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Isolation and Molecular Characterization of a Newly Isolated Strain of *Bacillus* sp. HMB8, With a Distinct Antagonistic Potential Against *Listeria monocytogenes* and Some Other Food Spoilage Pathogens

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Abstract

Antagonistic *Bacillus* sp. strain HMB8 and its concomitant *Micrococcus* sp. strain HMB7 were isolated from a healthy oral cavity of a volunteer. Phenotypic characteristics and 16S rRNA gene sequencing similarity confirmed that strain HMB8 belongs to *Bacillus* genus, whereas strain HMB7 belongs to *Micrococcus* genus. Isolated *Bacillus* sp. HMB8 exhibited considerable *in vitro* antagonistic activity against a broad range of indicator strains, including food spoilage bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli*. Partial characterization of the bioactive compounds from the culture supernatant of the *Bacillus* sp. HMB8 was carried out using three extraction methods; ammonium sulfate, chloroform-methanol, and ethyl-acetate. All extracts retained good antibacterial activity against all indicator strains, but to varying levels. Overall, the ethyl-acetate extract of *Bacillus* sp. HMB8 demonstrated the highest inhibitory activity against most indicator strains, notably against *L. monocytogenes*. The findings of the present study suggest that *Bacillus* sp. HMB8 bioactive compounds may have potential biotechnological applications as food biopreservatives.

Keywords: *Listeria monocytogenes*, food-borne diseases, antagonistic activity, *Bacillus* spp., human oral bacteria, biopreservative.

1.Introduction

Food-borne diseases due to the consumption of contaminated and unsafe food products are among the leading causes of mortality worldwide, accounting for 20 million cases annually (Pal *et al*., 2016). In a comprehensive study, carried in 2012, food-borne illness was estimated to affect approximately 9.4 million people in the United States, leading to 1351 deaths each year (Scallan *et al*., 2011). Food poisoning occurs directly by bacterial food-borne pathogens, such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, or indirectly by their toxins (Kadariya *et al*., 2014; Saraoui *et al*., 2016).

Listeria monocytogenes is an opportunistic pathogenic psychrotrophic bacterium with public health significance. It is the etiologic agent of listeriosis, a severe food-borne illness with high rates of hospitalization and mortality (20% to 30%) (Saraoui *et al*., 2016; Seman *et al*., 2002). The majority (99%) of listeriosis cases have a food-borne source (Ivanek *et al*., 2005). Listerial contamination associated health problems have a significant economic impact, for example, listeriosis can impact Ready-To-Eat (RTE) foods costing more than a billion dollars a year (Ünlü *et al*., 2016). *L. monocytogenes* can tolerate and grow in many different unfavorable conditions, such as high salt concentrations, presence of CO_2, and freezing and storage at −18 °C. These features constitute challenges during conventional food preservation methods (Ivanek *et al*., 2005; Saraoui *et al*., 2016).

Bacillus genus is widely distributed in the environment and is of great significance in biotechnological and applied microbiology, especially for producing pharmaceuticals and chemicals for food production (Baruzzi *et al*., 2011; Borriss *et al*., 2011; Sumpavapol *et al*., 2010). Considerable research work has been oriented towards the isolation and the

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characterization of new novel bacteriocins and bacteriocin-like inhibitory substances from *Bacillus* genus. Bacteriocins offer several advantages that make them suitable for food biopreservation; they are effective against many food-borne pathogens, including *Listeria monocytogenes*, and become inactive when exposed to proteolytic enzymes in the gastrointestinal tract (Duarte *et al*., 2013; Sharma *et al*., 2011).

The human oral cavity and colon host the most diverse microbiome in the human body (Wade, 2013). The human oral cavity contains numerous distinct microbial niches, such as teeth, lips, tongue, cheek, hard and soft palates, and tonsils, which are densely populated by microbes, including viruses, fungi, protozoa, archaea, and bacteria (Dewhirst *et al*., 2010; Wade 2013). Based on traditional 16S ribosomal DNA investigation, approximately 690 different taxa of the human oral microbiome became publicly available on "The Human Oral Microbiome Database" website (www.homd.org). More than half of these taxa were officially named, while 32% are still considered as uncultivated phylotypes (Chen *et al*., 2010).

