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Identification and antibacterial activity of *Thamnolia vermicularis* and *Thamnolia subuliformis*

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ABSTRACT

Thamnolia vermicularis (Tv) and *Thamnolia subuliformis* (Ts) are two species from the same habitat with such similar external morphological characteristics that researchers often confuse the two in their scientific work and do not distinguish between them. This study applies three different methods to distinguish them. The diversity of endophytic fungi was also compared and their antibacterial activity *in vitro* was evaluated. The results show that all three methods can distinguish Tv and Ts, and can be used to cross-validate each other. The ultraviolet fluorescence method and the chemical colour change method are simpler strategies, while thin-layer chromatography is relatively complicated but can more clearly distinguish the chemical composition of the two species. In the analysis of the endophytic fungi community structure of Tv and Ts, it was found that the diversity of endophytic fungi in Tv was more abundant and had wider antibacterial activity and better inhibitory activity against gram-positive bacteria *in vitro*.

1. Introduction

Drug-resistant microorganisms already pose a serious threat to public health. Unfortunately, there are relatively few approved new antibiotics at present, which undoubtedly exacerbates the global crisis. Therefore, there is an urgent need to develop low-toxicity, efficacious new antimicrobial drugs (Gultom et al., 2021; Shi et al., 2021). As a class of organisms with great medicinal potential, lichen has attracted widespread attention from all walks of life (Sepahvand et al., 2021). >50% of lichens have good antibacterial activity, and the substances that exert functional activity are mainly lichen acid metabolites, which are produced by their symbiotic fungi (Dieu et al., 2020; Ranković and Kosanić, 2015; Wethalawe et al., 2021).

Lichen *Thamnolia spp.* is distributed throughout China, among which the samples collected in Yunnan Province are mainly from Shangri-La, Yulong Snow Mountain, Meili Snow Mountain, and Cangshan Malong Peak (Yang et al., 2015). It was found that crude extracts *of Thamnolia spp.* had remarkable antibacterial activity against a variety of pathogens, the antimicrobial activity of the crude extract was very stable and had the potential to be developed as antimicrobial drugs (Ren, 2022). There are two species of *Thamnolia spp*: *Thamnolia vermicularis* (Tv) and *Thamnolia subuliformis* (Ts), moreover, field research found that they have extremely similar external morphological characteristics and living environments. (Yang et al., 2015). Due to they have middle transition forms and mixed growth, many researchers often mix them up in scientific research (Qi et al., 2019; Cheng, 2015), which causes problems in evaluating their physiological activity and identifying their active substances. To establish a simple and rapid method to distinguish them, three different methods were tested in this study. After distinguishing Tv and Ts, their microbial community compositions were evaluated with the aim of finding the root cause of the differences in their microbial composition levels. Furthermore, an *in vitro* antibacterial activity test was conducted to further characterise their differences in chemical composition and physiological activity.

2. Materials and methods

2.1. Sample collection

The mixed lichen samples were collected at an altitude of 3800 m at Malong Peak in Cangshan, Dali, Yunnan Province, China, and their growth substrate was a thin soil layer on rock or in crevices (the proportion of Ts and Tv was about 1:1 *w*/w). After cleaning, the samples were dried in the shade with ventilation, placed in a well-sealed sample

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bag and stored at 4 °C.

2.2. UV fluorescence method

The cleaned and dried mixtures were observed using a ZF-90 UV projector reflectometer (Shanghai Ji-hui Analytical Instrument Co., Ltd., Shanghai, China) under dark conditions and excitation by 365 nm UV light. According to Yang's et al. (2015) previous summary, the height and diameter of Tv and Ts were significantly different. Therefore, 100 individuals of Tv and Ts were randomly selected for diameter and height measurements using a vernier calliper.

2.3. Chemical chromogenic reaction method

Two common chemical reagents were used to test for a colour change following chemical reaction with Ts and Tv. 10% potassium hydroxide solution and 5% *p*-phenylenediamine ethanol solution were gently dripped onto the surface of the cortex with a thin capillary tube, and the colour change was observed. The results were further verified by the UV fluorescence method.

