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RESEARCH ARTICLE

# REVISED Exploring *Actinomycetes*-derived natural products for activity against *Streptococcus suis*

[version 2; peer review: 2 approved with reservations]

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## Abstract

### Background

*Streptococcus suis* is a major swine pathogen and an emerging zoonotic agent capable of causing severe systemic infections, leading to significant health and economic losses. In recent years, antimicrobial resistance in *S. suis* has increased substantially, with frequent reports of multidrug-resistant strains driven by extensive antimicrobial use in pig production. This situation is further exacerbated by the role of *S. suis* as a reservoir of resistance genes within a One Health context. Consequently, there is a growing need for new antimicrobial compounds. *Actinomycetes* represent a well-known source of bioactive compounds, and the exploration of rare and underexplored taxa offers opportunities to discover novel molecules with unique mechanisms of action.

### Methods

A screening of 800 extracts derived from *Actinomycetes* was performed against two virulent *S. suis* strains to evaluate antimicrobial activity. Extracts showing inhibitory effects were further subjected to fractionation by chromatography to isolate and characterize active components. Subsequent analyses focused on identifying fractions with inhibitory activity against the target strains using high-resolution

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mass spectrometry.

**Results**

The initial screening identified numerous *Actinomycetes*-derived extracts with inhibitory activity against *S. suis*, including extracts with activity against one or both strains. Based on activity spectrum and taxonomic origin, nine extracts were selected for further analysis. Fractionation enabled the identification of active fractions with consistent inhibitory effects against both strains. Compound characterization revealed bioactive molecules related to verucopeptin, hydroxyrifamycin and thiopeptides. In one extract, the active compound could not be identified, with analysis suggesting a complex composition of its active fraction.

**Conclusions**

These findings support *Actinomycetes* as a valuable source of novel antimicrobials against *S. suis.* The identified bioactive molecules further emphasize the potential of underexplored microbes for the discovery and development of novel antimicrobials, especially relevant in the context of increasing antimicrobial resistance.

## Plain Language summary

*Streptococcus suis* is a bacterium that causes serious disease in pigs and can also infect humans. It produces important health problems and economic losses. In recent years, it has become harder to treat because many strains are resistant to several antibiotics, largely due to their overuse in pig farming. This makes it important to find new types of treatments.

In this study, we tested 800 natural extracts from *Actinomycetes*, a group of bacteria known for producing useful compounds, to see if they could inhibit the growth of *S. suis.* Several extracts showed promising activity. Nine of these were selected for analysis.

The chemical complexity of extracts was reduced through resolution into different components, leading to the identification of molecules responsible for the antibacterial activity. Some active compounds were structurally related to known antibiotics, while one extract appeared to contain a complex mixture of active molecules that could not be fully identified.

Overall, the results show that *Actinomycetes* are a promising source of new antimicrobial compounds. These findings could help develop new treatments to control *S. suis* infections and address the growing problem of antibiotic resistance.

## Keywords

Streptococcus suis; antimicrobial resistance; Actinomycetes; antimicrobial screening; natural products




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**REVISED** **Amendments from Version 1**

The revised version highlights the use of an arbitrary inhibition cutoff and acknowledges the limitation of including only two strains in the screening.

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## Introduction

*Streptococcus suis* is an encapsulated, facultative anaerobic, Gram-positive coccus that commonly colonizes the upper respiratory tract of pigs, particularly the tonsils and nasal cavity.[1–3] Although it is part of the normal respiratory microbiota of its host, *S. suis* is also one of the most important bacterial pathogens in swine production. When this pathogen spreads systemically, it can be responsible for a wide range of pathological manifestations, including meningitis, septicemia, arthritis, and endocarditis, leading to significant health, welfare and economic losses.[2,4]

*S. suis* displays high phenotypic and genomic diversity, comprising numerous capsular serotypes and genetic lineages that vary in pathogenic potential, with serotype 2 most frequently associated with invasive disease in pigs and zoonotic infections in humans.[1,2,5] The clinical outcome of the *S. suis* infection depends on the virulence of the infecting strain and the host context.[3,6,7]

Beyond its impact on animal health, *S. suis* is an emerging zoonotic pathogen, particularly in Asia, where it has been recognized as a major cause of invasive human infection.[2,8] This dual role highlights its relevance within a One Health framework, linking animal, human, and environmental health.[9]

In this context, antimicrobial resistance (AMR) in *S. suis* has become an increasing concern,[4,10] since multidrug-resistant (MDR) strains have increasingly been reported.[11] Control of this pathogen in swine production systems has historically relied heavily on antimicrobial use, due to the limited availability of broadly effective vaccines that restricts preventive strategies.[4] As a result, selective pressure has contributed to the emergence and dissemination of resistant strains, particularly high rates against tetracyclines, macrolides, and lincosamides.[4,10]

From a global perspective, AMR in *S. suis* is part of the broader problem of MDR bacteria, defined as non-susceptibility to multiple antimicrobial categories.[12] Under a One Health approach, the use of antimicrobials in animal production, together with the horizontal transfer of resistance genes mediated by mobile genetic elements, facilitates the spread of resistance traits among animals, humans and the environment.[9] In this scenario, *S. suis* not only represents a pathogenic threat, but also acts as a reservoir and vehicle for resistance determinants, reinforcing its epidemiological importance.[10,13,14]

In light of the growing challenge of antimicrobial resistance, the search for new anti-infective compounds has regained considerable attention, particularly those derived from natural sources.[15] *Actinomycetes* have historically represented one of the most important reservoirs of naturally occurring antimicrobial compounds and continue to be a privileged source of biosynthetic diversity for the discovery of new inhibitory molecules.[16–19] In recent years, research efforts have increasingly focused on rare *Actinomycetes* and other underexplored bacterial taxa, as these microorganisms offer a greater likelihood of producing metabolites with novel chemical scaffolds and less redundant mechanisms of action than those observed in more conventional microbial collections.[17,20,21] This shift in focus is largely driven by the need to overcome the persistent issue of rediscovery of known compounds, which has historically limited the efficiency of traditional antimicrobial screening approaches.[17,19] Within this framework, the exploration of rare and underrepresented bacteria presents several strategic advantages and the possibility of diversifying current anti-infective development pipelines.[17,19,21] On one hand, it expands the accessible chemical space for the identification of bioactive compounds with inhibitory activity against pathogenic microorganisms.[22,23] On the other hand, it enables the integration of complementary approaches, such as taxonomic diversity, metabolomic profiles, and dereplication tools, to prioritize extracts and strains with a higher probability of yielding truly novel metabolites.[19,24]

Despite the recognized potential of *Actinomycetes* as a source of antimicrobial compounds,[19] relatively limited attention has been given to their application against *S. suis*, particularly in the context of antimicrobial-resistant strains.[4] This gap highlights the need for targeted studies aimed at identifying novel bioactive metabolites with activity against this pathogen. Therefore, the aim of this study was to evaluate the antimicrobial activity of *Actinomycetes*-derived extracts against *S. suis*, with the objective of identifying novel inhibitory molecules with potential relevance for the control of this clinically and economically important pathogen.