Recently, considerable attention has been given towards the isolation of new bacterial strains from different habitats, that may enable the discovery novel antimicrobial substances that can be used as natural food biopreservatives (Al-Dhabi *et al*., 2016; Axel *et al*., 2016; Crowley *et al*., 2013; Gómez-Sala *et al*., 2016; Manrique *et al*., 2016) and to combat multi-drug resistant pathogens (Asencio *et al*., 2014; Motta *et al*., 2004). The aim of the present study is the isolation and identification of *Bacillus* spp. from a healthy oral cavity of a volunteer, and the evaluation of the isolate's antagonistic potential towards *Listeria monocytogenes* and some other pathogenic species involved in food spoilage.

2.Materials and Methods

2.1. Indicator Strains

All the strains used as indicators belong to the collection of the Laboratory of Microbiology at Al-Hussein Bin Talal University, Jordan. All indicator strains were cultivated on Nutrient agar (Sigma-Aldrich) at 37 ºC for 18 hours, and stock cultures were maintained in Nutrient broth at –20 ºC with the addition of 40% glycerol. Indicator strains are listed in Table 1.

Table 1. Indicator strains used in this study

No	Indicator strains
1	*Listeria monocytogenes* (ATCC® 19115™)
2	*Staphylococcus aureus* (ATCC® 25923™)
3	*Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC® 14028™)
4	*Pseudomonas aeruginosa* (ATCC® 10145™)
5	*Escherichia coli* (ATCC® 25922™)

2.2. Bacterial Isolation from Human Oral Cavity

Isolates were collected from the roof of the oral cavity of a healthy volunteer. Samples were obtained using sterile swab soaked with 0.85 % NaCl. The swab was streaked directly onto Nutrient Agar (NA) media (Sigma-Aldrich). After 24 hours of incubation at 37ºC, morphologically different colonies were isolated and sub-cultured by streaking on the surface of NA agar plate for purification and preservation.

2.3. Preliminary Antagonistic Activity Screening

Each isolated bacterial strain was preliminary screened for antagonistic activity towards all other concomitant isolated strains. Each isolated bacterium was individually grown in 15 mL in Mueller–Hinton (MH) broth (Oxoid) at 37 ºC for 16 h. Five microliters of each culture having and inoculum concentration of around 5×10^5 CFU ml^{-1} were spotted onto MH agar (Oxoid) plates. After 18 h at 37 ºC, the bacteria were killed by chloroform fumes, and the plates were sprayed with all other concomitant isolated bacterial cultures (100 µL of a previously grown culture in 15 mL of MH broth). The plates were incubated for a further 16 h at 37 ºC and any inhibition zones were reported.

2.4. Antagonistic Activity of Strain HMB8 towards Listeria Monocytogenes and Some Other Indicator Strains

Screening of inhibitory activity was studied by the agar spot test that involved growing of the bacterial strain HMB8 isolated from the human oral cavity in 15 mL of MH broth at 37 ºC for 16 h. Five microliters of HMB8 culture with around 5×10^5 CFU ml^{-1} inoculum concentration were spotted onto MH agar plates. After 18 h at 37 ºC, the bacteria were killed by chloroform fumes and the plates sprayed with the indicator strains cultures (Table 1) (100 µL of a previously grown culture in 15 mL of MH broth). The plates were incubated for a further 16 h at 37 ºC and the diameter of the inhibition zones was measured (in mm). Three replicate plates were used for each indicator strain.

2.5. Phenotypic Characterization of the Isolated Strains

Only strains that demonstrated an antagonistic potential were morphologically and biochemically characterized using standard techniques (Gram stain, colony shape, size, and color on NA plates, catalase, and oxidase test, etc.) according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Growth characteristics at different temperatures and pH values were also studied.

2.6. Antibiotic Resistance Profiling

Isolates were subjected to antibiotic resistance profiling using the disc diffusion method on MH agar according to the CLSI procedures (Wayne, 2009). The following antibiotics (Oxoid) were used: neomycin 30 µg, chloramphenicol 30 µg, penicillin 10 µg, erythromycin 15 µg, tobramycin 10 µg, streptomycin 10 µg, kanamycin 30 µg, ampicillin 10 µg and gentamycin 10 µg.