2.4. Thin-layer chromatography method

According to the classification of Tv and Ts by the UV fluorescence method and chemical chromogenic reaction method, lichen at the designated growth stage (1 cm long) was selected for cleaning and shearing. Each sample of lichen was then placed in a 500-uL centrifuge tube and acetone was added to cover the sample, which was soaked for 5 min. The secondary metabolites in the sample were dissolved. On a silica gel plate, the location of the spot was marked in advance, and the general interval between the two spots was 1 cm. A fine capillary tube was used to spot the sample, and each group had 3 replicates, namely Tv: V1-V3, Ts: S1-S3. Spots were placed on both sides and in the middle, and *Lethariella cladonioides (L)* was used as the standard (White and James, 1985). After dispensing, the silica gel plate was carefully placed above the liquid level in the chromatographic cylinder for about 10 min, saturated, and then placed below the liquid level of the solvent by about l cm. This was done such that the origin of the sample was about 0.5 cm away from the solvent liquid level, and the original line was parallel to the liquid level to expand the layer. The silica gel plate was removed after the solvent front edge migrated about 8.5 cm from the origin and was quickly blown dry with a blower. The position and colour of chromatographic spots were observed and marked, the fluorescence of chromatographic spots was observed under 365 nm and 254 nm UV light, and the spot position, fluorescence colour and intensity were recorded. The silica gel plate was then wetted with 10% sulfuric acid solution, the spots were observed and marked carefully, and then placed in an oven at 110 °C for 10 min until the plate was well-coloured. The tangent line was taken on the upper and lower edges of the spot after colour rendering, with the area between the upper and lower tangents forming the first zone. In the same way, the fourth and seventh zones were demarcated at the spots of atranorin and norstictic acid of the partition standard. A straight line was drawn between zones 1 and 4, dividing them up to produce zones 2 and 3. Another straight line was drawn between zones 4 and 7, dividing them to produce zones 5 and 6. Finally, zone 8 was defined above zone 7. The components were identified based on the location of each chromatographic spot, its colour in daylight and ultraviolet light emission before and after colour development, and the intensity of its fluorescence.

2.5. Determination of the diversity of endophytic fungi in Tv and Ts

Healthy and complete samples were selected and impurities were removed (application of the naked eye to remove leaves and sediment). Then, samples were rinsed under flowing water for 30 min, blotted dry with filter paper, and placed on an ultra-clean workbench. Samples were rinsed three times more in sterile water, irradiated from above and below with ultraviolet radiation for 15 min, wiped dry again, and finally aliquoted into sterile centrifuge tubes. Tv and Ts were each processed in triplicate.

DNA extraction was performed first, and after the genomic DNA extraction was completed, the quality of the extracted genomic DNA was determined by 1% agarose gel electrophoresis. Then, PCR was performed to amplify the hypervariable region of the fungal ITS1F-ITS2R gene region with the primer pair 1F (CTTGGTCATTTAGAGGAAGTAA) and 2R (GCTGCGTTCTTCATCGATGC) using an ABI GeneAmp® 9700 PCR thermocycler (Applied Biosystems, Union City, CA, USA). The PCR cycling conditions for the ITS gene were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min, with samples held at 4 °C. Each PCR mixture contained 2 μL 10 × TransStart FastPfu buffer (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China), 2 μL 2.5 mM dNTPs, 0.8 μL forward primer (5 μM), 0.8 μL reverse primer (5 μM), 0.2 μL rTaq Polymerase, 0.2 μL BSA, 10 ng template DNA, and finally ddH_2O to make up to 20 μL. PCR reactions were performed in triplicate. For the synthesis of specific primers with barcodes, a random selection of representative samples for pre-experimentation was analysed. This ensured that most samples could be amplified to produce products of appropriate concentrations in the minimum number of cycles. PCR products from the same sample were mixed and detected by 2% agarose gel electrophoresis, and PCR products were recovered using an AxiPrep DNA gel recovery kit (Axygen Biosciences, Union City, CA, USA). After that, with reference to the preliminary quantitative results of electrophoresis, the PCR product was detected and quantified with the QuantiFluor-ST™ Blue Fluorescence Quantification System (Promega Corporation, Madison, WI, USA), and the TruSeqTM DNA Sample Prep Kit was used to prepare the sequencing library. Purified amplicons were pooled in equimolar concentrations and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw ITS gene sequencing reads were demultiplexed (Chen et al., 2018), quality-filtered using fastp version 0.20.0 and merged using FLASH version 1.2.7 with the following criteria (Magoč and Salzberg, 2011). (i) 300-bp reads that were truncated at any site received an average quality score of <20 over a 50 bp sliding window, and truncated reads shorter than 50 bp were discarded. Reads containing ambiguous characters were also discarded. (ii) Only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was 0.2. Reads that could not be assembled were discarded. (iii) Samples were distinguished according to the barcode and primers, the sequence direction was adjusted, exact barcode matching was performed, and a 2-nucleotide mismatch was set as the threshold for primer matching. Operational taxonomic units (OTUs) with a 97% similarity cut-off were clustered using UPARSE version 7.1(Edgar, 2013), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analysed by RDP Classifier version 2.2 against the ITS database (UNITE 8.0), using a confidence threshold of 0.7 (Wang et al., 2007). Chao index, Ace index, Shannon Wiener index, Simpson index and the coverage index were calculated based on OTU data. The data was processed by the software of Mothur (https://www.mothur.org/wiki/Download_mothur) and the calculation formulas refer to Graper's report (Graper et al., 2021).