## Methods

### Bacterial strains and cultivation of bacterial strains

Two *S. suis* strains representing the two serotypes most commonly associated with clinical cases in Europe were used in this study. The reference strain P1/7 (serotype 2, frequent in pigs and associated with clinical cases in humans) was kindly provided by Dr. Marcelo Gottschalk (Université de Montreal, Canada) and has been previously described.[25] The second strain, 895-LCR (serotype 9), originates from CReSA's collection and was isolated from cerebrospinal fluid obtained from pigs with meningitis. Both strains were initially cultured on chocolate PolyViteX agar plates (bioMérieux, France) and subsequently grown for the assays in Todd-Hewitt Broth (T1438, Sigma-Aldrich, Merck, Germany) supplemented with 2% (w/v) yeast extract (70161, Sigma-Aldrich, Merck, Germany) (THY).[26]

### Antimicrobial susceptibility testing

Antimicrobial susceptibility of *S. suis* strains was performed by determining the minimum inhibitory concentration (MIC) of selected antibiotics using the broth microdilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). As no species-specific clinical breakpoints are available for *S. suis*, MIC results were interpreted following the breakpoints of the Viridans group streptococci (or *Streptococcus pneumoniae* when the value was not available for the Viridans group) found in CLSI document M100–27 (2017).

Briefly, serial dilutions appropriate for each antibiotic were prepared from stock solutions at 10 mg/mL and diluted in THY. Stock solutions were prepared in appropriate solvents according to their solubility, including dimethyl sulfoxide (DMSO) (D8418, Sigma-Aldrich, Merck, Germany) when required. To evaluate the potential effect of DMSO on bacterial growth, the solvent was tested separately over a range of concentrations under the same experimental conditions.

For the assay, each well was inoculated with 90 µL of a bacterial suspension adjusted to a final concentration of 1 x $10^5$ CFU/mL and 10 µL of the corresponding antibiotic dilution. Wells containing no antibiotic served as growth controls, while wells containing medium only were used as sterility controls. Plates were incubated at 37 °C for 24 h in a Synergy 2 microplate reader (Biotek), and optical density was measured at 600 nm ($OD_{600}$). The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible bacterial growth compared to the growth control.

The antibiotics tested included representatives from different antimicrobial classes: penicillin G and cefuroxime (β-lactams), chloramphenicol (amphenicols), clindamycin (lincosamides), erythromycin (macrolides), linezolid (oxazolidinones), levofloxacin (quinolones), rifampicin (ansamycins), tetracycline hydrochloride (tetracyclines), and telithromycin (macrolide-ketolides). All antibiotics were obtained from commercial sources (Table S1; Sigma-Aldrich, Merck, Germany) and prepared according to the manufacturer's instructions.

### *Actinomycetes* extracts

The NAICONS strain collection, its origin and composition, have been described before.[17] Full extracts from randomly selected strains were prepared as described.[24] Crude extracts were obtained through standardized high-quality extraction procedures from different *Actinomycetes* genera (e.g., *Streptomyces*, *Nocardia*, *Micromonospora*, among others). For plate preparation, dried extracts derived from strains of the NAICONS microbial collection (NAICONS, Milan, Italy) were employed. Extract plates were prepared following a protocol adapted from previously described methodologies.[24,27]

Briefly, a 10 mL culture was processed to generate both whole and fractionated extracts. For crude extracts, 2 mL of culture were mixed with 4 mL of ethanol, incubated under agitation for 1 h at 30 °C and subsequently centrifuged (4000 rpm, 8 min), with the supernatant collected as full extract.

For fractionation, the remaining 8 mL of culture, were separated into mycelium and supernatant. The mycelial pellet was sequentially extracted with ethanol at increasing concentrations (20%, 50% and 100%; 4 mL each), with shaking for 1 h at 30 °C and centrifugation (4000 rpm, 8 min) after each step to obtain mycelium-associated fractions.

In parallel, the supernatant was incubated with a HP20 resin (0.8 mL, 1 h, 30 °C) to adsorb extracellular metabolites. After centrifugation (3000 rpm, 1 min), the resin was sequentially eluted with ethanol solutions of increasing concentration (5%, 30% and 80%) with incubation steps (15 min, 30 °C) and centrifugation (3000 rpm, 1 min) between each elution.

All extracts were dried overnight at room temperature and stored at 4 °C until use.

### Screening of *Actinomycetes* extracts for antimicrobial activity against *S. suis*

A total of 800 *Actinomycetes*-derived extracts were screened with *S. suis* strains P1/7 and 895-LCR. Dried extracts were initially resuspended in 12.5 µL of dimethyl sulfoxide (DMSO) per well in a 96-well plate. Subsequently, 112.5 µL of autoclaved MilliQ water were added to obtain a stock solution containing 10% (v/v) DMSO.

For the antimicrobial assays, 10 µL of each stock solution of full extracts were transferred to a new 96-well plate containing 90 µL of bacterial culture adjusted to 1 x $10^5$ CFU/mL. This resulted in a final volume of 100 µL per well and a final DMSO concentration of 1% (v/v). Assays were performed independently for each *Streptococcus suis* strain. Each plate included a growth control consisting of 10 µL of 10% DMSO and 90 µL of bacterial culture, and a second control consisting of a chloramphenicol dilution series at known concentrations. Plates were incubated at 37 °C under static conditions and bacterial growth was monitored by measuring $OD_{600}$ at 0 and 24 h. Growth inhibition was calculated by normalizing the $OD_{600}$ values to the growth control as indicated in the formula below:

$$\text{Inhibition}\,(\%) = 100\,\text{x}\,\left(1 - \left(\Delta OD_{\text{sample}} / \Delta OD_{\text{growth control}}\right)\right), \text{where}\ \Delta OD = OD_{24h} - OD_{0h}.$$

An 80% inhibition threshold was used to define active extracts. This arbitrary cut-off was selected to prioritize extracts showing strong antibacterial activity and to facilitate hit identification. In some cases, OD decreased with incubation time, yielding an inhibition value higher than 100%. For clarity, these values are reported as 100% inhibition.

### Selection and fractionation of active extracts

Selected extracts were subjected to fractionation following a previously described HPLC-based approach.[27] Briefly, the extracts were separated into fractions by reverse-phase high-performance liquid chromatography (HPLC). Fractionation was performed using a Dionex UltiMate 300 system (ThermoFisher) equipped with a Merck LiChrospher RP-18 reverse-phase column (5 µm, I.D. x L 4.6 x 125 mm, Merck, Darmstadt, Germany) maintained at 50 °C. The mobile phase consisted of 0.05% formic acid in water (phase A) and acetonitrile (phase B), with a flow rate of 1 mL/min. Compounds were eluted using a linear gradient from 10% to 95% acetonitrile over 18 min, followed by a 5 min hold at 95%. UV absorbance was monitored at 230 and 270 nm. For each selected extract, 24 fractions were collected at one-minute intervals. The fractions were then dried using a speed vacuum concentrator and subsequently resuspended in 10% DMSO for biological evaluation using the screening assay described above.