2.7. Molecular Characterization of the Isolated Strains DNA Extraction

DNA extraction of isolated samples was performed using the G-spin Total DNA Extraction Mini Kit (iNtRON Biotechnology, Suwon, Korea) according to manufacturer's instructions. The extracted DNA was used as a template for PCR to amplify 16S rRNA genes.

2.8. Amplification of 16S rRNA Gene and Phylogenetic Analysis

The SSU rRNA gene was amplified with the bacterial forward primer 27F (3'- AGRGTTYGATYMTGGCTCAG-5') and reverse primer 1492R (5′-RGYTACCTTGTTACGACTT-3′). Amplification of 16S rRNA was performed in a total volume of 50 µL containing 2 µL Genomic DNA, 25 µL of 2x PCR Master-mix Solution (i-MAX II, iNtRON), 1 µL Forward Primer, 1 µL Reverse Primer, and 31 µL of sterile distilled water (sdH2O). PCR mixture with the addition of known bacteria genome, *Salmonella enterica* (ATCC® 14028™), was used as positive control, whereas PCR mixture with the addition of water in place of genomic DNA was used as negative control.

The PCR reaction mixtures, were incubated at 94°C for 3 minutes as an initial denaturation, cycled 30 times through denaturation for 1 minute at 94 °C, annealing for 1 minute at 60 °C, and elongation for 5 minutes at 72°C, and a final extension for 5 minutes at 72°C .Next, 10 µL of each PCR product was mixed with 2 µL of Blue/Orange 6x loading dye and analyzed via 1% agarose gel electrophoresis. In addition, 6 µL of 1 Kb Hyper ladder loading was used to identify band sizes. PCR quick-spin PCR Product Purification Kit (QIAGEN, Germany) was used to purify PCR products according to the manufacturer's protocol. The purified PCR products were sequenced using a commercial service provided by MACROGEN, Korea.

The resulting 16S rRNA gene sequences were compared with those in GenBank using the blast program (National Center for Biotechnology Information; NCBI) and the EzTaxon database (http://eztaxon-e.ezbiocloud.net/) (Kim *et al*., 2012). The 16S rRNA gene sequences of isolated strains were aligned with corresponding sequences of closely related type species (retrieved from the GenBank/EMBL/DDBJ database) using clustal x1.83 (Thompson *et al*., 1997).

Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987) contained in MEGA6 software (Tamura *et al*., 2013). Bootstrap values (more than 40%) based on 1000 replications were listed at nodes.

2.9. Nucleotide Sequence Accession Number

The nucleotide sequences of 16S rRNA documented in the present study were deposited in the NCBI nucleotide sequence database under Accession Numbers KX859179 (HMB7) and KX859180 (HMB8).

2.10. Partial Purification of Bioactive Compounds

To extract the bioactive compounds from the bacterial broth culture supernatant during the growth cycle, three methods of extraction were applied; ammonium sulfate extraction, chloroform-methanol extraction, and ethyl-acetate extraction. Strain HMB8 was inoculated into 1500 mL sterile nutrient broth and incubated on a shaker at 37°C for 72h. Cells were collected by centrifugation (4000 rpm for 15 min) and the supernatant recovered and passed through a 0.22 µm filter. The resultant supernatant was used in each of the extraction methods.

2.11. Ammonium Sulfate Extraction

Solid ammonium sulfate $(NH_4)_2SO_4$ was slowly added to 500 mL of the supernatant at 4°C to achieve a 70% saturated solution with constant stirring overnight. Precipitated bioactive compounds were pelleted by centrifugation (4000 rpm for 30 min, 4°C). The resultant pellet was re-suspended in 1/100 of the original culture volume in SP buffer (25 mM sodium phosphate buffer, pH 7). This solution was designated as a crude antibacterial preparation (ASE), and tested for its antimicrobial activity against indicator strains.

2.12. Ethyl-acetate Extraction

Five hundred milliliters of ethyl-acetate solvent were added to 500 mL of culture supernatant filtrate. The mixture was shaken vigorously for 15 min and kept stationary for another 15 min to separate the organic phase from the aqueous phase. The resulting mixture separated in two layers, the organic layer which contained the secondary metabolites and the aqueous layer. The crude extract (0.97 g) was obtained after concentrating the solvent using a rotary evaporator at 60 °C. This extract was designated as (EAE) and stored at 4 °C for the antimicrobial assay (Al-Dhabi *et al*., 2016). For determination of the extact's antimicrobial activity, it was re-suspended in dimethyl sulfoxide (DMSO).