2.6. In vitro antibacterial activity of Tv and Ts

Fresh samples were cleaned and dried (45 °C, 10 h), then crushed and sieved (0.25 mm). 75% methanol was added to a solid-liquid ratio of 1:10 (g/mL). After ultrasonic extraction for 30 min, the supernatant was collected after repeated extraction. Samples then underwent rotary

evaporation at 50 °C to 10 mL. Powders were prepared by vacuum freeze-drying for 12 h (−25 °C, 50 Pa), then sealed and stored at −20 °C.

Tested gram-positive bacterial strains included Methicillin-resistant *Staphylococcus aureus* (ATCC 43300), purchased from Beijing Bei-Na Chuang-lian Biotechnology Research Institute (Beijing, China), and *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Listeria seeligeri* (CICC 21671) and *Listeria monocytogenes* (CICC 21663), which were donated by the Dali Quality and Technical Comprehensive Supervision Testing Center (Dali, China).

Tested gram-negative bacterial strains included *Salmonella paratyphi* A (BNCC 336664), *Salmonella paratyphi* B (BNCC 103169), *Escherichia coli* (CMCC(B) 441027), *Klebsiella pneumoniae* (ATCC 4352) and *Pseudomonas aeruginosa* (ATCC 27853), donated by the Dali Quality and Technical Comprehensive Supervision Testing Center. Bacteria were inoculated into LB medium and placed at a constant temperature in a shaking incubator at 37 °C for 24 h.

The *in vitro* antibacterial activities of methanol extracts of Tv and Ts were investigated by the pre-pour plate method combined with the punch method (Ren, 2022; Tramer and Fowler, 2010). The methanol extracts were dissolved in dimethyl sulfoxide (DMSO). The bacterial suspension of the strain to be tested was prepared with sterile normal saline at 1×10^5 CFU/mL (colony forming unit/mL), and then the nutrient agar (Guangdong Huankai Microbial Sci & Tech. CO. LTD., Guang Zhou, China) and bacterial solution were mixed at a ratio of 20:1 for further use. Wells (6 mm) were drilled into the solidified agar, and 20 μL of Tv or Ts methanol extract was added to each well. The positive control was treated with 0.5 mg/mL levofloxacin hydrochloride (20 μL), and the negative control was DMSO. Five replicates were tested for each treatment. After diffusion at 4 °C for 2 h (Tan et al., 2016), the plates were placed in a 37 °C incubator and cultured for 24 h to observe the formation of the inhibition zone.

The antibacterial activity of Ts and Tv was judged according to criteria described by (Li et al., 2019). Inhibition zones with diameters >6 mm were considered to demonstrate antibacterial activity and those that reached 6 mm in all five replicates qualified. The negative control should have no inhibition zone, otherwise, the test is invalid. Substances resulting in inhibition zones with diameters >6 mm but <8 mm indicated low sensitivity; those with diameters >8 mm and <14 mm were defined as moderately sensitive; those with diameters >14 mm but <20 mm were considered highly sensitive; those with diameters over 20 mm were extremely sensitive.

2.7. Statistical analysis

The test results were expressed as the mean ± standard deviation. The data were processed and analysed using SPSS 26.0 software (International Business Machines Corporation, Armonk, NY, USA). Hypothesis testing was performed using the *t*-test, with $P < 0.05$ indicating a significant difference.

3. Results

3.1. Identification of Tv and Ts by three different methods

The ultraviolet fluorescence method detects whether the secondary metabolites of the lichen medullary layer exhibit fluorescence. The results of the test mainly depend on differences in the composition of the medullary layer of Tv and Ts (Fig. 1). Ts has yellow fluorescence, while Tv does not appear fluorescent (Lord et al., 2013).

The chemical chromogenic reaction method involves directly adding reagents to the site to be examined in the lichen, resulting in the production of different colours by different lichen bodies encountering different reagents. Both Tv and Ts showed positive reactions to potassium hydroxide solution and *p*-phenylenediamine ethanol solution. Both turned yellow after potassium hydroxide staining and could not be distinguished by the naked eye. However, there was a significant difference between the two after staining with *p*-phenylenediamine ethanol solution (Fig. 2). According to a data table for the colours of common lichen secondary metabolites (Wei, 1982), the orange colour of the Tv cortex indicates that it is rich in thamnolic acid, and the yellow colour of the Ts cortex suggests that it is rich in squamatic acid and baeomycesic acid.