Fractions displaying antimicrobial activity were selected for further analysis by high-resolution mass spectrometry (HRMS) to identify the active compound(s). To help identify the active compounds, adjacent inactive fractions (immediately preceding and following the active fraction) were also included in the LC-HRMS analyses, which were performed as described before,[24] using a Vanquish UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer, equipped with a YMC-Triart ODS column. The mobile phase consisted of 0.1% formic acid in water (A), LC-MS grade acetonitrile (B), and LC-MS grade isopropanol (C), using a gradient program as previously described.[23] The flow rate was set to 0.8 mL/min, with an injection volume of 8 µL and a column temperature of 40 °C. During separation, the flow was split, directing 75% to a diode array detector and 25% to the mass spectrometer, which operated with a heated electrospray ionization source in both positive and negative modes. MS/MS data were subsequently analyzed and compared against the Molecules Gateway and external spectral databases to facilitate compound identification.[24,28–31]

## Results

### Antimicrobial susceptibility profiles and DMSO concentration optimization in *S. suis*

Preliminary assays with antibiotics from different classes at a range of concentrations were performed to optimize the screening conditions for *S. suis* (Figure S1 and S2). These experiments were also used to establish the maximum non-interfering concentration of DMSO for subsequent assays.

The reference strain *S. suis* P1/7 exhibited high susceptibility to all antibiotics tested (Figure S1; Table 1), consistent with previous reports.[32] In contrast, the clinical isolate 895-LCR displayed an MDR phenotype (Figure S2; Table 1), showing resistance to erythromycin (macrolides), clindamycin (lincosamides), and tetracycline (tetracyclines), together with higher MIC for penicillin (penicillins), cefuroxime (cephems) and telithromycin (macrolide-ketolides) than P1/7. Other antimicrobial agents, including linezolid, rifampicin, and fluoroquinolones, retained activity against this isolate. This pattern is consistent with resistance profiles commonly described in clinical *S. suis* isolates[32] and suggests the presence of acquired resistance mechanisms.

The effect of DMSO on bacterial growth was evaluated across a range of concentrations in both strains, revealing a dose-dependent inhibitory effect (Figure S1 y S2). High concentrations resulted in marked growth inhibition, which

**Table 1. Minimum inhibitory concentration (MIC) of antibiotics for reference *Streptococcus suis* strain P1/7 and clinical isolate 895-LCR.**

| Antibiotics | MIC (µg/mL) | |
|---|---|---|
| | P1/7 | 895-LCR |
| Penicillin | 0.06 (S) | ≥ 1[#] |
| Cefuroxime | 0.03 (S) | 0.125 (S) |
| Erythromycin | ≤ 0.06 (S) | > 32 (R) |
| Telithromycin* | ≤ 0.06 (S) | 1 (S) |
| Clindamycin | 0.03 (S) | > 1 (R) |
| Chloramphenicol | 1 (S) | 2 (S) |
| Linezolid | 1 (S) | 1 (S) |
| Levofloxacin | 1 (S) | 1 (S) |
| Rifampicin* | ≤ 0.06 (S) | ≤ 0.06 (S) |
| Tetracycline | 0.25 (S) | 32 (R) |

*Note*: The breakpoints to classify the strains as susceptible or resistant were obtained from *Streptococcus* spp. Viridans group or *Streptococcus pneumoniae* when necessary.R, resistant; S, susceptible.
[#]Highest concentration tested
*MIC breakpoints taken from *Streptococcus pneumoniae.*

progressively decreased as the solvent concentration was reduced. At 2.5% DMSO, inhibition was minimal, while at lower concentrations it was virtually absent. Based on these results, 1% DMSO was considered suitable for solubilization of extracts and was selected for all subsequent experiments.

## Screening of *Actinomycetes* extracts against *S. suis*

A direct screening approach was performed to identify extracts with antimicrobial activity against the *S. suis* strains P1/7 and 895-LCR. The inhibitory activity of the tested extracts showed a broad distribution, ranging from non-detectable effect to strong growth inhibition (Figure S3).

To prioritize candidates for further characterization, extracts displaying high antimicrobial activity were selected using a threshold of >80% growth inhibition. Based on this criterion, 142 extracts were identified as highly active against the reference strain P1/7. A similar analysis performed with the clinical strain 895-LCR resulted in the identification of 123 extracts exceeding the same inhibition threshold. In both cases, highly active extracts formed a clearly defined subset, distinguishable from the majority of extracts showing low or moderate inhibitory effects. From the active extracts, 117 inhibited both strains, while 25 extracts inhibited only P1/7 and 6 inhibited only 895-LCR. These findings highlight the presence of a significant fraction of bioactive compounds within the screened collection and provide a focused set of candidates for subsequent analyses.

## Selection and fractionation of active extracts

Out of the 117 active extracts that inhibited both *S. suis* strains (P1/7 and 895-LCR), 14 extracts did not contain - as listed in the Molecules Gateway[24] - known antimicrobial compound that could account for the observed activity. For further analysis, we selected four extracts (from the 14 above) from rare *Actinomycetes* taxa (73990, 78443, 78450 and 78471), four additional extracts from *Streptomyces* (72356, 77387, 78495 and 88546) and one extract that contained deferoxamine, a chelating compound (78455).

These selected extracts were subjected to HPLC fractionation, generating 24 fractions per extract, which were subsequently evaluated for antimicrobial activity against the P1/7 strain (Figure 1). Analysis of the bacterial growth curves in the presence of fractions derived from extracts 78443, 78455, and 88546 showed that antimicrobial activity was restricted to specific fractions, while most fractions did not inhibit bacterial growth and displayed growth curves comparable to the untreated control. For extract 78471, no fractions exhibiting full inhibitory activity were identified, and only one fraction caused a delay in bacterial growth, indicating partial inhibitory effects. Finally, none of the fractions derived from extracts 72356, 73990, 77387, 78450 and 78495 inhibited bacterial growth.