2.13. Chloroform-Methanol Extraction

Five hundred milliliters of methanol were added to 500 mL of culture supernatant filtrate with vigorous stirring, then 125 mL of chloroform were added with vigorous stirring and incubated for 20 min on ice, and then the suspension was centrifuged at 14000 g for 5 mins. The upper aqueous layer was carefully removed, and the lower phase was dried. The crude extract was designated as (CME) and stored at 4 °C.

For determination of the extract's antimicrobial activity, it was re-suspended in SP buffer.

2.14. Extracts' Antibacterial Activity Determination

The antimicrobial activity of the resultant crude extracts was determined using the agar disk diffusion method (Kimura *et al*., 1998) against several indicator strains (Table 1), as well as against the concomitant isolated HMB7 strain. Dissolved extracts (ECE, CME and ASE) were sterilized by filtration through a 0.22 µm syringe filter (Millipore, Bedford, MA, USA). An aliquot (20 µL) of each dissolved extract was applied on discs (6 mm) and the discs were placed on MH agar plates previously inoculated with 100 µL of each of the ATCC indicator strains with around 5 × 105 CFU ml^{-1} inoculum concentration. After incubation at 37 °C for 16 h, MH plates were observed for the appearance of growth-inhibition zones around the discs.

3.Results

3.1.Isolation of Presumptive Antagonistic Bacteria from a Healthy Human Oral Cavity

Eight different bacterial colonies were isolated and sub-cultured on nutrient agar media on the basis of colony morphology. Next, each isolated strain was preliminary screened for an antagonistic activity towards all other concomitant isolated strains using the agar spot test. Only one strain designated as (HMB8) exhibited an antagonistic activity against another isolated strain designated as (HMB7) figure (1).

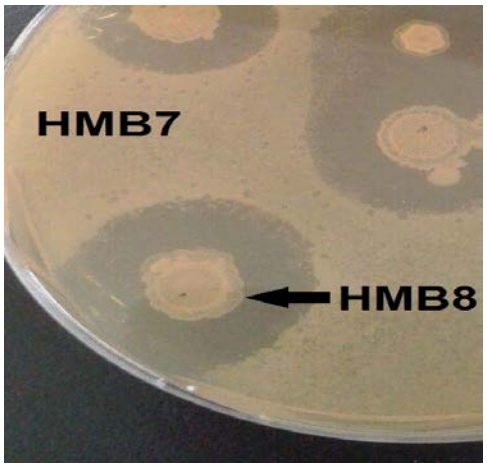

Figure 1. Screening of antagonistic activity of isolated bacteria as determined by the agar spot test. Bacteria designated (HMB8) showed an antagonistic activity towards one concomitant bacteria designated as (HMB7).

3.2.Antagonistic Activity of Strain HMB8 towards Listeria Monocytogenes and Other Indicator Strains

Strain HMB8 was tested for antagonistic activity against five ATCC bacterial food spoilage indicator strains, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli*. As shown in figure (2), HMB8 showed significant antagonism against all indictor strains except Salmonella Typhimurium; *E. coli* (26.1 ± 0.2 mm), *L. monocytogenes* (23 ± 0.9 mm), *S. aureus* (23 ± 0.9 mm), and *P. aeruginosa* (20.6 ± 0.7 mm).

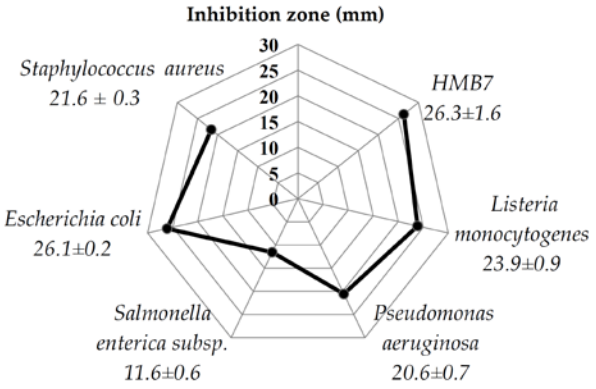

Figure 2. Inhibitory spectrum of HMB8 against *Listeria monocytogenes* and other indicator strains as determined by the agar spot test. The values are means of three replicates ± standard deviation.

