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Isolation, Characterization, and Antibacterial Activity of Actinomycetes from Sheep Feces

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ABSTRACT

Actinomycetes are a vital group of Gram-positive bacteria known for producing a wide range of bioactive secondary metabolites, including important antibiotics. These microorganisms play an essential role in the degradation of organic matter and nutrient cycling, contributing significantly to soil health and fertility. Their capacity to synthesize diverse compounds and the presence of key biosynthetic pathways involving polyketide synthases and non-ribosomal peptide synthetases highlights their potential in antibiotic discovery, particularly against antibiotic-resistant pathogens. This study aimed to isolate and characterize *Actinomycetes* from fresh sheep feces collected in Ilam Province, Iran, focusing on their antibacterial activity and biosynthetic potential. A total of 86 actinomycete isolates were obtained from fecal samples collected from sheep in 2021. Morphological characterization confirmed all isolates as Gram-positive and filamentous. Molecular identification through PCR amplification of the 16S rRNA gene yielded a product of approximately 640 base pairs for all isolates. Antibacterial screening revealed that 17 isolates exhibited activity against various pathogens, with the highest efficacy observed against *Bacillus cereus* (62.1%). Molecular analysis also indicated the presence of biosynthetic gene clusters, with 31 isolates (36.05%) bearing non-ribosomal peptide synthetase (*NRPS*) gene, 15 isolates (17.44%) containing polyketide synthase I (*PKS-I*), and 16 isolates (18.6%) with polyketide synthase II (*PKS-II*) genes. This study highlights the significant antibacterial properties and biosynthetic capabilities of *actinomycetes* from sheep feces, suggesting their potential use in therapeutic, agriculture, and biotechnological applications.

Keywords

Actinomycetes, Polyketide Synthase, Non-Ribosomal Peptide Synthetase, Antibacterial Activity, Sheep Feces

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Abbreviations

PKS: Polyketide Synthase
NRPS: Non-Ribosomal Peptide Synthetase
BGC : Biosynthetic Gene Clusters
PCR: Polymerase Chain Reaction
NA: Nutrient Agar
SCA:Starch Casein Agar

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Introduction

Actinomycetes, particularly members of the genus *Streptomyces*, represent a significant group of Gram-positive bacteria renowned for their capacity to produce a diverse array of bioactive secondary metabolites, including many clinically relevant antibiotics. These metabolites are crucial in pharmaceutical applications, especially in addressing the escalating issue of antibiotic resistance by providing new compounds for drug development [1].

Actinomycetes' ecological diversity facilitates their thriving in various environments, contributing to their ability to produce different classes of bioactive compounds such as antibacterials, antifungals, and anticancer agents [2]. Notably, actinomycetes account for approximately 45% of all known bioactive microbial metabolites, with over 10,000 compounds reported [3]. Their unique chemical structures and biological activities render them invaluable in the search for new therapeutic agents [4].

Actinomycetes inhabit various ecological niches, with soil and organic matter serving as key reservoirs. They thrive in diverse environments, including marine ecosystems, freshwater habitats, and extreme conditions such as deserts and high-salinity areas [5]. Furthermore, actinomycetes have been isolated from unique habitats like mangrove forests, caves, and endophytic niches within plants, underscoring their adaptability and ecological significance [6, 7]. These microorganisms engage in complex interactions within their ecosystems, significantly influencing their metabolite production. Understanding the ecological context of actinomycetes is essential for discovering novel bioactive compounds, as their secondary metabolites are often linked to environmental interactions [8]. The ongoing exploration of these diverse habitats continues to unveil new actinomycete species with potential therapeutic applications [9].