For the thin-layer chromatography method, acetone extracts of Tv and Ts were applied as spots on the plate and allowed to migrate under the influence of the solvent. Their positions following chromatography and the presence or absence of fluorescence were observed under a UV projector (Fig. 3). It was found that the Ts extract had strong fluorescence in the third region and weak fluorescence in the fifth region, and the corresponding chemical colour change reaction was carried out. Combined with the data of chemical composition zoning, the chemical

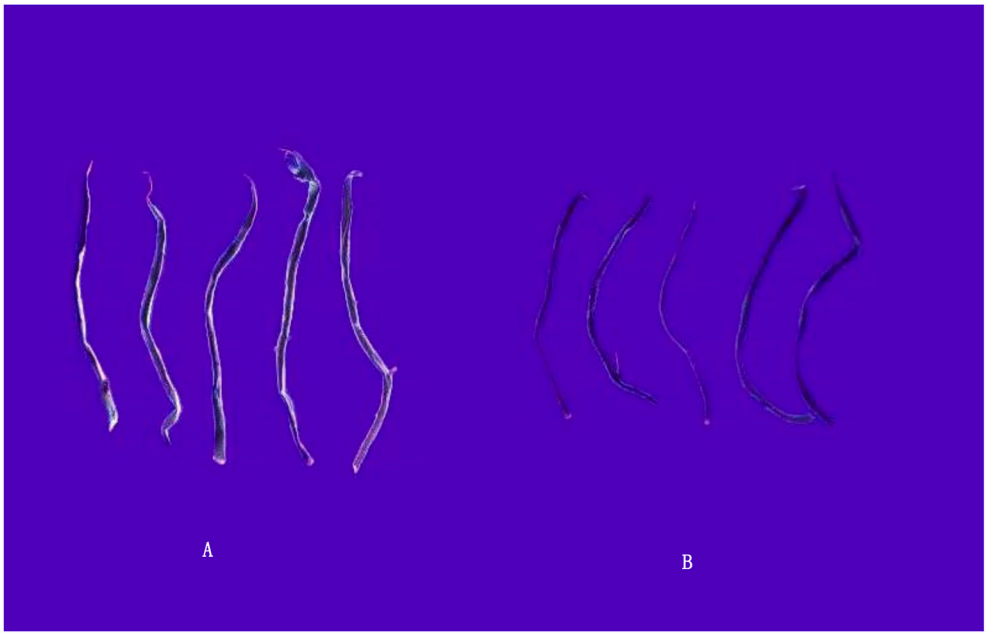

Fig. 1. Differences in the ultraviolet fluorescence (365 nm) of Ts (A) and Tv (B).

	K	P
Tv	+ (yellow)	+ (orange)
Ts	+ (yellow)	+ (yellow)

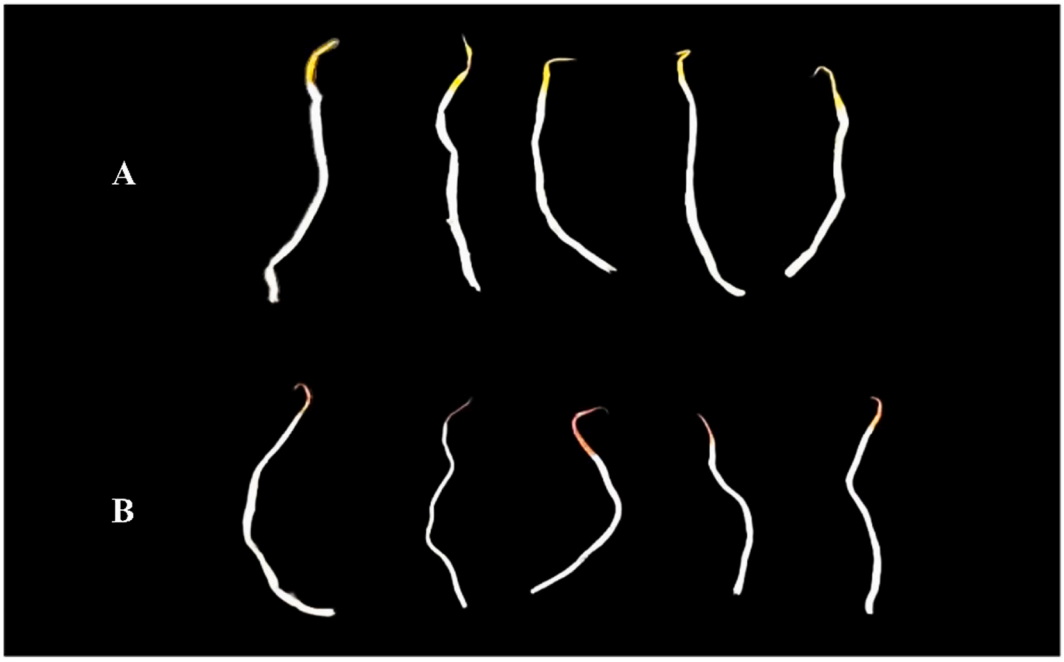

Fig. 2. Results of the colour change reaction test for Ts (A) and Tv (B) using the *p*-phenylenediamine ethanol solution.

composition in these areas was identified as consisting of squamatic acid and baeomycesic acid, respectively. Meanwhile, Tv had spots in the second region, and, combined with the chemical colour change reaction and zoning data, it was determined that the chemical composition of the lichen in this region was primarily thamnolic acid. In addition, it was also found that Tv had fluorescent spots in the third and fifth regions, which corresponded to the fluorescent spots of Ts, but the fluorescence intensity was relatively weak, suggesting that Tv contains a small amount of squamatic acid and baeomycesic acid.