3.3.Phenotypic Characterization of the Isolated Strains

On the basis of the results obtained during *in vitro* antagonistic activity screening for against food spoilage pathogens, both HMB8 and its concomitant inhibited HMB7 strains were morphologically and biochemically characterized using standard techniques. Growth parameters at different temperatures and pH values were also determined. The morphological and physiological characteristics of the selected strains are listed in Table 2.

Strain HMB8 was a Gram-positive bacillus. The colonies on agar media appeared white, circular, dry, flat, and irregular, with lobate margins. The optimum conditions for growth were 37°C and pH 8.0. In contrast, strain HMB7 was a Gram-positive coccus that produced yellow-pigmented circular colonies which were convex with entire margins. The optimum conditions for growth were 37°C and pH at 7.0. Biochemically, strain HMB8 was catalase and oxidase positive, had distinct fermentation profiles of different carbon sources, and was unable to produce urease. On the basis of the observed phenotypic characteristics, the strain was grouped into genus *Bacillus*. Strain HMB7 was catalase-positive, both oxidase and urease negative, and was able to use citrate; it was used as a sole carbon and energy source. Hence, the strain was grouped into genus *Micrococcus*.

Strain HMB8 was susceptible to neomycin, chloramphenicol, erythromycin, tobramycin, streptomycin, and gentamycin, and resistant penicillin and ampicillin. Strain HMB7 was susceptible to neomycin, streptomycin, kanamycin, ampicillin, gentamycin, erythromycin, and tobramycin, and resistant to both chloramphenicol and penicillin.

Table 2. Physiological and biochemical characteristics of strains HMB8 and HMB7.

Characteristics	HMB7	HMB8
Gram stain	Positive	Positive
Morphology	Coccus	Bacillus
Optimum growth pH	7.0	8.0
Optimum growth temp	37˚C	37˚C
Catalase production	Positive	Positive
Citrate utilization	Positive	Positive
Oxidase production	Negative	Positive
Urease production	Negative	Negative
Utilization of carbon sources		
D-Galactose	Negative	Positive
Sucrose	Positive	Positive
D-Fructose	Positive	Positive
D-Xylose	Negative	Positive
D-mannitol	Positive	Positive
Standard antibiotics (inhibition zone in mm)		
Neomycin 30 µg	19.3±0.2	30.9±1.3
Chloramphenicol 30 µg	Resistant	30.9±2.7
Penicillin 10 µg	Resistant	Resistant
Erythromycin 15 µg	9.3±1.3	23.9±1.9
Tobramycin 10 µg	14.9±0.2	31.1±0.7
Streptomycin 10 µg	33.5±0.6	37.8±1.1
Kanamycin 30 µg	32.8±0.6	41.4±2.2
Ampicillin 10 µg	24.1±2.8	Resistant
Gentamycin 10 µg	28.6±0.5	33.3±1.2

The antibiotics resistance profiles were determined using the disk diffusion assay. The values are means of three replicates ± standard deviation.

3.4. Molecular and Phylogenetic Analysis of the Isolated Strains

In assays for molecular identification of HMB7 and HMB8, PCR products of approximately 1.5 kbp were amplified from the 16S rRNA gene (Figure 3), and partial sequences of 961 bp and 1349 bp, respectively, were obtained (Genbank Accession Numbers: KX859179 (HMB7) and KX859180 (HMB8)). BLAST sequence comparisons suggested that HMB7 belongs to the genus *Micrococcus* and has a close relationship with *Micrococcus yunnanensis* YIM 65004$^{(T)}$, *Micrococcus aloeverae* AE-6$^{(T)}$, *Micrococcus endophyticus* YIM 56238$^{(T)}$ and *Micrococcus luteus* NCTC 2665$^{(T)}$. Nucleotide similarity was 99.27%, 99.17%, 98.96% and 98.96%, respectively. On the other hand, analyses of the 16S rRNA gene sequence of HMB8 indicated that it belongs to the genus *Bacillus* and was closely related to *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429$^{(T)}$ and *Brevibacterium halotolerans* with 99.93% nucleotide similarity, each, followed by *Bacillus mojavensis* with 99.85% nucleotide similarity, and *Bacillus subtilis* subsp. *spizizenii* with 99.78% nucleotide similarity. The phylogenetic trees of both strains are illustrated in figures 4 and 5.