Sheep feces, rich in organic nutrients, provide an optimal environment for the growth of actinomycetes, thereby promoting their metabolic diversity. The nutrient composition of sheep feces supports a diverse microbial community, including various actinomycete species that thrive in such organic-rich substrates [10]. *Actinomycetes* in fecal environments play a vital role in decomposing organic matter and cycling nutrients, thereby enhancing their metabolic capabilities [11]. The presence of these microorganisms in feces can lead to the production of bioactive compounds, including antibiotics, which are beneficial for ecological balance and potential pharmaceutical applications [12]. Additionally, the interaction between actinomycetes and other microbial communities in feces can further enrich their metabolic profiles, making fecal matter a valuable resource for discovering novel actinomycete strains with unique properties [13].

The microbiota of sheep feces, including actinomycetes, significantly contributes to organic matter decomposition and nutrient cycling. *Actinomycetes* are key players in breaking down complex organic materials, such as cellulose and lignin, facilitating the conversion of organic waste into simpler compounds that plants and other microorganisms can utilize [14, 15]. During the composting process, actinomycetes, bacteria, and fungi function as chemical decomposers, transforming organic matter into stable products like compost, which enriches soil fertility [16]. Their metabolic activities enhance nutrient availability and contribute to forming soil aggregates, improving soil structure and health [17]. Overall, the presence of actinomycetes in sheep feces highlights their ecological importance in maintaining soil health and promoting sustainable agricultural practices through effective nutrient recycling [18].

Polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are essential enzymes involved in the biosynthesis of secondary metabolites, encompassing a wide range of bioactive compounds. PKS and NRPS are characterized by their modular organization, where each module incorporates specific substrates into the final product. This modularity enables the synthesis of structurally diverse compounds through the assembly of various building blocks [19]. Typically, these enzymes are organized in biosynthetic gene clusters (BGCs), facilitating the coordinated expression of the genes required for synthesizing these complex molecules. The arrangement of genes within these clusters can vary significantly, with some clusters containing hybrid PKS/NRPS systems that combine functionalities of both types of synthases [20]. This organization enhances biosynthesis efficiency and allows for the evolution of new compounds through gene rearrangements and modifications [21].

This study focused on the isolation and characterization of actinomycetes from sheep feces, assessing their antibacterial potential and screening for *PKS* and *NRPS* genes. *Actinomycetes* are known for their

Abbreviations - cont'd

TSB: Tryptic Soy Broth
EDTA: Ethylenediaminetetraacetic Acid
SOM: Soil Organic Matter
MH: Mueller-Hinton
bp: Base Pairs
RNA: Ribosomal RNA
SOM: Soil Organic Matter;
B. cereus: Bacillus cereus
E. coli: Escherichia coli
S. aureus: Staphylococcus aureus
P. aeruginosa : Pseudomonas aeruginosa

ability to produce a variety of bioactive secondary metabolites, making them valuable for biotechnological applications, particularly in antibiotic discovery [22]. Research has demonstrated that *Actinomycetes* isolated from various environments, including fecal matter, can exhibit significant antimicrobial activity. For example, studies have reported that a substantial percentage of isolated Actinomycetes possess PKS and NRPS genes, indicating their potential for producing secondary metabolites with antibacterial properties [23]. The presence of these BGCs is crucial for developing new antibiotics, especially in light of rising antibiotic resistance [24]. Thus, this study aimed to isolate *Actinomycetes* from sheep feces and investigate their potential for producing novel antimicrobial compounds, with a focus on the presence of BGCs such as *PKS* and *NRPS*, which are critical for the development of new antibiotics in response to increasing antibiotic resistance.

Results

Isolation and Identification of Actinomycetes

Eighty-six *Actinomycetes* isolates were obtained from sheep feces. These isolates exhibited diverse colony morphologies, including variations in texture and pigmentation. All isolates were Gram-positive and filamentous, characteristic of *Actinomycetes*. The identity of all isolates was confirmed through PCR amplification of the 16S rRNA gene, resulting in a product of approximately 640 base pairs (Figure 1).

