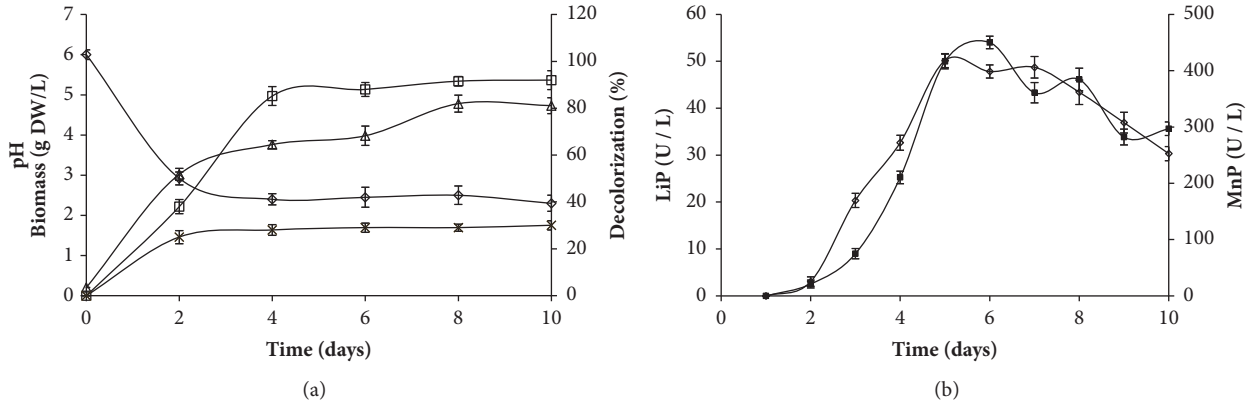

Figure 2: Time course of pH (◊), Biomass (△), and decolorization (□: with activated *A. niger*, x: with inactivated *A. niger*) (a); LiP (▲) and MnP (■) (b) during azo dye CR treatment by *A. niger*. The error bars represent the standard deviation of measurements for 3 samples.

150 to 200 rpm, the MnP activity decreased but LiP activity remained almost constant. Mahmoud et al. [15] reported an increase in the decolorization rate of direct red azo dye by *A. niger* with increasing the agitation speed. The maximum removal efficiency was recorded at agitation speed of 250 rpm, after which no significant increase was noticed. On the contrary, some authors noticed decolorization decreased under shaking condition due to the competition between azo dyes and oxygen for reduced electron carriers [16].

CR decolorization is also affected by medium pH. *A. niger* showed high CR removal (85%) and enzyme activities under slightly acidic pH varying from 5 to 6 (differences are not significant $P > 0.05$) (Figure 1(b)). In fact, MnP activity notably dropped at high pH values (pH > 7), which can be due to enzymes stability. This enzyme may be stable only at acidic pH. Michaels and Lewis 1986 showed that medium pH is one of the critical environmental factors that affects azo biodegradation. Kumar et al. 2011 obtained methyl violet biodegradation by *Aspergillus* sp. only in slightly acidic conditions (pH 5.5) and that the decolorization was inhibited at a pH higher than 6.5.

From Figure 1(c), it can be deduced that a temperature around 30°C is the most favorable temperature to carry out this decolorization and a significant decrease was observed at higher temperature. This can be explained by cell viability reduction at high temperature or to the inactivation of the enzymes responsible for RC decolorization. Parshetti et al. [17] showed that optimum temperature for dye decolorization for most fungi varied between 25 and 35°C.

Figure 1(d) shows that CR removal was slightly inhibited at 0.5gL-1, and the best result was found with 0.25 $g.L^{-1}$ with a decolorization rate of 96% and enzyme production of 56 and 470 $U.L^{-1}$ for LiP and MnP, respectively. When the initial dye concentration increased to 1g.L-1, a significant reduction in the decolorization rate was observed (73%), which can be due to the dye toxicity at high concentrations [18]. Same result was reported by Parshetti et al. [17], who showed that reactive blue-25 at high concentration affected the decolorization performance of *Aspergillus ochraceus*.

3.2. Time Effect on CR Decolorization and Enzymatic Activities. Optimal conditions previously set up were used to study the biodegradation and the adsorption ability of *A. niger*. RC removal and enzymatic activities were conducted with 2 g of fresh activated or inactivated mycelia, inoculated into 100 mL of synthetic media containing 250 mg. L-1 of CR dye, and incubated at 30°C. Decolorization, biomass production, pH, LiP, and MnP activities were monitored during 6 days (Figure 2).

The biomass increased and pH decreased the first six days and then stabilized**.** The maximum mycelium dry weight obtained was 6.4 $g.L^{-1}$. *A. niger* growth induced a significant decolorization that reached 97% after 6 days of incubation. However, inactivated cells led to 27% color removal during the same period (Figure 2(a)). Then, the decolorization could be attributed to both biodegradation and dye adsorption on the fungal mycelium. Therefore, 97% of decolorization is the result of two-process combination, the enzymatic activity responsible for the molecule degradation and adsorption phenomena. In fact, 1g of fresh biomass can eliminate 27% of CR dye by adsorption mechanism and 70% by enzymatic biodegradation.