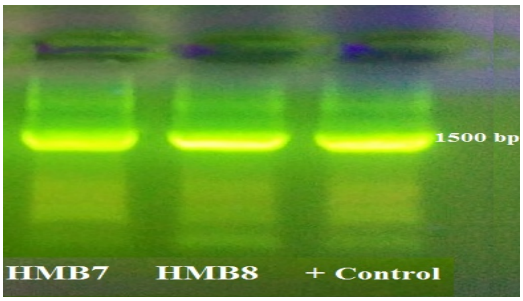

Figure 3. Agarose gel electrophoresis revealing successful amplification of 16S rRNA genes from HMB7 and HMB8.The 16S rRNA gene of *S.* Typhimurium was amplified as positive control.

In figure 4, the accession number of each type strain is shown in parenthesis. The tree was generated using the Neighbor-Joining (NJ) method using the MEGA6 software package and using *Nesterenkonia alba* DSM 19423 as an outgroup. Bootstrap values (more than 40%) based on 1000 replications are listed at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

In figure 5, the accession number of each type strain is shown in parenthesis. The tree was generated using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) using the MEGA6 software package (Tamura *et al*., 2013) and *Geobacillus thermoglucosidasius* NBRC 107763 as an outgroup. Bootstrap values (more than 40%) based on 1000 replications are listed at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

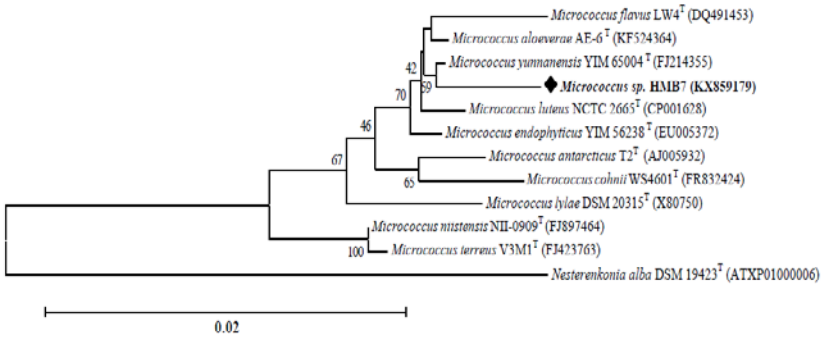

Figure 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between *Micrococcus* sp. HMB7 and the related type species of the genus *Micrococcus.*

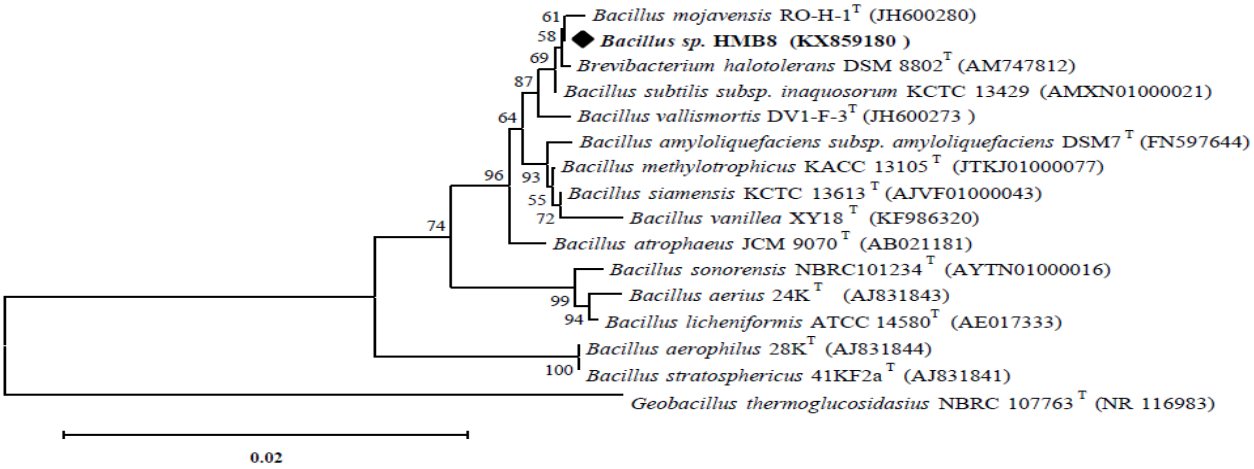

Figure 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between the strain *Bacillus* sp. HMB8 and the related type species of the genus *Bacillus*.