3.2. Diversity of endophytic fungi in Tv and Ts

The five diversity indices for the microbial communities in Tv and Ts are shown in Table 1. The Chao index and Ace index of Tv are higher than those of Ts, indicating that the species richness of Tv is higher than that of Ts. In contrast, there was very little difference between Ts and Tv in terms of the Shannon Wiener index and Simpson index, suggesting that the individual distribution of Ts and Tv in the microbial community was relatively uniform. In addition, the coverage index of the two samples was close to 1, indicating that the coverage rate of the Tv and Ts sample libraries was high, which confirms that these sequencing results reliably reflect the fungal diversity of Tv and Ts.

There were 739 and 673 OTUs in Tv and Ts, respectively (Fig. 4), of which 398 OTUs were common, accounting for >50%. This suggests that the fungal compositions of Tv and Ts were similar. At the same time, there were 341 and 245 unique OTUs in Tv and Ts, respectively, indicating that there were also some differences in their microbial community compositions. To investigate these differences, we further analysed the fungal community structure in Tv and Ts at the phylum and genus levels (Fig. 5A). There were 4 dominant fungal phyla (relative abundance ≥1%) in Tv and Ts, namely *Ascomycota, Basidiomycota*, unclassified fungi, and *Olpidiomycota*, in addition to less common phyla. Among them, the fungal communities in Tv and Ts were mostly accounted for by *Ascomycota* and *Basidiomycota* at the phylum level, but there was some difference in their relative abundances. The relative abundance of *Ascomycota* in the Ts samples was >50%, while the relative abundance of *Ascomycota* in Tv was as high as 70% to 90% (Fig. 5A). However, the relative abundance of *Basidiomycota* was higher in Ts than in Tv, suggesting that although Tv and Ts belong to the same genus and grow in the same conditions, there are still some differences in their fungal community structures. The fungal community structure of Tv and Ts at the genus level (Fig. 5B). The dominant fungal genera in Tv and Ts were mainly *Cladophialophora,* unclassified *Hyaloscyphaceae,* unclassified *Ascomycota, Tremella* and unclassified *Cystobasidiomycetes*. Among them, *Cladophialophora* accounted for 19% to 45% in Tv and 3% to 19% in Ts, and unclassified *Hyaloscyphaceae* accounted for 3% to 39% in Tv and 0.3% to 8% in Ts. In addition, unclassified *Ascomycota* accounted for 3% to 14% in Ts, while the proportion in Tv was only 3% to 5%. Similarly, the proportions of *Tremella* and unclassified *Cystobasidiomycetes* in Ts were relatively high, at 5% to 11% and 5% to 15%, respectively. However, *Tremella* and unclassified *Cystobasidiomycetes* accounted for only 2% to 4% and 1% to 2% in Tv, respectively. The abundances of the remaining genera were not different between Ts and Tv.

3.3. In vitro antibacterial activity of Tv and Ts

According to Table 2, different strains showed different sensitivities to Tv and Ts extracts. Specifically, the gram-positive bacterium *S. aureus* showed high sensitivity to the Tv methanol extract (the inhibition zone diameter reached 20 mm), while only moderate sensitivity to the Ts methanol extract. Therefore, the antibacterial activity of Ts was less than that of Tv for this taxon ($P < 0.01$). For MRSA, moderate sensitivity