Time course for LiP and MnP produced by *A. niger* was achieved in order to evaluate the maximum enzyme activity during CR removal by *A. niger* (Figure 2(b)). LiP activity was detected after 2 days and reached a maximum at the 5th of the culture. However, maximum MnP activity was obtained in the 6th day. The highest MnP and LiP activities were 53.2 ± 1.41 $UI.L^{-1}$ and 450.13 ± 11 $UI.L^{-1}$, respectively. However, laccase activity was not detected. Since the MnP activity level produced was much higher than the LiP activity, MnP seemed to play the most important role in the decolorization. The presence of ligninolytic extracellular enzymes in culture supports biological decolorization alongside the nonbiological color removal by adsorption [19]. Several studies have shown that ligninolytic fungal enzymes are efficient for dye decolorization [20, 21]. Parshetti et al. [17] have shown

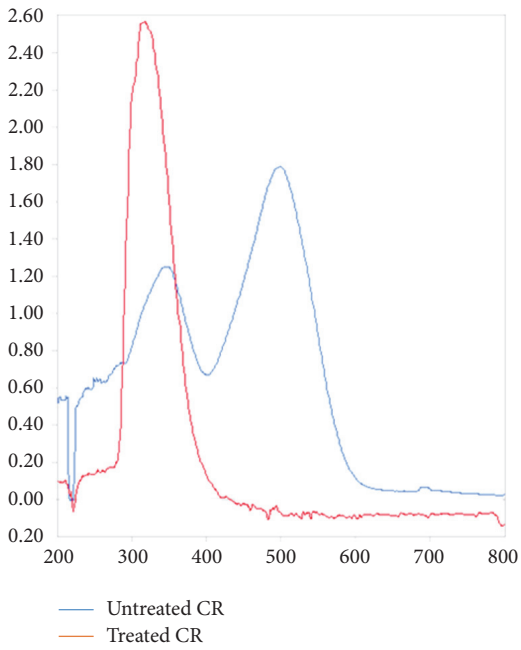

Figure 3: Changes in UV-visible absorption spectrum of CR (200 mg.L-1) before and after CR decolorization by *A. niger* treatment at shaking condition (200 mg. L^{-1} CR dye at pH 6.0 ± 0.2 shaked at 150 rpm incubated at 30°C for 8 days).

the presence of lignin peroxidase, laccase, and tyrosinase produced by *Aspergillus ochraceus* during reactive blue 25 decolorization.

3.3. CR Color Removal By A. niger. CR color removal was confirmed by UV-visible analysis (200-800 nm) (Figure 3). CR UV-Vis scanning showed two peaks. One extended at 340 nm, which is due to the interaction between aromatic hydrocarbon or polycyclic aromatic hydrocarbon groups and other chromophore and another at 495 nm, which had a relation to azo double bond and large conjugated system for the whole dye molecule. After 96 h treatment, a decrease in the major peak intensity at 495nm was observed. It indicated that dye structure especially that of chromophore was transformed during *A. niger* treatment. However, the absorbance at 340 nm was increased which evinced that interaction of the aromatic hydrocarbons or polycyclic aromatic hydrocarbon groups and some chromophore were partly destroyed, or new aromatic compounds might appear [22]. These changes suggested that *A. niger* is able to transform the CR dye to other compounds. This result suggested the chromophore group's breakdown. Wang et al. [23] reported that the degradation of the aromatic hydrocarbon or polycyclic aromatic hydrocarbon groups completely seemed to be more difficult than the destruction of the azo double bond and the large conjugated system.

3.4. CR Adsorption on A. niger Biomass. In order to elucidate the nature of the functional groups responsible for the biosorption, FTIR analysis of the lyophilized biomass was carried out before and after incubation in CR solution (200 mg.L-1) (Figures 4(a) and 4(b)). The FTIR spectra of *A. niger* biomass before treatment showed the characteristic band at 3275 cm−1, which is attributed to O–H bending vibrations. The peak at 2937 cm−1 corresponds to asymmetric and symmetric stretching of the C- H bond of -CH2 group. The band at 1641 cm−1 is due to the bending of N–H groups of chitin on the cell wall structure of fungal pellets [9]. The peaks around 1559 cm−1 indicated the presence of amide which resulted from NH deformation. The bands at 1420 cm−1, 1139 cm-1, and 1090 cm−1 are representing -CH3 wagging (umbrella deformation), symmetric –SO3 stretching, and C–OH stretching vibrations, respectively, which were due to several functional groups present on the fungal cell walls [22]. The peak at 579 cm−1 is corresponding to C–O bending vibrations. The adsorption of CR on the fungal biomass induced an increase in some peaks intensity, in particular, those around 3287, 2933, 1649, 1154, and 1034cm-1. An appearance of new peaks at 2859, 1379, 1262, and 1154 cm-1 was due to introduction of new functionalities on the surface of biosorbent which confirmed the CR adsorption on fungal biomass. Similar FTIR results were observed for the phenolic compounds' biosorption on various fungus biomass [23].

3.5. Bioconversion of CR by A. niger. LC-MS/MS spectra of both treated and nontreated CR dye solution display different patterns the major several compounds obtained after CR decomposition at different m/z ratios confirming the CR biodegradation by *A. niger* (Figure 5). According to LC-MS/MS spectrum, the possible degradation pathway for CR dye is showed in Figure 6. The degradation of the CR dye may occur via the following steps: (i) the simultaneous total deamination and oxygenation of (CR) forming the compound (A) with m/z value of 698. When the total deamination is followed by the loss of both sodium atoms, the degraded products (B) and (C) with m/z values of 663 and 619, respectively, are formed. (ii) The partial deamination of (CR) and the peroxidase asymmetric cleavage of C-N bond between the aromatic ring and the azo group with the loss of a sodium atom afford the intermediates (H) and (D) with m/z values of 227 and 429, respectively. (iii) The peroxidase asymmetric cleavage of C-N bond followed by deprotonation leaded to the intermediate (G) with m/z value of 271. (iv) The benzene ring opening and dehydrogenation formed intermediates (E) and (F) with m/z values of 371 and 321, respectively. (v) The peroxidase cleavage produces also low molecular weight of stable degraded products, the sodium naphthalene sulfonate (I) (m/z = 227) and the cycloheptadienylium (J) (m/z=91). In addition to LiP and MnP as degrading agents, Figures 5 and 6 showed that the most abundant intermediates of degradation (A, B, C, H, and D) resulted from deamination of the CR dye. This is in agreement with the literature which reported the GlcN6P desaminase role of *A. niger* [24].