Overall, only four out of the nine fractionated extracts yielded fractions with detectable antimicrobial activity (Figure 1), whereas no active fractions were identified for the remaining five extracts. This suggests that, in some cases, the

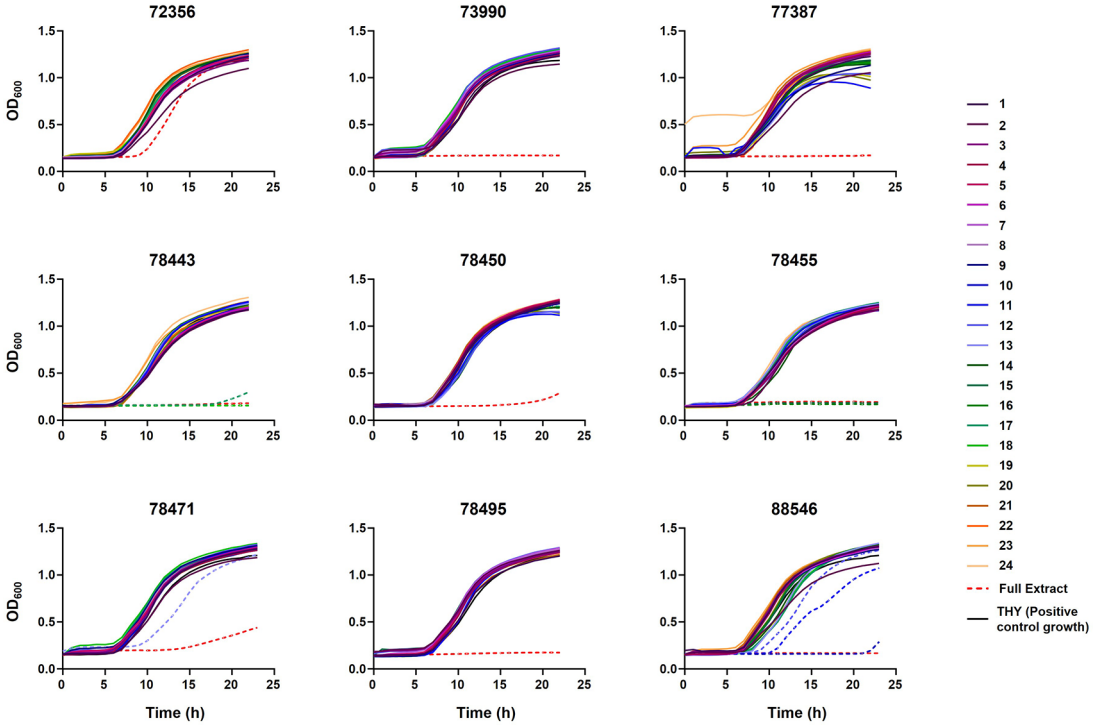



**Figure 1. Growth curves of *Streptococcus suis* strain P1/7 in the presence of extract fractions.** *S. suis* strain P1/7 was cultured in the presence of the 24 fractions obtained from the selected extracts. Bacterial growth was monitored over 24 hours by measuring optical density at 600 nm ($OD_{600}$). Dashed lines represent fractions that exhibited partial or complete inhibition of bacterial growth. The following controls were included: untreated P1/7 grown in THY (positive growth control) and P1/7 cultured in the presence of the full extract. The title of each graph corresponds to the extract analyzed.

antimicrobial activity observed in the raw extracts may depend on the combined or synergistic effect of multiple components, which could be lost upon fractionation. Alternatively, the active compounds were not eluted from the column under the conditions used, may be present at concentrations below the detection threshold after separation or may exhibit reduced stability during the fractionation process.

Based on the activity profiles obtained for the P1/7 strain, the fractions of the four extracts showing antimicrobial activity were selected for further analysis against the clinical strain 895-LCR. Instead of testing all 24 fractions per extract, a subset of eight fractions per extract was selected, including those displaying complete or partial inhibition, as well as their immediately adjacent fractions, to better delineate the active regions (Figure 2). In contrast to the results observed for the P1/7 strain, only one of the tested fractions (from extract 88546) resulted in complete growth inhibition in 895-LCR. The remaining fractions did not fully inhibit bacterial growth but instead induced a delay in growth, indicating a partial inhibitory effect. These results suggest that, although the identified fractions retain antimicrobial activity against both strains, their effect is less pronounced in the clinical isolate 895-LCR.

## Identification of bioactive compounds by HRMS

A total of eight selected bioactive fractions, together with the corresponding inactive flanking fractions, were subjected to high-resolution mass spectrometry (HRMS) analysis to identify the compounds responsible for the observed antimicrobial activity (Table 2).

For extract 78443, fractions 17 and 18 were analyzed (Figure 3). LC-MS analysis of these fractions revealed a major ion at *m/z* 918.515$^{+}$ with a retention time of 12.91 min, absent in the inactive fractions. The corresponding full mass acquired in negative ionization mode showed the presence of a signal at m/z 894.5194$^{-}$. The comparison of these data revealed that *m/z* 918.515 was a sodium adduct, corresponding to a neutral mass of 895.5253 Dalton (Da). This value matches the reported molecular mass of verucopeptin, an antitumor antibiotic isolated from *Actinomadura verrucosospora* in 1993.[33] The obtained MS and MS2 behaviors were consistent with the proposed structure, providing additional support for its

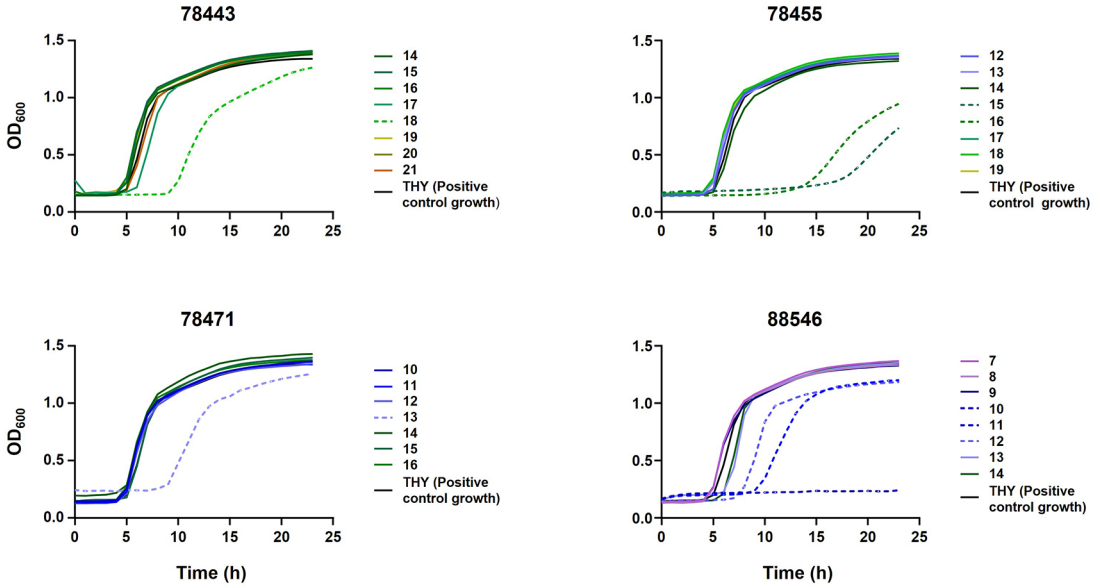



**Figure 2. Growth curves of *Streptococcus suis* strain 895-LCR in the presence of selected extract fractions.** *S. suis* 895-LCR was cultured in the presence of selected fractions from the four extracts that previously exhibited antimicrobial activity against strain P1/7. For each extract, 7 to 8 fractions were evaluated, including those that had shown complete or partial inhibition of bacterial growth in P1/7, together with their immediately adjacent fractions. Bacterial growth was monitored over 24 hours by measuring optical density at 600 nm ($OD_{600}$). Dashed lines represent fractions that exhibited partial or complete inhibition of bacterial growth. The following control was included: untreated 895-LCR grown in THY (positive growth control). The title of each graph corresponds to the extract analyzed.