3.5. Bioactive Compounds Partial Purification and Antibacterial Activity

Table 3. Anti-bacterial activity of *Bacillus* sp. HMB8 semi-purified bioactive compounds.

Indictor strains	Strain number	Inhibition zone (mm)			
		Ethyl-acetate Extract (**EAE**)	Ammonium Sulfate Extract (**ASE**)	Chloroform-methanol Extract (**CME**)	Kanamycin 30 µg
L. monocytogenes	(ATCC® 19115™)	10.9±0.8	9.4±2.4	9.8±0.6	17.0
S. aureus	(ATCC® 25923™)	11.9±2.1	8.3±0.4	7.0±0.2	15.0
S. Typhimurium	(ATCC® 14028™)	8.0±4.1	7.2±1.0	6.8±0.3	15.0
E. coli	(ATCC® 25922™)	13.3±0.9	12.8±1.4	12.6±2.6	16.0
P. aeruginosa	(ATCC® 10145™)	9.3±0.2	11.4±0.6	9.1±0.2	7.0
Micrococcus sp. HMB7	Acc No (KX859179)	15.7±1.4	13.6±2.4	13.8±1.3	22.0

The values are means of three replicates ± standard deviation

After *Bacillus* sp. HMB8 strain was polyphasically characterized, an attempt was made to partially extract the bioactive compounds from its broth culture supernatant. Three methods of extraction were applied; ammonium sulfate extraction, chloroform-methanol extraction, and ethyl-acetate extraction. Resulting extracts were tested against all ATCC indicator strains (Table 1) as well as against the concomitant isolated *Micrococcus* sp. HMB7 using the agar disk diffusion method.

Results revealed also that the extracts retained a considerable antibacterial activity against all indicator strains and a good one towards the concomitant isolated *Micrococcus* sp. HMB7 (Table. 3).

4. Discussion

Bacillus spp. are widespread in natural environments (Porwal *et al.*, 2009) and have been and continue to be extensively screened for their potential production of useful bioactive products (Baruzzi *et al*., 2011; Borriss *et al*., 2011; Sumpavapol *et al*., 2010). In the present study, an attempt was conducted to isolate bacteria from a healthy human oral cavity with an antagonistic potential against *Listeria monocytogenes* and other food spoilage pathogens.

The human oral cavity is densely colonized by different bacterial species (Wade, 2013). It has been speculated that the population sizes of microorganisms in the human oral cavity are controlled by the antagonistic activity of the oral bacterial flora (Strahinic *et al*., 2007). Based on the above, we hypothesized that the human oral cavity is a source of bacterial strains with antagonistic activities, particularly towards food spoilage pathogens, as it is the gateway through which the food enters, and in which it gets chewed and mixed with saliva.

Strain HMB8 was isolated from the oral cavity and exhibited a noticeable antagonistic activity against another isolated strain designated as HMB). As shown in Figure (2), when HMB8 was subjected to further investigation against

five selected ATCC bacterial pathogens involved in food spoilage including *L. monocytogenes*, *S. aureus*, *S.* Typhimurium, *P. aeruginosa*, and *E. coli*, a wide spectrum of good antagonistic activities were observed.