3.6. Phytotoxicity Analysis. Phytotoxicity analysis revealed the toxicity of CR and its products to *Zea mais* and *Solanum lycopersicum* seeds (Table 1). Compared to water treatment, CR before degradation reduced significantly the germination rate, shoot, and root length of both *Zea mais* and *Solanum*

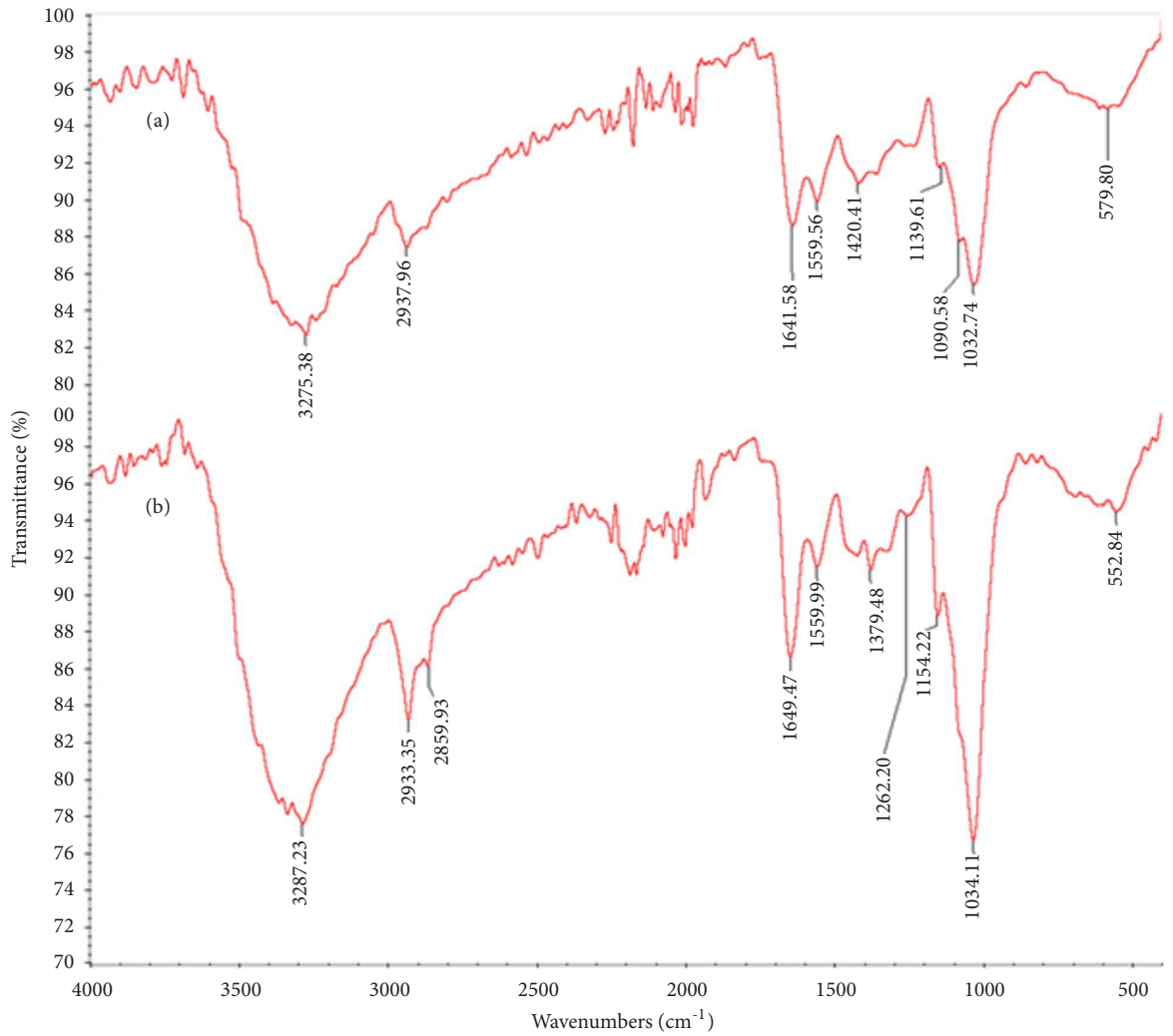

Figure 4: FTIR spectra of inactive *A. niger* before (a) and after biosorption of CR dye (b) (100 mL broth medium and 200 mg. L^{-1} CR dye inoculated by 2g of autoclaved fresh fungal biomass of *A. niger* (inactivated biomass) incubated at 30°C for 48 h under stirred conditions (120 rpm)).

Table 1: Phytotoxicity study of CR before and after biodegradation by *A. niger on Zea mais* and *Solanum lycopersicum*.

Parameters	*Zea mais*			*Solanum lycopersicum*		
	Water	Congo red	Transformation intermediates	Water	Congo red	Transformation intermediates
Germination (%)	91± 2.02	60 ± 3.52 (∗)	82 ± 3.05 (Ns)	88± 2.08	60± 3.78 (∗)	81±2.72 (Ns)
Shoot length (cm)	12.2±1.02	6.5±0.36 (∗)	10.9±0.52 (Ns)	5.16±0.44	1.8±0.15 (∗∗)	4.66±0.44 (Ns)
Root length (cm)	4.26±0.37	1.7±0.14 (∗∗)	3.16±0.21 (Ns)	3.13±0.12	2.06±0.17 (∗)	3.2±0.15 (Ns)

Ns: differences are not significant; ∗: differences are significant at $P \leq 0.05$, ∗∗: differences are significant at $P \leq 0.01$ according to ANOVA statistical analysis.

lycopersicum ($p < 0.01$). Nevertheless, the metabolites generated after the CR biodegradation are less toxic than the crude dye. In fact, there is no difference between the shoot and root length of both *Zea mais* and *Solanum lycopersicum* treated with treated CR and with water (differences were not significant with $P > 0.05$). Babu et al. [5] showed that the degradation metabolites of CR are comparatively less toxic than the crude CR to *Artemia franciscana*. Therefore, in addition to its degradation, the CR dye is detoxified by *A. niger* indicating the degradation of the amines in the solution. This result is in accordance with the deaminating effect of GlcN6P deaminase in *A. niger* observed in LC-MS/MS data.