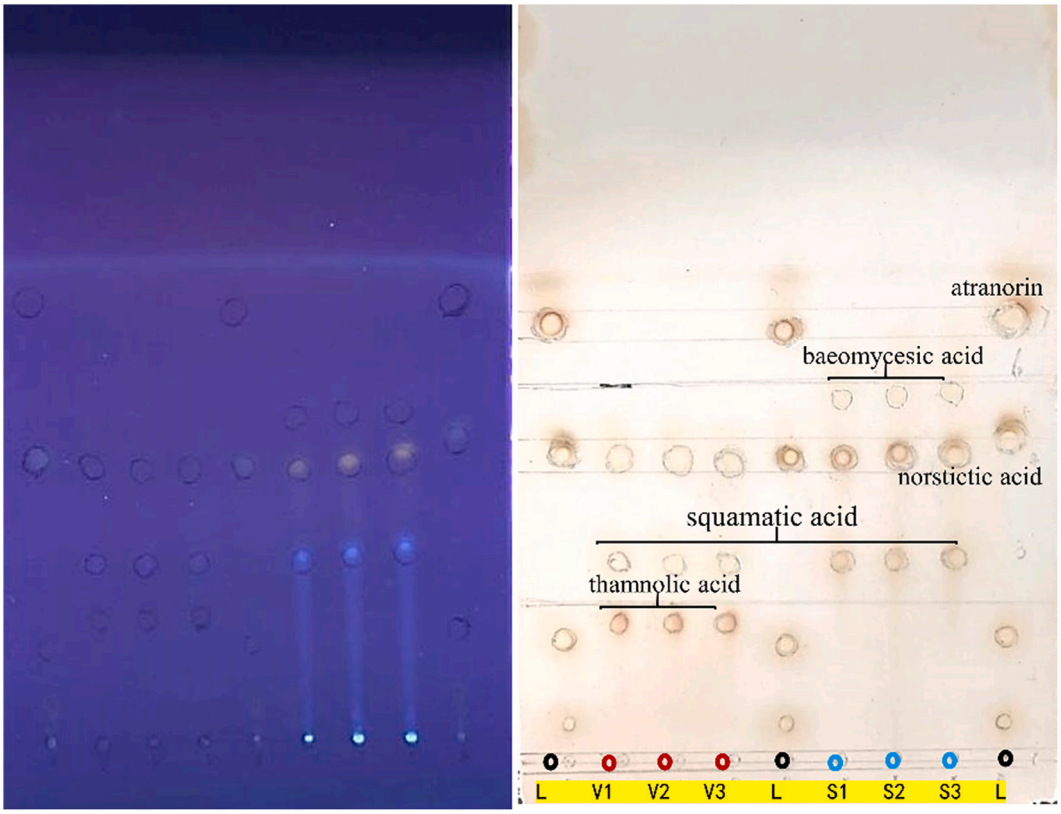

Fig. 3. Thin-layer chromatography of secondary metabolites in Ts and Tv.
L: *Lethariella cladonioids*; V1-V3: three replicates of *Thamnolia vermicularis;* S1-S3: three replicates of *Thamnolia subuliformis.*

Table 1
Alpha diversity analysis of microbial communities in Tv and Ts.

Sample	Chao index	Ace index	Simpson index	Shannon-Wiener index	Coverage
V1	554.708	550.671	0.042	3.952	0.999417
V2	350.571	352.587	0.207	2.499	0.999385
V3	440.929	447.212	0.107	3.011	0.998980
S1	361.040	359.487	0.056	3.562	0.999692
S2	394.103	391.759	0.045	3.787	0.999549
S3	309.500	311.737	0.196	2.789	0.998787

Note: V1-V3 are three replicates of *Thamnolia vermicularis;* S1-S3 are three replicates of *Thamnolia subuliformis.*

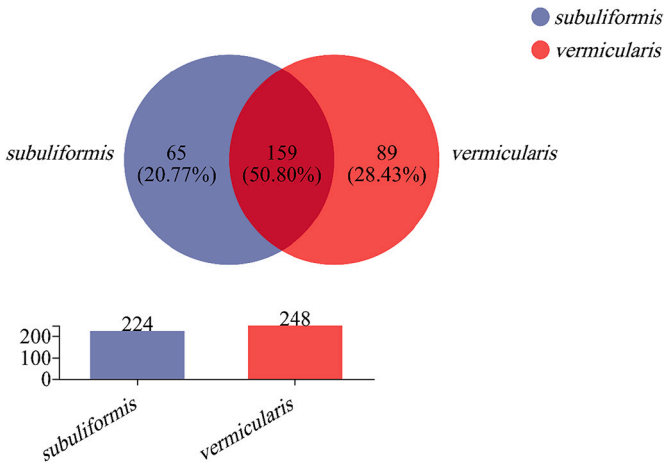

Fig. 4. Venn diagram of the OTUs in Ts and Tv.

was observed to both the Tv and Ts methanol extracts. *S. epidermidis* exhibited extreme sensitivity to the Tv methanol extract, with the inhibition zone diameter exceeding 20 mm, while the Ts methanol extract had no inhibitory activity against *S. epidermidis*. *Listeria spp.* is another major genus of gram-positive bacteria, which also showed different sensitivities to the Tv and Ts methanol extracts. Specifically, *L. monocytogenes* showed moderate sensitivity to Ts and L. *seeligeri* showed moderate sensitivity to Tv. Gram-negative bacteria were also tested but were less sensitive to the Tv and Ts methanol extracts than gram-positive bacteria. Some common gram-negative bacteria such as *S. paratyphi* B, *E. coli, K. pneumoniae* and *P. aeruginosa* were screened; these strains were not sensitive to the Tv and Ts methanol extracts. However, *S. paratyphi* A exhibited a high sensitivity to the Tv methanol extract and moderate sensitivity to the Ts methanol extract, but there was no statistical difference between Tv and Ts ($P > 0.05$).