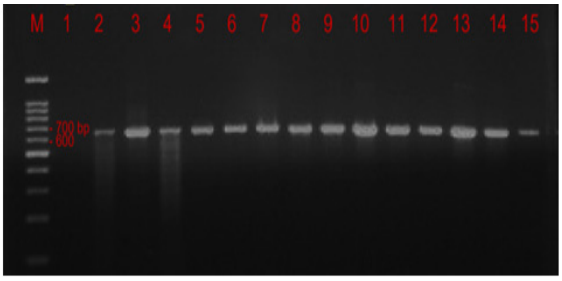

Figure 1.
Agarose gel electrophoresis of 16S rRNA PCR products from bacterial isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from bacterial isolates showing a 640 bp band representing the amplified 16S rRNA gene.

Antibacterial Activity

Out of 86 tested isolates, 17 strains showed antibacterial activity against one or more pathogens. The activity distribution was as follows: against *Staphylococcus aureus:* 16.1% (1 isolate), against *Escherichia coli*: 65.4% (4 isolates), against P*seudomonas aeruginosa*: 32.2% (2 isolates), and against *Bacillus cereus:* 62.1% (10 isolates). The most potent activity was observed against *Bacillus cereus*, underscoring the therapeutic potential of these isolates.

Molecular Identification and Gene Screening

PCR analysis revealed the following distribution of biosynthetic genes: 31 isolates (36.04%) carried *NRPS*, 15 isolates (17.44%) harbored *PKS-I,* and 16 isolates (18.6%) contained *PKS-II* genes (Figures 2-4). These findings indicate substantial biosynthetic potential among the isolates for secondary metabolite production.

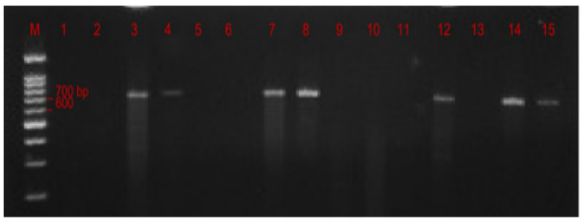

Figure 2.
Agarose gel electrophoresis of *NRPS* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from actinomycete isolates, displaying bands between 700–750 bp, indicative of the amplified *NRPS* gene.

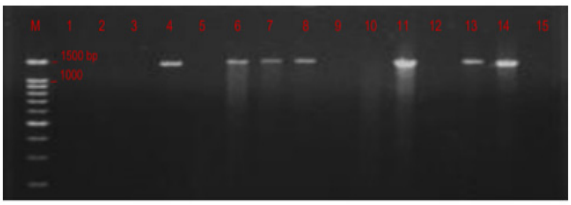

Figure 3.
Agarose gel electrophoresis of *PKS-I* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from actinomycete isolates showing a band at 1200-1400 bp, representing the amplified *PKS-I* gene.

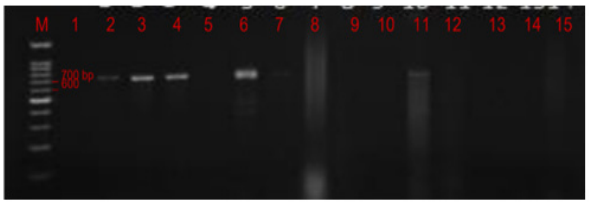

Figure 3.
Agarose gel electrophoresis of *PKS-II* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes: 2–15: PCR products from actinomycete isolates exhibiting a band at approximately 600 bp, corresponding to the amplified *PKS-II* gene.

Discussion

The study of *Actinomycetes* isolated from sheep feces has unveiled their potential as a source of antibacterial compounds, which is particularly relevant in the context of rising antibiotic resistance. *Actinomycetes* are renowned for their ability to produce bioactive metabolites that can effectively combat both Gram-positive and Gram-negative bacteria, including notorious pathogens such as *B. cereus* and *E. coli.*

Actinomycetes are recognized for their production of secondary metabolites with antimicrobial properties, making them important candidates in the search for new antibiotics [25, 26]. The successful isolation of 86 *Actinomycetes* strains, with 17 exhibiting notable antibacterial activity, underscores the ecological richness of this niche and the potential for discovering novel antibacterial agents [27]. Previous studies have demonstrated that various *Actinomycetes* strains produce metabolites that inhibit the growth of critical pathogens, suggesting their valuable role in developing new antibiotics, particularly against resistant strains [28].