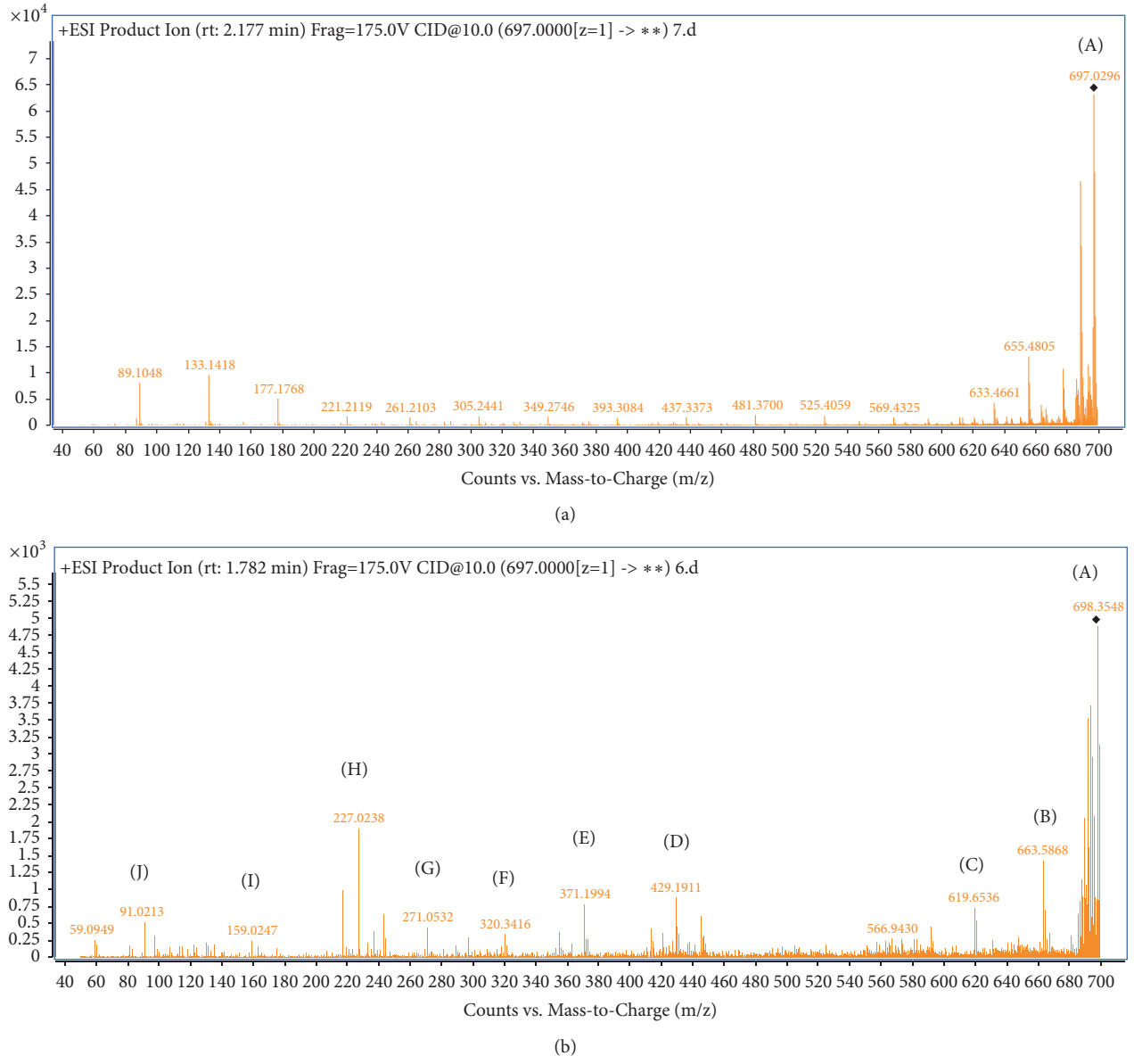

FIGURE 5: LC-MS/MS spectrum of crude CR (a) and degraded product formed during the CR decolorization process by *A. niger* (b) (200 mg. L^{-1} CR dye at pH 6.0 ± 0.2 shaked at 150 rpm incubated at 30°C for 8 days).

3.7. Microtoxicity Analysis. Microbial toxicity of crude CR solution (200 mg.L-1) and treated one was tested against *B. cereus* ATCC 11778 and *E. coli* ATCC 10536 (Figure 7). Inhibition was obtained with untreated CR dye solution. In fact, after 8 hours of incubation *B. cereus* and *E. coli* density remained very lower in the media containing crude CR than the control ones, with a significant difference ($p < 0.05$), while an improvement of bacterial growth was observed in the media containing treated dye. Density values of *B. cereus* and *E. coli* were 1.34 and 1.68, respectively, on treated CR and 2.019 and 2.37, respectively, on nutrient broth. Therefore, the CR degradation seems to detoxify the azo dye.

4. Conclusion

This study revealed that the CR was successfully decolored and biodegraded by *Aspergillus niger.* High decolorization efficiency (97%) was obtained after six days of culture. 1g of fresh biomass can eliminate 27% of CR dye by adsorption mechanism and 70% by enzymatic biodegradation. This degradation is due to the combined action of three enzymes LiP, MnP, and probably deaminase. UV-Vis, FTIR, and LC-MS/MS analysis as well as phytotoxicity and microtoxicity tests have proven the effective role of degrading and detoxifying CR dye by *A. niger*. This biological process is recommendable for further development as a potential technology for wastewater treatment.