4. Discussion

Lichen is rich in a variety of unique chemicals, which mainly include aliphatic and phenol metabolites (Ten et al., 2020). There are three major pathways for the biosynthesis of lichen chemicals, including the *acetate-polymalonate pathway*, mevalonate pathway and shikimic acid pathway (Xie et al., 2016). Among them, the acetate-polymalonate pathway is the main pathway for the formation of lichen chemicals, and most of its secondary metabolites are phenolic acids, especially lichen diols and β-lichen diol derivatives (Alam et al., 2020; Sharma and Mohammad, 2020). It was also found that these substances were almost only present in lichen, so they attracted great interest from researchers (Devashree et al., 2021; Alam et al., 2020). According to relevant literature, lichen is rich in squamatic acid can show yellow fluorescence at 365 nm, which was confirmed in Ts in this study (Wang and Qian, 2012). Meanwhile, Tv did not show a fluorescence reaction under UV light due to its abundant thamnolic acid. Hence, the presence or absence

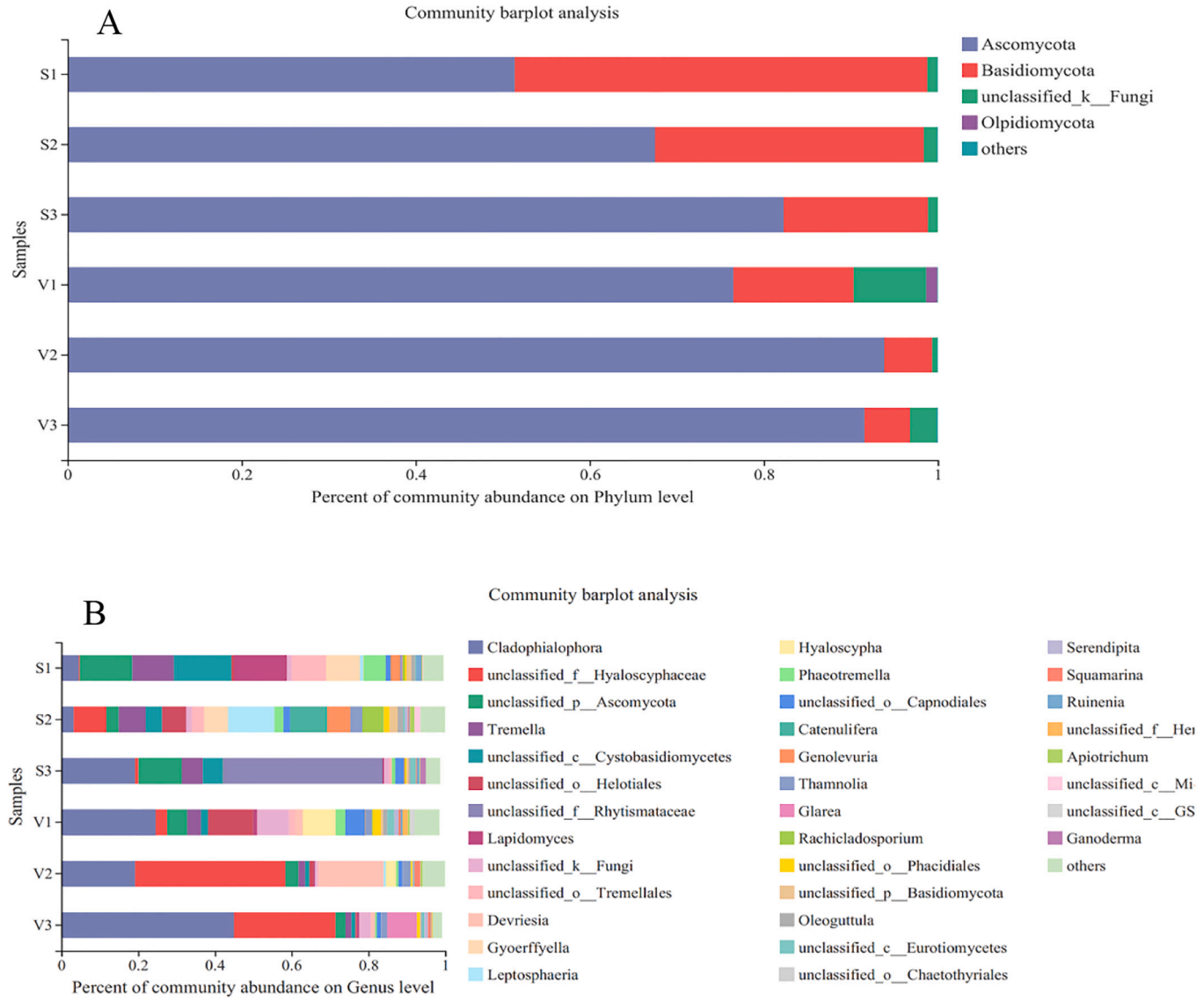

Fig. 5. Fungal community structure of Ts and Tv.
A: phylum level; B: genus level; V1-V3: three replicates of Tv; S1-S3: three replicates of Ts.

Table 2
Results of antibacterial activity test of Tv and Ts.