The microbiota present in sheep feces, particularly enriched with *Actinomycetes*, plays a crucial role in organic matter decomposition and nutrient cycling, which enhances soil fertility [16]. *Actinomycetes* facilitate the breakdown of complex organic materials, contributing to soil organic matter (SOM) and nutrient availability [29, 30]. The diversity of *Actinomycetes* isolated from various habitats, including extreme environments, indicates unique antibacterial activities [31, 32]. This ecological significance supports the notion that environments rich in *Actinomycetes* serve as reservoirs for microorganisms capable of producing bioactive compounds with applications in agriculture and medicine [33].

Molecular characterization of isolates has revealed their potential as producers of bioactive compounds. Research indicates that a significant percentage of isolates possess genes associated with secondary metabolite production, highlighting their capacity for synthesizing antimicrobial compounds [34]. The presence of *PKS* and *NRPS* genes further suggests a robust biosynthetic machinery capable of generating diverse secondary metabolites [20]. Notably, the discovery of hybrid PKS systems has led to the identification of novel aromatic polyketides with therapeutic applications [35].

The antibacterial activity of isolated *Actinomycetes* against *B. cereus* has important implications for food safety and industrial contamination management. For instance, certain strains have shown potential as biocontrol agents in the food industry, effectively inhibiting the growth of *B. cereus* and downregulating its toxin-related genes [36]. Similarly, natural compounds, such as olive oil polyphenol extract, have demonstrated efficacy in reducing *B. cereus* populations in dairy products [37]. The inhibition of *E. coli* also holds significant implications in both veterinary and medical fields, with advancements in monoclonal antibodies and novel small-molecule inhibitors showing promise as alternatives to traditional antibiotics [38, 39].

Future research should prioritize the purification and characterization of bioactive compounds exhibiting antibacterial effects. Techniques such as metabolomic profiling and bioassay-guided fractionation are essential for elucidating the chemical structures and mechanisms of action of these metabolites [40, 41]. Additionally, exploring the regulatory pathways governing the expression of *PKS* and *NRPS* genes is crucial for optimizing metabolite yield and diversity [42, 43]. The study of unconventional habitats like sheep feces for microbial bioprospecting is gaining traction, revealing diverse microbial communities with potential applications in agriculture and biotechnology [44, 45].

In conclusion, combining molecular and microbiological approaches is vital for developing novel antimicrobial agents to combat emerging drug-resistant pathogens. Innovative strategies, including the use of antimicrobial peptides, nanoparticles, and new classes of antibiotics, represent significant advancements in addressing the urgent challenge of antibiotic resistance [46, 47, 48]. The exploration of natural antibacterial agents from sources like *Actinomycetes* not only enhances food safety but also contributes to sustainable practices in veterinary pharmacology and agriculture.

Materials & Methods

Sample Collection

Fresh sheep feces samples were collected from 28 sheep grazing in various geographical regions of Ilam Province, Iran, between March and June 2021.

Sample Preparation and Enrichment

Fresh sheep feces were aseptically collected using sterile forceps. The sample was immediately placed in a sterile zipped bag and transported to the laboratory on ice within two hours of collection.

In the laboratory, each sheep feces sample was thoroughly mixed, and 1 gram of the homogenized sample was serially diluted in sterile distilled water (10-fold dilutions up to *10^-6^*). A volume of 100 µL from each dilution was spread-plated onto two different media: nutrient agar (NA) is a general-purpose medium used for the growth of a wide variety of microorganisms, while starch casein agar (SCA) is a selective medium that enhances the growth of actinobacteria by incorporating starch and casein as sources of carbon and nitrogen.