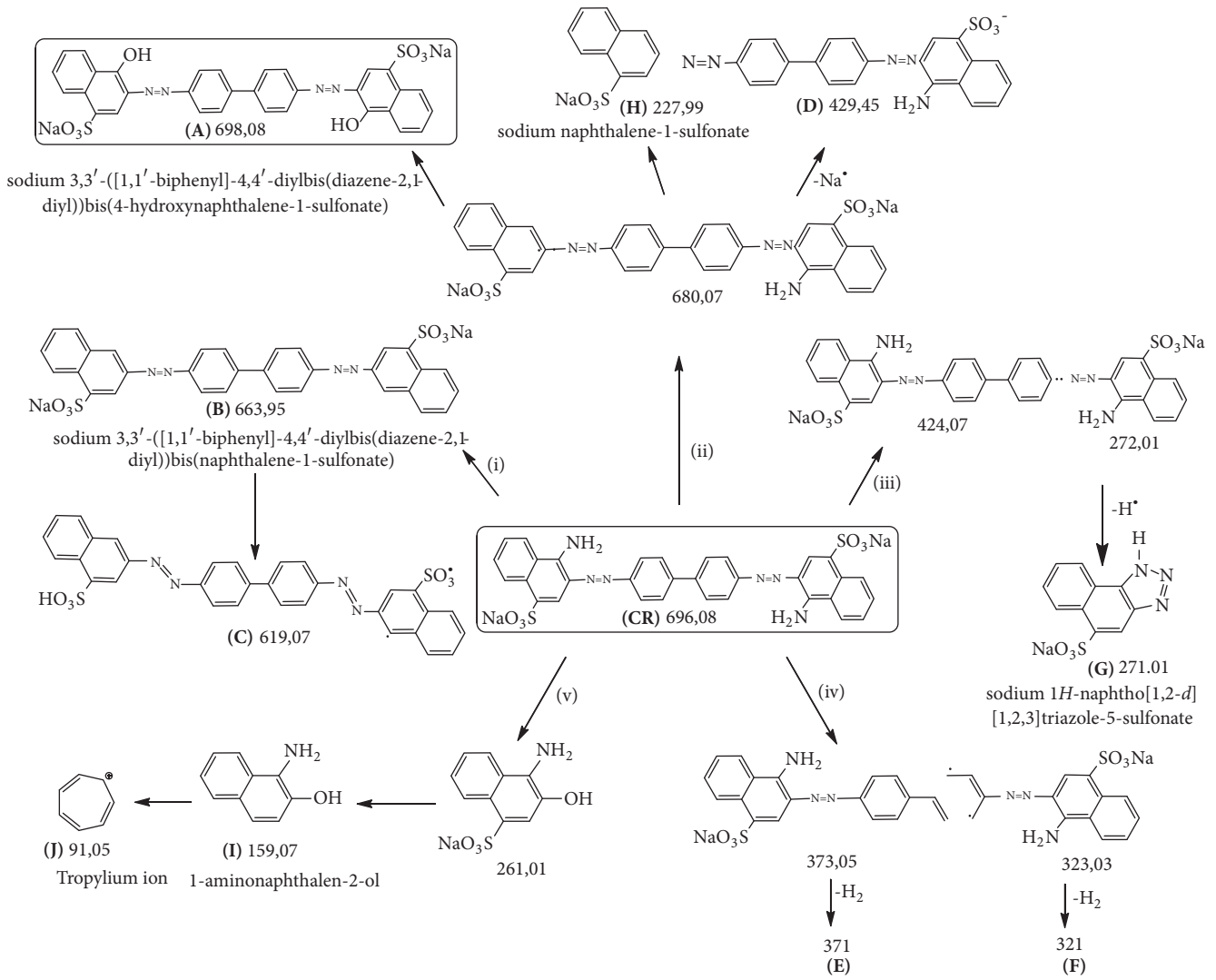

Figure 6: Proposed biodegradation pathway of CR using *A. niger*, with the identification of different degradation intermediates by LC-MS/MS.

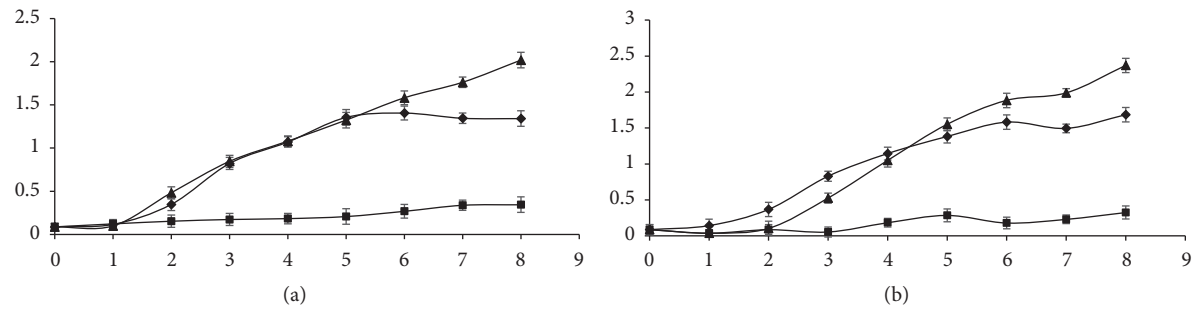

Figure 7: Kinetics growth of *Bacillus cereus ATCC 11778 (a) and Escherichia coli ATCC 10536 (b)* on nutrient broth, CR (200 mg.L^{-1}), and its transformation metabolites. The error bars represent the standard deviation of measurements for 3 samples.

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 paper.

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Review Article

Green Tea Catechins: Their Use in Treating and Preventing Infectious Diseases

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Green tea is one of the most popular drinks consumed worldwide. Produced mainly in Asian countries from the leaves of the *Camellia sinensis* plant, the potential health benefits have been widely studied. Recently, researchers have studied the ability of green tea to eradicate infectious agents and the ability to actually prevent infections. The important components in green tea that show antimicrobial properties are the catechins. The four main catechins that occur in green tea are (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG). Of these catechins, EGCG and EGC are found in the highest amounts in green tea and have been the subject of most of the studies. These catechins have been shown to demonstrate a variety of antimicrobial properties, both to organisms affected and in mechanisms used. Consumption of green tea has been shown to distribute these compounds and/or their metabolites throughout the body, which allows for not only the possibility of treatment of infections but also the prevention of infections.