**Table 2. Selected extracts for fractionation and further analysis, together with their main characteristics (percentage of inhibition of the growth of *S. suis* strains P1/7 and 895-LCR, *Actinomycetes* genus used to produce the extract [producer] and compound identified).**

| | | % of growth inhibition | | |
|---|---|---|---|---|
| **Extract** | **Producer** | **P1/7** | **895-LCR** | **Compound** |
| 73990 | *Amycolatopsis* | 100 | 100 | N.D. |
| 78443 | *Actinomadura* | 100 | 100 | Verucopeptin |
| 78450 | *Micromonospora* | 100 | 100 | N.D. |
| 78455 | *Micromonospora* | 100 | 100 | Sch 18640 |
| 78471 | *Micromonospora* | 100 | 100 | 8-Deoxy-3-hydroxyrifamycin S |
| 72356 | *Streptomyces* | 100 | 100 | N.D. |
| 77387 | *Streptomyces* | 100 | 95 | N.D. |
| 78495 | *Streptomyces* | 100 | 100 | N.D. |
| 88546 | *Streptomyces* | 100 | 100 | Not clear |

N.D. Not determined

identification. Furthermore, taxonomic annotation supports this assignment, since extract 78443 was prepared from a culture of an *Actinomadura* strain as the original verucopeptin producer (Figure 3).

For extract 78455, fractions 15 and 16 were analyzed (Figure 4). LC-MS analysis revealed an ion at m/z $772.7280^{+2}$ with a retention time of 9.7 min and $841.7505^{+2}$ with a retention time of 10.3 min. Based on the observed masses and fragmentation patterns, these compounds are tentatively assigned as Sch 18640 thiopeptide and its congener lacking 2 dehydroalanines at the C-terminal position. These compounds are thiostrepton-type peptides isolated from a *Micromonospora arborensis* strain in 1981[34] (Figure 4).

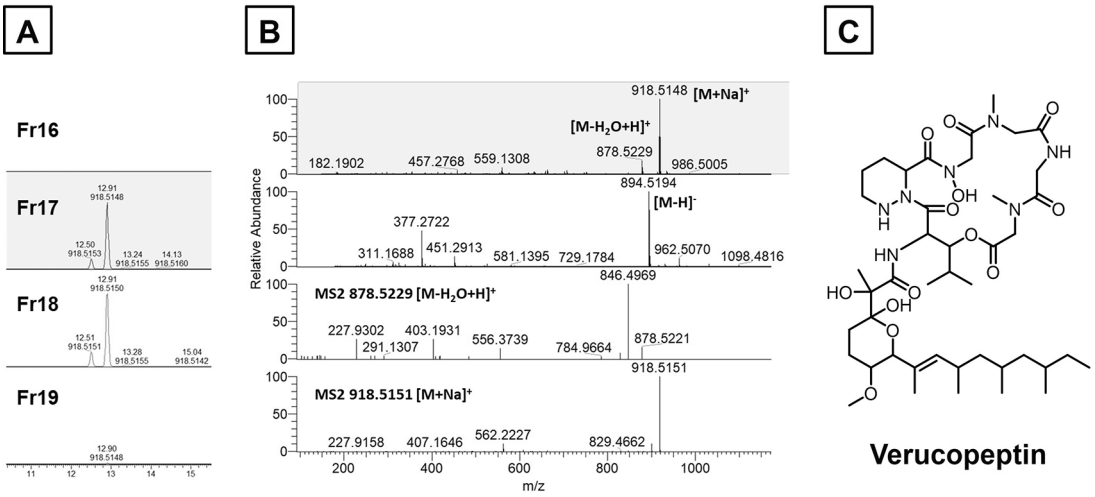



**Figure 3. Identification of verucopeptin in extract 78443.** Panel A: Extracted ion chromatograms of m/z 918.5148$^{+}$ from active (17 and 18) and inactive (16 and 19) fractions from extract 78443. Panel B, from top to down: Positive and negative Full Mass spectra at 12.9 minutes in fraction 18, MS2 spectra of the dehydrated and sodium adducts. Panel C: Chemical structure of verucopeptin.

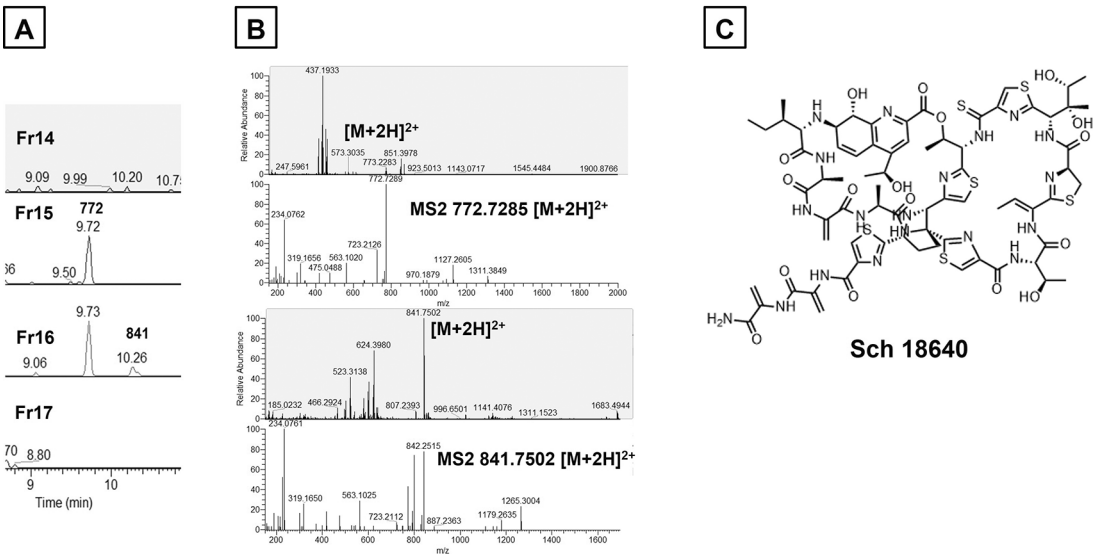



**Figure 4. Identification of compound Sch 18640 in extract 78455.** Panel A: Extracted ion chromatograms of m/z 772.7280$^{2+}$ and m/z 841.7502$^{2+}$ from active (15 and 16) and inactive (14 and 17) fractions from extract 78455. Panel B, from top to down: Positive Full Mass spectrum at 9.73 minutes in fraction 16, MS2 spectrum of m/z 772.7285$^{2+}$, positive Full Mass spectrum at 10.26 minutes in fraction 16, MS2 spectrum of m/z 841.7502$^{2+}$. Panel C: Chemical structure of Sch 18640.