To inhibit the growth of fungi and other bacteria, both media were supplemented with 50 µg/mL and 50 µg/mL of cycloheximide and

nalidixic acid, respectively.

Isolation and Purification of Actinomycetes

The inoculated plates were incubated at 28°C for 7-14 days. Distinct colony morphologies were observed and selected for further purification. Single colonies were subcultured onto NA and SCA plates until pure isolates were obtained.

Morphological Characterization of Actinomycetes

Isolate identification was based on morphological traits, including colony size, shape, color, margin, elevation; microscopic features such as Gram stain reaction, mycelium formation, and spore morphology; and pigmentation, which may be diffusible or non-diffusible.

DNA Extraction and Molecular Identification of Actinomycetes

Genomic DNA was extracted from pure cultures using a modified method based on Peng et al. (2013) [49]. Briefly, 2 mL of a 48-hour culture grown in TSB at 37°C was centrifuged at 6,000 g for 2 minutes. The supernatant was discarded, and the pellet was resuspended in 300 µL of lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl). This suspension was incubated at 55°C for 60 minutes with gentle shaking, followed by centrifugation at 16,000 g for 5 minutes. The supernatant containing the DNA was transferred to a new tube and stored at -20°C for further analysis. Polymerase chain reaction (PCR) targeting the 16S rRNA gene was performed using Actinomycetes-specific primers, as listed in Table 1 and described previously [50].

Detection of BGCs

Using specific primers listed in Table 1, the presence of *PKS-I*, *PKS-II* and *NRPS* genes was investigated by PCR as described elsewhere [51, 52].

Antibacterial Activity Assay

All actinomycetal isolates were fermented, and their resulting extracts were screened according to previous research without modifications [53].

The antibacterial activity of the actinomycetal strains was evaluated using reference strains of two Gram-negative and two Gram-positive pathogens (Table 2). The bacteria were cultured overnight at 37°C in Mueller-Hinton (MH) broth, and the culture was then adjusted to a turbidity level of 0.5 McFarland standard.

Following the procedure described by Hajizadeh et al. (2023) [53], bacterial lawns were created on MH agar with 6 mm wells, into which 100 µL of crude extracts were added. The plates were left at room temperature for one hour before being incubated at 37°C. After 24 hours, the inhibition zones were assessed in millimeters (mm), utilizing 100

Table 2.
Gram-positive and Gram-negative test pathogens included in the study for evaluating antibacterial activity

Reference strain	Accession number
Bacillus cereus	PTCC 1015
Escherichia coli	PTCC 1330
Pseudomonas aeruginosa	PTCC 1430
Staphylococcus aureus	ATCC 33591

Authors' Contributions

T.M., F.P., and G.H. conceived and planned the experiments. T.M. and F.P. carried out the experiments. T.M, and F.P. contributed to sample preparation. T.M., F.P., and K.S. contributed to the interpretation of the results. F.P. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Table 1.
List of oligonucleotide primers used in the study

Primer name	Sequence (5'-3')	Gene	Product size (bp)	Reference
ACT235f	CGCGGCCTATCAGCTTGTTG	16S rRNA	640	50
ACT878r	CCGTACTCCCCAGGCGGGG			
A3F	GCSTACSYSATSTACACSTCSGG	*NRPS*	700-800	52
A7R	SASGTCVCCSGTSCGGTAS			
KIF	TSAAGTCSAACATCGGBCA	*PKS-I*	1200-1400	51
M6R	CGCAGGTTSCSGTACCAGTA			
PKS-II-A	TSGCSTGCTTCGAYGCSATC	*PKS-II*	600	52
PKS-II-B	TGGAANCCGCCGAABCCGCT			

Conflict of interest

The authors declare that there is no conflict of interest.

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