1. Introduction

Infectious diseases are a leading cause of morbidity and mortality worldwide. HIV/AIDS and malaria are among the top ten infectious diseases in the world; and the most common types of infections are respiratory tract and diarrheal diseases [1]. With the advent of antimicrobial agents in the mid-1900s came the hope that eradication of infectious diseases was close. Unfortunately, the microorganisms involved were able to become resistant to the antimicrobial agents, and that only made it harder to fight these organisms. The CDC has estimated that each year more than two million people in the US suffer from antibiotic-resistant infections and that as many as 23,000 people die each year from these infections [2]. This results in not only increased morbidity and mortality, but also increased healthcare costs, which can be a huge financial burden for many countries. A recent analysis of the medical costs from healthcare-associated infections (those infections acquired in a healthcare facility) alone estimated that the annual costs of these infections in the US are between 28 and 45 billion dollars [3]. Antimicrobial resistance issues continue to impact these costs. One study found that the cost of antimicrobial resistance associated illnesses in the US could be as high as 55 billion dollars (20 billion dollars for healthcare costs and 35 billion dollars for lost productivity) annually [4]. To help in the fight against infectious diseases, researchers are looking at the possibilities of using natural plant products, which could turn out to provide a tremendous cost savings in healthcare. One of the plants that is currently being widely studied is the tea plant, looking especially at green tea.

Tea is one of the most commonly consumed beverages in the world, and green tea is becoming increasingly popular, accounting for around 20% of total global tea production. Tea is produced from the *Camellia sinensis* plant and is grown in over 30 countries. The best areas for growing tea plants are in specific tropical and subtropical regions. There are four main tea types produced: white, green, Oolong, and black tea. The type of tea is determined by how the tea leaves are processed, specifically by drying and fermentation methods. White tea is processed the least and uses very young leaves and leaf buds. Green tea is produced from more mature leaves with no fermentation. Oolong tea is produced by partially fermenting the leaves and black tea by fully fermenting the leaves [5–7]. Green tea is most commonly consumed in China, Japan, and Korea. Black tea is most commonly consumed in the US and the UK [8].

Green tea has been shown to have anticarcinogenic, anti-inflammatory, antimicrobial, and antioxidant properties and is beneficial in cardiovascular disease (CVD), diabetes and obesity, and neurologic and oral health. The anticarcinogenic properties include controlling cell proliferation, apoptosis and angiogenesis in tumor cells [9–12]. Inflammation is a component of many conditions and diseases including aging, arthritis, cancer, CVD, diabetes, and obesity. The general anti-inflammatory properties of green tea include the ability to decrease the denaturation of proteins and increase the production of anti-inflammatory cytokines [7, 13]. Oxidative stress results from the damaging effects of reactive oxygen species (ROS). The antioxidant properties of green tea include the ability to limit the amount of free radicals by binding to ROS, upregulating basal levels of antioxidant enzymes, and increasing the activity of these antioxidant enzymes [6, 14, 15]. The effects of green tea on CVD include the anti-inflammatory and antioxidant effects. In addition, the consumption of green tea has been shown to inhibit atherosclerosis, reduce total lipid levels, and improve the ratio of LDL to HDL [16, 17]. Diabetes and obesity are closely associated with a spectrum of disorders known as metabolic syndrome (MetS) which includes increased waist diameter, elevated plasma triglycerides, decreased HDL, increased fasting blood glucose, and elevated blood pressure [18, 19]. Type 2 diabetes is also associated with insulin resistance and sometimes decreased insulin production. Green tea has been shown to increase insulin receptor sensitivity and stimulate glucose-induced insulin secretion [20, 21]. Obesity is a result of an increase in fat mass which is caused by increase in the size of fat cells. Green tea has been shown to inhibit digestive enzymes and absorption of fat, which leads to decreased body waist circumference, intra-abdominal fat, plasma total and LDL cholesterol, triglycerides, and blood pressure [22–24]. The challenges of inflammation and oxidative stress can lead to DNA damage, protein misfolding, and loss of ATP production in mitochondria. This can result in cell death and loss of cognitive functions in the brain. The anti-inflammatory and antioxidant properties of green tea also protect neurons, and green tea metabolites have been shown to cross the blood brain barrier [25–29]. Green tea has been shown to be antimicrobial against most oral bacteria. In addition, it has been shown to improve oral health by increasing the activity of oral peroxidases, preventing the development and progression of periodontitis, and reducing dentin erosion and tooth loss, and it has a role in improving bad breath [30–34].

2. Green Tea Composition

The components in green tea that are the most medically relevant are the polyphenols. The most pertinent polyphenols are the flavonoids; and the most pertinent flavonoids are the catechins. The catechins comprise 80-90% of the flavonoids and around 40% of the water-soluble solids in green tea. Green tea contains more catechins than the other teas, mainly because of the way it is processed after harvesting. The amount of catechins in green tea can also be affected by where the tea is grown, the growth conditions, when it is harvested, how the leaves are processed, and the brewing temperature and length of time of brewing. These factors lead to a huge variation in catechin content among the varieties and brands of green tea consumed [35–45].

The four main catechins found in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG). The most abundant catechin is EGCG (~60%), and the next most abundant is EGC (~20%), then ECG (~14%), and EC (~6%). EGCG is the most studied in association with health, but EGC and ECG have been studied as well. As mentioned above, there can be a wide variation in the amount of catechins in any particular green tea beverage, although standardized extracts are available for use as supplements [7, 46, 47].

In order to be effective in the body these catechins need to be bioavailable after consumption. Once in the body, the catechins undergo metabolic processing in the liver and small intestine and colon. This processing produces glucuronide and sulfate conjugates or methyl epicatechins. Native forms of ECG and EGCG and metabolites of EC and EGC can be detected and measured in blood plasma. No forms of ECG and EGCG can be detected in urine, only metabolites of EC and EGC [48, 49]. Catechins are generally most stable in solution at a pH range of 4-6. It is now known that human serum albumin acts as a stabilizer, binding to the catechins and then transporting them [50]. Various studies in humans have found that the peak concentrations of catechins and their metabolites occur in blood plasma between 1.5 and 2 hours after ingestion and in urine between 4 and 6 hours after ingestion. The levels of these peak concentrations are affected by an individual's metabolism and of course by the amount of catechins in the ingested type of green tea. Commonly, the levels found in the body are directly proportional to the amount of catechins consumed [51–53]. Tables 1 and 2 show examples of blood plasma and urine concentration studies in humans.