For extract 78471, fraction 13 was analyzed (Figure 5). LC-MS analysis revealed an ion at m/z 694.2877, corresponding to a neutral mass of 695.2942 Da, consistent with the [M-H]$^{-}$ adduct. This mass, fragmentation pattern and retention time matched that of the original standard of 8-deoxy-3-hydroxyrifamycin S.

For extract 88546, fractions 10, 11 and 12 were analyzed. LC-MS analysis indicated a complex chemical profile, with several detected ions lacking clear database matches, suggesting that the activity may be due to a novel compound.

## Discussion

AMR constitutes a major One Health concern, affecting both humans and animals, where interactions between human health, animal health and the environment play a key role in its emergence, maintenance, and dissemination.[35–37] In this

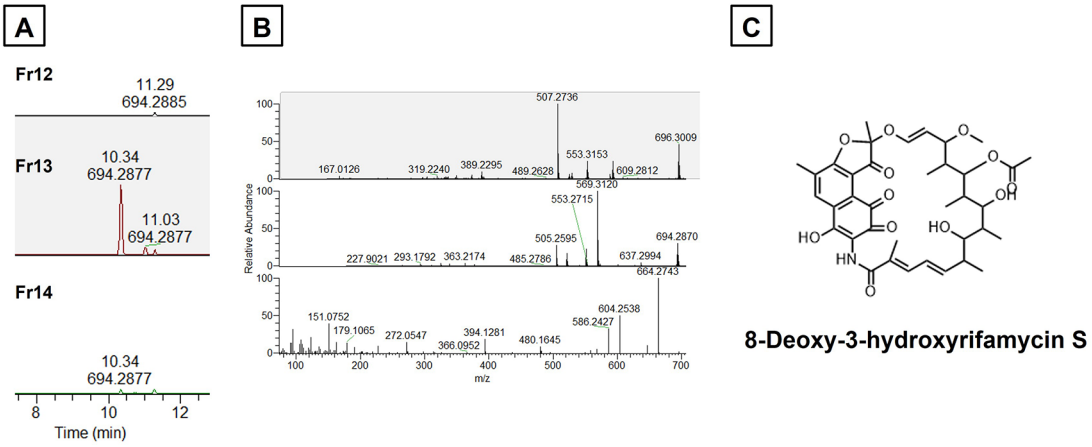



**Figure 5. Identification of 8-Deoxy-3-hydroxyrifamycin S in extract 78471.** Panel A: Extracted ion chromatograms of m/z 694.2877$^-$ from active (13) and inactive (12 and 14) fractions from extract 78471. Panel B, from top to down: Positive and negative Full Mass spectra at 10.3 minutes in fraction 13, MS2 spectrum of m/z 696.3009$^+$. Panel C: Chemical structure of 8-Deoxy-3-hydroxyrifamycin S.

context, MDR represents a critical challenge, since it restricts therapeutic options, promotes the persistence of resistant pathogens across different reservoirs, and contributes to the spread of resistance determinants among interconnected ecosystems.[37,38] In parallel with efforts to reduce antimicrobial use in animal production and human health, and consequently limit the emergence of new resistances,[39,40] there is an urgent need to develop or discover new antimicrobials. Within this framework, *S. suis* represents a particularly relevant model due to its significance as a porcine pathogen and an emerging zoonotic agent.[2] The high burden of *S. suis* in swine production promotes the use of antibiotics in livestock, thereby contributing to the selection of resistant strains and increasing the risk of transmission and infection in humans.[10,13,41,42] In this study, we performed a screening of *Actinomycetes* extracts against *S. suis*, with particular emphasis on underexplored genera within this phylum and we identified extracts with inhibitory activity that cannot be assigned to already-known compounds. Although based on only two S. suis strains, these promising findings warrant further validation using a broader collection of strains to better reflect the diversity of this bacterial species.

Our results allowed the identification of Sch 18640, a compound with inhibitory activity against *S. suis*, belonging to the thiopeptide family. Thiopeptides are sulfur-rich natural peptide antibiotics classified as RiPPs (ribosomally synthesized and post-translationally modified peptides), as they originate from a ribosomal precursor peptide that undergoes extensive enzymatic modifications.[43–45] Their biological relevance lies in their potent activity against Gram-positive bacteria, mainly through inhibition of protein synthesis. Depending on the specific compound, they can bind to the 50S ribosomal subunit or interfere with the elongation factor EF-Tu.[43,44,46] Given that *S. suis* is a Gram-positive bacterium and therefore falls within the typical target spectrum of thiopeptides, the observed activity is consistent with the known antibacterial profile of this family.[43,44] This supports its potential as a promising candidate for further structural and functional characterization, especially to address problems of solubility, bioavailability, and chemical synthesis, which have constrained the clinical development of thiopeptides.[43,44]

Another relevant finding was that the extract 78455, according to the Molecules Gateway, contained deferoxamine, a chelating compound used in the treatment of iron intoxications. In an attempt to explore the repurposing of this drug as an antibiotic, we tested this extract against *S. suis* and, in fact, the extract inhibited the growth of the bacterium. However, upon fractionation, this compound was not identified as the agent responsible for the inhibition of *S. suis.* This apparent discrepancy might be explained by the presence of another active compound in the extract. Deferoxamine (also known as desferrioxamine B) is a hydroxamate siderophore with a high affinity for ferric iron ($Fe^{3+}$), a property that underlies its clinical use as an iron-chelating agent.[47] Indeed, it is an approved drug for the treatment of acute iron intoxication and chronic iron overload.[48] This molecule is also of microbiological interest, as it was originally described as a metabolite of *Streptomyces pilosus*, where it plays a natural role in iron acquisition under conditions of limited metal availability.[49] Deferoxamine can also function as an exogenous xenosiderophore in some bacterial species, promoting iron uptake and bacterial growth or even virulence.[47,50,51] However, this capability is not universal and depends on the presence of specific uptake systems for siderophore-bound iron.[51] In contrast, our results suggest that *S. suis* does not exploit deferoxamine and neither its presence in the medium adversely affects the bacterium. This is consistent with previous reports indicating that *S. suis* does not produce siderophores and instead relies on alternative strategies to cope with iron

limitation.[52] Consequently, deferoxamine does not appear to contribute to the observed antimicrobial activity, which is more likely driven by other compounds present in the extract that were not clearly identified in our analysis.