Encouraged by these promising results, the two isolated strains were polyphasically characterized, the bioactive compounds were partially extracted from strain HMB8, and the antibacterial activities of the extracts were determined. On the basis of morphological, cultural, and some biochemical characteristics presented in table 2, strain HMB8 was initially identified as *Bacillus* spp., whereas HMB7 could be preliminary grouped into *Micrococcus* genus based on its morphology, and colony characteristics (creamy-yellow-color) (Kocur *et al*., 2006). Both strains were subjected to 16S rRNA gene amplification and sequencing. Molecular analysis results confirmed that HMB8 belongs to genus *Bacillus* and that it had the highest identity (99.93%) with *B. subtilis* subsp. *inaquosorum* and *Brevibacterium halotolerans*, followed by *Bacillus mojavensis* with 99.85% nucleotide similarity, and *Bacillus subtilis* subsp. *spizizenii* with 99.78% nucleotide similarity. All aforementioned species are considered members of the 'subtilis-group' or 'subtilis spectrum' (Rooney *et al*., 2009), which are difficult to differentiate as they possess similar phenotypic characteristics and share a high degree of 16S rRNA gene sequence similarity (Gatson *et al*., 2006; Roberts *et al*., 1996; Rooney *et al*., 2009). To reach a final accurate identification of strain HMB8, DNA–DNA hybridization and fatty acid composition of all closely related species should be evaluated. Unfortunately, such techniques are not available in our research lab. As clearly shown in figure 4, the detailed molecular phylogenetic analysis revealed that strain HMB7 represents a member of the genus *Micrococcus*. Although, high level of 16S rRNA gene sequence similarities is reported among *Micrococcus* spp. (Whitman, 2015), a combination of phenotypic and phylogenetic studies suggested that strain HMB7 is closely related to *Micrococcus yunnanensis* YIM 65004$^{(T)}$. Members of *Bacillus* and *Micrococcus* genera are common human oral cavity normal flora members and are frequently isolated from its different parts (Anesti *et al*., 2005; Bergan and Kocur, 1982; Chen *et al*., 2010).

An Initial attempt to purify the bioactive components from the HMB8 using three different extraction methods indicated that extracts retained a noticeable antibacterial activity against all indicator strains as well as against isolated *Micrococcus* sp. HMB7 (Table 3). The ethyl-acetate extract tended to produce the highest inhibitory effects against most used indicator strains, notably *L. monocytogenes* where a 10.9±0.8 mm zone of inhibition was observed. No obvious difference in the inhibitory effects of the extracts was found between Gram-positive and Gram-negative bacteria, suggesting that the bioactive compounds have a broad spectrum of activity.

One of the main characteristics shared among *Bacillus* spp. is the capacity to produce a large number of antimicrobial substances with different chemical compositions (Tabbene *et al*., 2009), *B. subtilis* subgroup is one of the key producers of these substances in the genus (Földes *et al*., 2000). A strain designated as *Bacillus subtilis* IFS-01 was isolated from the rhizosphere of cereals and showed antagonistic activity *in vitro* against various phytopathogenic, food-borne pathogenic, and spoilage micro-organisms (Földes *et al*., 2000). In another study, a new *Bacillus* strain producing a bacteriocin-like substance, with a broad inhibitory range for pathogenic and food spoilage bacteria, such as *L. monocytogenes*, was reported (Motta *et al*., 2007).

Due to the involvement in a number of food-borne diseases, *L. monocytogenes* constitutes a microorganism of high public health importance (Saraoui *et al*., 2016; Seman *et al*., 2002). The fairly broad inhibitory spectra of our of *Bacillus* sp. HMB8 strain against a number of serious and challenging food-borne pathogens/spoilage-causing bacteria, warrants further investigations to determine the possibility of using the extracted bioactive compounds as effective and safe biopreservative in food. Several attempts have been made to purify and characterize active compounds from *Bacillus* spp. and to evaluate their application in food biopreservatives (Baruzzi *et al*., 2011), for example, an extracellularly produced bacteriocin with desirable preservation attributes was isolated from food-grade *Bacillus subtilis* R75 (Sharma *et al*., 2011).

There have been several studies in the literature reporting antagonism among oral streptococci (Dempster and Tagg, 1982; Donoghue and Tyler, 1975; Strahinic *et al*., 2007). However, to our knowledge, no attempts were done to explore the potential antagonism among oral normal *Bacillus* spp. and to investigate their capacity to produce active substances that might be used in the food industry as biopreservatives.

The results obtained in the present study highlight the isolation oral *Bacillus* sp. HMB8 which demonstrated an ability to produce broad-spectrum inhibitory compounds that may prove useful as biopreservatives in different food products. Future studies should be conducted for the identification of the compounds found in the extract and to investigate their antagonistic activity (if any), to clarify their nature, and mode of action.

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