3. Antimicrobial Properties

The antimicrobial effects of green tea catechins (GTCs) on microorganisms have been studied for many years. Green tea has been shown to combat these organisms in various ways, directly and indirectly, and has been shown to work synergistically with some antibiotic agents. Other known health benefits of green tea such as the anti-inflammatory and antioxidant effects may also contribute to the antimicrobial effects. Studies conducted on *Escherichia coli* found that exposure to green tea polyphenols (GTPs) resulted in major gene expression changes for 17 genes, with upregulation occurring in nine genes and downregulation in eight genes [75–77]. Table 3 shows a summary of the antimicrobial effects of green tea on bacteria.

4. Effects on the Bacteria Cell Membrane

One of the major properties of GTCs is the ability to bind to bacterial cell membranes. This binding can lead to interference in various bacterial processes and can damage the cell

Table 1: Amount of EGCG in blood plasma after a single dose.

EGCG dose (mg)	C_{max} (ng/ml)	Reference
200	73.7	[54]
400	111.8	
600	169.1	
800	438.5	
400	137.6	[55]
800	234.9	
88	135	[56]
140	34.7	[57]
225	300	[58]
375	1970	
525	2020	
50	130.4	[59]
100	180.4	
200	332.2	
400	624.5	
800	1067.4	
1600	3391.6	
200	376.9	[60]
400	525.2	
800	1682.1	
110	119	[61]
219	326	
329	321	

Table 2: Amount of EGC in 24-hour urine collection.

EGC dose (mg)	Amount in 24 hour urine (mg)	Reference
37	0.4	[54]
74	1.4	
111	3.5	
148	3.7	
82	~3.0	[56]
154	3.5	[57]
148	10.5	[62]
102	~3.0	[61]
204	~4.0	
306	~4.8	

membrane resulting in increased permeability and leading to cell lysis. Because EGCG is negatively charged it can combine with the positively charged bacterial cell membrane, especially in gram positive bacteria. The lipopolysaccharide (LPS) on the outer membrane of gram negative bacteria makes them more resistant to binding by GTCs [53, 63, 64, 66]. Studies with *E. coli* and *Pseudomonas aeruginosa* have shown that EGCG binding to the bacterial cell membrane can result in generation of H_2O_2 which is involved in damage to the cell membrane [63, 74]. Studies with *Staphylococcus aureus* have shown that this assault on the cell membrane causes a major cell wall stress response, resulting in upregulation of peptidoglycan biosynthesis genes and an alteration in cell wall structure. In methicillin-resistant *Staphylococcus aureus* (MRSA) strains, this change in peptidoglycan biosynthesis genes results in the production of PBP2 (penicillin-binding protein 2), which is what confers resistance to β-lactam drugs. Production of PBP2 is also inhibited by EGCG [64, 78, 79]. An important result of green tea binding is the loss of bacterial ability to bind to host cells. Studies using human and mammalian cells lines have shown that various bacteria such as *Fusobacterium nucleatum*, *Staphylococcus epidermidis*, and *Helicobacter pylori* have significantly decreased adherence to these cells [66, 67, 80]. Other important results are the loss of the ability for quorum sensing and biofilm formation of *P. aeruginosa*, *F. nucleatum*, and *Streptococcus mutans* [66, 68, 81]. Damage to the cell membrane also results in loss of function to transmembrane transporter proteins which are responsible for secretion of toxins and efflux of substances such as antimicrobial agents [53, 65, 69, 70].

5. Effects on Other Bacterial Cell Functions

There are a wide variety of other effects that GTCs have on bacterial functions. An important one which can affect most bacteria is the ability of GTCs to inhibit bacterial fatty acid biosynthesis by inhibiting enzymes involved in the biosynthetic pathway. Because this is an essential pathway for most bacteria, researchers are looking at targeting this pathway in antimicrobial drug development. Fatty acids are important for building cell membranes, as an energy source, and are involved in the production of toxic bacterial metabolites [53, 73]. Another target is the folate biosynthesis pathway. The enzyme dihydrofolate reductase (DHFR) is essential in this pathway, and is known to be a target for certain sulfa drugs. EGCG has also been shown to inhibit DHFR activity [53, 82]. Other important effects against enzymes include inhibition of bacterial DNA gyrase, inhibition of bacterial ATP synthase activity, and inhibition of bacterial protein tyrosine phosphatase and cysteine proteases [53, 71, 83]. Some specific bacterial effects include reducing bacterial H_2S production and inhibiting hemolytic activity of *F. nucleatum*, inhibiting the ability of *Listeria monocytogenes* to escape from the macrophage phagosome by inhibiting activity of listeriolysin O, and inhibiting the ability of *E. coli* to transfer plasmid content via conjugation [66, 72, 84].

6. Synergism

Since GTCs are known to have antimicrobial action, researchers have begun assessing the potential synergism of these catechins with other known antimicrobial agents. Green tea catechins have now been shown to act in synergy with imipenem against MRSA; with metronidazole against *Porphyromonas gingivalis*; with azithromycin, cefepime, ciprofloxacin, chloramphenicol, doxycycline, erythromycin, nalidixic acid, piperacillin, or tobramycin against *E. coli*; with ampicillin, Cefalotin, doxycycline, erythromycin, penicillin, or tetracycline against *Enterobacter aerogenes*; with chloramphenicol or tetracycline against *Pseudomonas aeruginosa*; and with aztreonam, ceftazidime, ciprofloxacin, gentamicin, meropenem, or tetracycline against *Acinetobacter baumannii*. The ability of GTCs to inhibit the function of

Table 3: Antimicrobial effects of green tea catechins.