One of the active metabolites identified in the screening was verucopeptin, produced by *Actinomadura*, a genus widely recognized as a source of chemically diverse bioactive compounds. Verucopeptin has been identified as a molecule of particular interest,[53] primarily for its antitumor activity. This compounds presents inhibitory activity against the vacuolar ATPase (v-ATPase) and research efforts have mainly focused on its chemical structure, biosynthesis, and mechanism of action.[33,54–56] Current evidence supports also its action as an antibacterial agent,[55] which can explain the activity found against *S. suis.*

Hydroxyrifamycin S compounds are hydroxylated derivatives of rifamycin S within the rifamycin family, primarily produced by *Amycolatopsis mediterranei* (formerly *Nocardia mediterranei* in earlier literature).[57–59] It is known that these molecules are generally described as biosynthetic intermediates, derived metabolites, or structural analogues employed to investigate structure-activity relationships, rather than as end products with well-established clinical or industrial applications.[60–62] Thus, their relevance lies in providing insight into the diversification of the rifamycin biosynthetic pathway and in elucidating how specific structural modifications influence biological activity.[63,64]

The specific compound found in this study, 8-deoxy-3-hydroxyrifamycin S, can be linked to two well-documented features: the occurrence of 3-hydroxyrifamycin S derivatives[61] and the existence of an 8-deoxy branch in rifamycin biosynthesis.[65–68] Indeed, Traxler & Ghisalba[69] explicitly positions this compound within the proposed biosynthetic scheme involving late-stage substitution at the C3 position, supporting its interpretation as a plausible intermediate within this metabolic network. Regarding its activity against *S. suis*, a mechanistic rationale supports its potential relevance, as members of the rifamycin family are known to target bacterial RNA polymerase.[63] Furthermore, structurally related derivatives have demonstrated activity against other bacterial species, such as *S. aureus,*[60,67] and we determined that our two *S. suis* strains were susceptible to rifampicin (rifamycin class).

## Conclusion

Overall, this study represents a step forward in the search for novel antimicrobials against the zoonotic pathogen *S. suis*, with potential implications not only for swine health but also for human medicine within a One Health framework. Although further research is needed to fully characterize the identified-compounds efficacy and safety, these findings lay the groundwork for the development of new therapies to combat this worldwide relevant pathogen.

## Author roles

**Ayelén Perez-Falcèn:** Data curation, Investigation, Visualization, Writing – Original Draft Preparation. **Marianna Iorio:** Conceptualization, Resources, Writing – Review & Editing. **Margherita Sosio:** Conceptualization, Resources, Writing – Review & Editing. **Florencia Correa-Fiz:** Conceptualization, Funding Acquisition, Resources, Supervision, Writing – Review & Editing. **Stefano Donadio:** Conceptualization, Funding Acquisition, Resources, Supervision, Writing – Review & Editing. **Virginia Aragèn:** Conceptualization, Funding Acquisition, Resources, Supervision, Writing – Review & Editing.

## Ethics and consent

Ethical approval and consent were not required.

## Data availability

### Underlying data

Zenodo: Exploring *Actinomycetes*-derived natural products for activity against *Streptococcus suis.* https://doi.org/10.5281/zenodo.22247751.[70]

The project contains the following underlying data:

- Underlying data.xlsx (Contains the raw $OD_{600}$ data of the bacterial growth in the different conditions).

### Extended data

Zenodo: Exploring *Actinomycetes*-derived natural products for activity against *Streptococcus suis.* https://doi.org/10.5281/zenodo.22247751.[70]

The project contains the following extended data:

- Extended data.docx (Contains a table S1 with the antibiotics used and all figures presented in the text as supplementary figures with their legends).

- Figure S1. jpg, Figure S2.jpg, Figure S3–1.jpg, Figure S3–2.jpg, Figure S3–3.jpg, Figure S3–4.jpg and Figure S3–5.jpg (Figures presented in the text as supplementary figures).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

## Acknowledgements

We would like to sincerely thank the entire team at Naicons for their support and collaboration throughout this work. We are especially grateful to Cristina Brunati and Stefania Serina for their invaluable assistance with laboratory activities and their support during the screening process.

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# Open Peer Review

## Current Peer Review Status: ? ?

**Version 1**

Reviewer Report 30 September 2026

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?

**Iqbal Ahmad**
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[2] Aligarh Muslim University, Aligarh, India

The authors have made a good attempt to screen large number of isolates of actinomycetes against *S. suis* strains.
Although, the number of strains should have been included more in number to get broad spectrum activity at least for most promising actinomycetes extracts.
The methodology is largely explained in detail except at some places where only reference are given. A total of 800 *Actinomycetes*-derived extracts were screened in this study. What is the source of these strains,? WHO have previously isolated or obtained these actinomycetes are not mentioned in the text. Please provide the details/reference.
What is basis of selection of 10% SDS for fractionation stock solution making for biological activity.
The fractions were then dried using a speed vacuum concentrator and subsequently resuspended in 10% DMSO for biological evaluation using the screening assay described above.
10% DMSO may show antibacterial activity. What is THY as control? Is it an antibiotic?
It is advisable to include some MDR strains for antimicrobial action to enhance its scope in combating AMR in this study or separately.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and does the work have academic merit?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Applied Microbiology and Natural product Research: anti-infectives

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 09 Oct 2026

**Ayelén Perez Falcón**

**Reviewer comment: The authors have made a good attempt to screen large number of isolates of actinomycetes against *S. suis* strains. Although, the number of strains should have been included more in number to get broad spectrum activity at least for most promising actinomycetes extracts. Response:** We thank the reviewer for this valuable comment and agree that the inclusion of a larger panel of *S. suis* strains would strengthen the evaluation of the antimicrobial activity of the selected extracts. We have addressed this point in the revised manuscript by explicitly acknowledging the use of only two *S. suis* strains as a limitation of the study in the Discussion section. **Manuscript changes:** We have added the following sentence to the Discussion: Although based on only two *S. suis* strains, these promising findings warrant further validation using a broader collection of strains to better reflect the diversity of this bacterial species.
**Reviewer comment: The methodology is largely explained in detail except at some places where only reference are given. A total of 800 Actinomycetes-derived extracts were screened in this study. What is the source of these strains,? WHO have previously isolated or obtained these actinomycetes are not mentioned in the text. Please provide the details/reference. Response:** We have included the information in the revision version. **Manuscript changes:** We have added the following sentence to the "*Actinomycetes* extracts" section in M&M: The NAICONS strain collection, its origin and composition, have been described before [17].
**Reviewer comment: What is basis of selection of 10% SDS for fractionation stock solution making for biological activity. The fractions were then dried using a speed vacuum concentrator and subsequently resuspended in 10% DMSO for biological evaluation using the screening assay described above. 10% DMSO may show antibacterial activity. What is THY as control? Is it an antibiotic? Response:** As described in the manuscript, the fractions were resuspended in 10% **DMSO** (not SDS) to prepare stock solutions; however, only 10 µL of each stock solution was added to 90 µL of bacterial suspension, resulting in a final DMSO concentration of 1% (v/v) in the assay. To ensure that the solvent did not affect bacterial growth, DMSO controls were included and are reported in the Supplementary Material. These controls confirmed that 1% DMSO did not exert antibacterial activity against *S. suis* under the conditions tested. Regarding the

reviewer's question about THY, this refers to Todd Hewitt Broth, the culture medium used for the growth of *S. suis* (please, see section: "Bacterial strains and cultivation of bacterial strains"). In the assay, THY without the presence of any extract was used as the growth control to represent uninhibited bacterial growth.
**It is advisable to include some MDR strains for antimicrobial action to enhance its scope in combating AMR in this study or separately.** We agree that evaluating the identified compounds and fractions against additional multidrug-resistant (MDR) strains is of great interest. In fact, 895-LCR is a MDR strain (please see table 1), but we acknowledge that the inclusion of more MDR strains could further broaden the relevance of this work in the context of antimicrobial resistance. We thank the reviewer for this suggestion and will consider this aspect in future studies.

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 21 September 2026

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Overall assessment

It is a relevant screening study of 800 extracts derived from Actinomycetes which were screened against two Streptococcus suis strains, followed by HPLC fractionation and HRMS analysis. The study is scientifically relevant, and has useful data for antimicrobial compounds.

Comments
The authors need to make it clear whether they used appropriate statistical analysis or explain why they did not use statistical testing.
The rationale for using the 80% inhibition threshold should be clarified.
The authors should differentiate between confirmed identification and tentative identification from HRMS/MS alone.
If any compound is detected in an active fraction, this does not necessarily mean that the compound is responsible for the antimicrobial activity.
Only 2 strains of S. suis are used in the study. The number of strains used should be recognized when discussing the general applicability of results.
The number that is more than 100% inhibition in Table 2 (104-109%) should be verified and clarified.

Minor comments
Spot and correct typographical and grammatical errors, such as 'S. suisinfection' or 'Finaly'.
Include adequate LC-HRMS method details to replicate
The manuscript has scientific merit, but minor revisions are required to clarify replication/statistics, compound identification, antimicrobial activity attribution, and the scope of the conclusions.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and does the work have academic merit?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Medical Microbiology; Actinobacteria; Natural Products; Antimicrobial Activity

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 09 Oct 2026

**Ayelén Perez Falcón**

**Reviewer comment: The authors need to make it clear whether they used appropriate statistical analysis or explain why they did not use statistical testing. Response:** The aim of this study was to perform a primary high-throughput screening of 800 natural extracts against two *Streptococcus suis* strains to identify potential active candidates. Growth inhibition was calculated relative to the growth control, and compounds showing $\geq$ 80% inhibition were classified as active hits. The screening was conducted using an arbitrary threshold (80%) intended for hit identification. This threshold was based on previous experience with similar tests (Al Nahhas et al. 2025) and experimentally validated with controls. Statistical analyses were not performed since they were considered unnecessary.
**Reviewer comment: The rationale for using the 80% inhibition threshold should be**

**clarified. Response:** The ≥ 80% inhibition threshold was arbitrarily selected for hit identification during the primary screening. Given the large number of extracts evaluated, a stringent cut-off was considered necessary to prioritize only extracts displaying strong antibacterial activity and to minimize the inclusion of weakly active candidates. Similar screening strategies have been previously applied to extract collections, where an ≥80% inhibition threshold was used to define positive candidates and was described as a balance between sensitivity and specificity for identifying biologically relevant hits (Al Nahhas *et al.* 2025, DOI: 10.12688/openreseurope.19988.2). **Manuscript changes:** We have added this explanation at the end of section "Screening of *Actinomycetes* extracts for antimicrobial activity against *S. suis*": This arbitrary cut-off was selected to prioritize extracts showing strong antibacterial activity and to facilitate the identification of biologically relevant hits.
**Reviewer comment: The authors should differentiate between confirmed identification and tentative identification from HRMS/MS alone. Response:** We agree that the distinction between confirmed and tentative identifications should be made clearer. In the present study, compound assignments were based on HRMS data, MS/MS fragmentation patterns, retention time information, and comparison with available database entries and literature reports. Since no additional structural confirmation techniques were performed, these identifications should be considered probable rather than experimentally-confirmed.
**Reviewer comment: If any compound is detected in an active fraction, this does not necessarily mean that the compound is responsible for the antimicrobial activity. Response:** We agree that the detection of a compound in an active fraction does not by itself demonstrate that the compound is solely responsible for the observed antimicrobial activity. Other metabolites present in the fraction, including minor constituents, as well as possible synergistic or additive interactions among different components, may also contribute to the detected activity. Nevertheless, the identification of the compounds by HRMS/MS, together with the agreement of their exact masses and fragmentation patterns with published data and specialized databases, allowed us to propose these compounds as the main candidates responsible for the observed activity. However, we acknowledge that definitive confirmation of their individual contribution would require further studies using purified compounds. **Reviewer comment: Only 2 strains of *S. suis* are used in the study. The number of strains used should be recognized when discussing the general applicability of results. Response:** We agree that the use of only two *S. suis* strains represents a limitation of the present study. While the selected strains were chosen because of their virulence and relevance to the study objectives, it is also clear that they do not capture the full genetic and phenotypic diversity of *S. suis*. Therefore, the antimicrobial activity observed in this work cannot be assumed to be broadly representative of all *S. suis* strains. We have added this limitation to the discussion section. **Manuscript changes:** We have added the following sentence to the Discussion section: Although based on only two *S. suis* strains, these promising findings warrant further validation using a broader collection of strains to better reflect the diversity of this bacterial species.
**Reviewer comment: The number that is more than 100% inhibition in Table 2 (104-109%) should be verified and clarified. Response:** We thank the reviewer for pointing out this issue. The inhibition values exceeding 100% were a result from the calculation method used, where inhibition was determined based on the change in optical density (ΔOD = OD24h - OD0h) relative to the growth control. These values do not indicate biological inhibition greater than 100% but rather reflect a decrease in OD relative to the initial

measurement. We agree that reporting inhibition values greater than 100% may be conceptually misleading. Therefore, these values have been capped at 100% in Table 2, and the reason for this adjustment was clarified in M&M section: **Manuscript changes:** We have added the following clarification in section "Screening of *Actinomycetes* extracts for antimicrobial activity against *S. suis*" of Material and methods: In some cases, OD decreased with incubation time, yielding an inhibition value higher than 100%. For clarity, these values are reported as 100% inhibition. **Minor comments Spot and correct typographical and grammatical errors, such as 'S. suisinfection' or 'Finaly'. Response:** We have corrected them. **Include adequate LC-HRMS method details to replicate Response:** We don´t agree with the reviewer. The LC-HRMS method has been described in detail previously (Simone et al. 2024; El Nahhas et al. 2025) and it is essentially repeated here.

***Competing Interests:*** No competing interests were disclosed.