Organism	Effects	References
Cell Membrane	Binding to bacterial cell membrane	[63–65]
Associated Effects	Damaging bacterial cell membrane	[63]
	Inhibits ability of bacteria to bind to host cells	[66, 67]
	Inhibits ability of bacteria to form biofilms	[66, 68, 69]
	Disrupts bacterial quorum sensing	[68]
	Interferes with bacterial membrane transporters	[65, 69, 70]
Bacterial Cell Functions	Inhibits bacterial DNA gyrase	[71]
Effects	Reduces bacterial H_2S production	[66]
	Inhibits bacterial hemolytic action	[66, 72]
	Inhibition of bacterial DHFR enzyme	[53]
	Inhibits bacterial fatty acid synthesis enzymes	[73]
	Increases bacterial internal ROS levels	[74]

Table 4: Synergism of green tea with antimicrobial agents.

Antimicrobial Action	Drug Synergism
Inhibit Cell Wall Synthesis	ampicillin
	ampicillin/sulbactam
	amoxicillin
	aztreonam
	cefalotin
	cefepime
	cefotaxime
	ceftazidime
	imipenem
	meropenem
	oxacillin
	penicillin
	piperacillin
Inhibit Protein Synthesis	amikacin
	azithromycin
	chloramphenicol
	doxycycline
	erythromycin
	gentamicin
	tetracycline
	tobramycin
Inhibit Nucleic Acid Synthesis	ciprofloxacin
	levofloxacin
	metronidazole
	nalidixic acid

bacterial efflux pumps (as mentioned previously) also plays a role in at least an additive antimicrobial effect for GTCs and many antimicrobial drugs, especially in gram negative bacteria that possess RND-type efflux pumps [53, 69, 70, 85–89]. Table 4 lists antimicrobial agents that have shown synergy with GTCs and the targets of these drugs.

7. Effects on Other Microorganisms

Green tea catechins have also been shown to be effective against a number of viruses, parasites, fungi, and even prions. The main antiviral effects include inhibiting the virus from binding to and entering host cells (adenovirus, enterovirus, HBV, HCV, HIV, HSV, influenza, and rotavirus); inhibiting viral RNA and DNA synthesis and viral gene transcription (enterovirus, EBV, HBV, HCV, and HIV); and destroying and functionally altering various viral molecules (adenovirus, HSV, and influenza) [64, 90–96]. Studies performed with adult healthcare workers to determine if green tea supplements could prevent infection with viruses causing influenza showed significantly fewer instances of influenza symptoms and a reduced incidence of laboratory-confirmed influenza cases versus the control group [97].

The main effect of GTCs on various parasite infections is a decrease in parasite numbers and growth. Other effects noted were fragmentation of parasite DNA and reduced fatty acid synthesis in the parasites. Studies with parasites include *Plasmodium falciparum*, *Babesia* spp., *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania braziliensis* [98–102].

Fungi that have been affected by GTCs include *Aspergillus niger*, *Candida* spp., *Penicillium* sp., *Microsporum canis*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. Research testing for synergistic effects found that EGCG showed synergism with amphotericin B, fluconazole, and miconazole in *Candida* spp.; and in *Candida tropicalis* strains that were resistant to fluconazole, EGCG, and fluconazole together induced apoptosis in the yeast cells [64, 103–107].

Prions are proteins that are considered to be infective agents because the abnormally structured (β-sheet) forms are able to induce normally structured (α-helix) forms to change shape. In the abnormal shape, protein function is lost and protein aggregation occurs in cells. Unlike other infectious agents, prions cannot be destroyed using autoclaving; the proteins have to be degraded to be noninfectious. Research using yeast cells found that EGCG could inhibit the β-sheet prions from changing the α-helical forms and could induce reversal of the β-sheet forms back to α-helical forms [108].

8. Antimicrobial Scope

There is a large amount of research that has assessed the antimicrobial effects of green tea catechins on a wide variety of microorganisms, including many gram negative and gram positive bacteria, some viruses, fungi, and prions. One of the most clinically important bacteria that has been researched is *S. aureus*, especially MRSA strains. The most studied gram negative bacteria is *E. coli* which is known for causing the majority of urinary tract infections. There are several recently published manuscripts that contain extensive information on which organisms are affected by green tea catechins [53, 64, 96, 109].

9. Prevention of Infection

Since it has been shown that GTCs have multiple types of antimicrobial abilities against so many organisms, it would be expected that green tea catechins could also prevent infections. One study was mentioned previously describing how green tea reduced the number of colds and influenza incidents. Another study involving adults showed that consuming green tea supplements twice daily for 3 months resulted in 32% fewer instances of cold or influenza symptoms and nearly 23% fewer illnesses of 2 or more days duration [110]. A study involving children found that, in school-aged children who consumed green tea on a regular basis, the number of incidents of influenza A or B was inversely associated with the number of cups of green tea consumed per day or per week [111]. Another study with Japanese nursey school children who gargled with green tea (or placebos) at least once each day found that there were up to 3 times fewer instances of illnesses with fevers in the green tea gargling group [112]. Two other studies with adults found that gargling with a green tea extract (GTE) solution resulted in at least half as many cases of influenza in the GTE gargling groups compared with the control groups [113, 114].

10. Conclusions

The research into the effects of green tea on human health has shown that it can be an important dietary factor in the prevention and treatment of various diseases such as arthritis, cancer, CVD, diabetes and obesity, infections, and in neurologic and oral health. Studies that were originally performed in animals and cell lines have become more frequently performed using humans. This type of research is vital if we are to fully discover what benefits GTCs can have in health issues. The more researchers that become involved in this, the clearer the answers. The studies on antimicrobial effects are providing very promising data, especially if GTCs prove to have synergistic abilities with many of the currently used antimicrobial agents and perhaps with drugs used to treat other diseases. The emergence of various multidrug-resistant bacteria, along with a dearth of effective antimicrobial drugs, makes the potential of green tea an extremely timely issue. There are also many areas across the globe where the cost of drugs is currently beyond the earning power of most of the population. Green tea is relatively inexpensive and fairly easy to obtain for most people. It could prove to be an answer for improving health on a global scale.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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