Category	Object	Tv extract (mm)	Ts extract (mm)
	MRSA	12.3 ± 0.5	11.6 ± 0.6
	S. aureus	20.0 ± 0.6	10.9 ± 1.1**
gram-positive bacteria	*S. epidermidis*	22.3 ± 1.0	–
	L. seeligeri	–	11.3 ± 1.0
	L. monocytogenes	12.9 ± 0.5	–
	S. paratyphi A	14.6 ± 0.6	13.6 ± 0.6
	S. paratyphi B	–	–
gram-negative bacteria	*E. coli*	–	–
	K. pneumoniae	–	–
	P. aeruginosa	–	–

Note: * represents $P < 0.05$ for the Ts or Tv inhibition zone diameter, * * represents $P < 0.01$ for the Ts or Tv inhibition zone diameter. If the diameter of the bacteriostatic circle is less than or equal to 6 mm, the extract has no bacteriostatic activity; if it is >6 mm and less than or equal to 8 mm, it indicates low sensitivity; if it is >8 mm and less than or equal to 14 mm, it indicates moderate sensitivity; diameters >14 mm and less than or equal to 20 mm indicate high sensitivity; diameters over 20 mm indicate extreme sensitivity.

of fluorescence can be used as a basis for the identification of Tv and Ts, which are two species of the genus *Thamnolia* (Lord et al., 2013; Yang et al., 2015). In this study, the fluorescence of Tv was not obvious, although a small amount of fluorescence was observed (Fig. 1). It is speculated that there may be a small amount of squamatic acid in Tv; this hypothesis has also been confirmed by a previous study (Guo et al., 2011). Furthermore, the diameter and height of Tv and Ts were also analysed in this study. It was found that these values for Tv and Ts were relatively discrete and did not obey the normal distribution or variance uniformity. This indicates that there were extreme values for the Tv and Ts samples regardless of height or diameter, resulting in no significant difference in morphology between Ts and Tv. At present, the definition of the subordinate units of genus *Thamnolia* has been controversial, and the root cause of the controversy is that there are many downward variants of genus *Thamnolia* in addition to Tv and Ts. That is, there are transitional forms, resulting in small differences between Tv and Ts that render them very difficult to distinguish. There is a consensus among scholars that the genus *Thamnolia* contains only two species, namely Ts and Tv (Yang et al., 2015). Platt's molecular phylogenetic analysis of the nr LSU fragment sequences of the two species also supports this view (Platt and Spatafora, 2000). Therefore, from our results, it is difficult to

quickly distinguish Ts and Tv by morphological observation alone.

Lichen can produce unique secondary metabolites that cannot be produced by many other fungi and plants (Goga et al., 2020). According to the different structures of these secondary metabolites, lichens can present different colours when they encounter different chromogenic agents (Frisch et al., 2020). In addition, related studies have confirmed that these colour change reactions are more sensitive in regions that are closer to the growth point of the lichen (Culberson, 1972), so the growth point was selected for colour change detection in this study. In this study, *p*-phenylenediamine ethanol solution can clearly distinguish between Tv and Ts, because the thamnolic acid in Tv can react with *p*-phenylenediamine ethanol solution which turned orange (Wei, 1982). It was also confirmed by the subsequent thin-layer chromatography method.

A third strategy involved separating the lichen chemical substances on a thin-layer chromatography plate by selecting an appropriate solvent system. Components were then identified according to the different partition data of lichen chemical components (White and James, 1985). Based on the thin-layer chromatogram combined with the corresponding fluorescent spots, it was speculated that there might be a small amount of squamatic acid and baeomycesic acid in Tv (Guo et al., 2011). The results further confirm the fact that the above-mentioned ultraviolet fluorescence detection of Tv demonstrates a weak fluorescence at 365 nm. By comparing the above methods, we found that the fastest way to distinguish between Ts and Tv was the ultraviolet fluorescence method. The chemical chromogenic method has been used in lichen classification and identification, but it has shortcomings. For some special lichen acids, such as lecanoric acid and olivtoric acid, although both can react with sodium hypochlorite to produce a red colour, it is often difficult to determine the category of specific lichen acids purely based on colour changes following chemical reactions. In contrast, thin-layer chromatography has the dual functions of separation and identification and can overcome such problems, so this method is widely used in lichen classification (Ahmer et al., 2021).

The above three identification methods were consistent with each other, indicating that Tv and Ts are two independent species of the genus *Thamnolia*. Although there were no differences in the habitat or external morphological characteristics between the two species, some differences were identified in their chemical components. To better understand their differences, the fungal diversities of Tv and Ts were also evaluated in this study. Mycobiome technology was employed to analyse Tv and Ts through the direct extraction of environmental microbial DNA for sequencing and bioinformatics analysis to determine the diversities of microbial composition. Currently, the most commonly used fungal molecular marker is 18S rDNA or ITS (Liu et al., 2015). Among them, the effective amplification of endogenous fungal DNA is mainly through PCR amplification of a 360–450 bp region in the 18S rDNA, the 18S nuclear ribosomal small subunit rDNA gene, is commonly used in phylogenetics, while the ITS region of the nuclear ribosomal DNA repeat unit is by far the most commonly sequenced region for taxonomic identification at and below the genus level (Xu et al., 2016). So the difference between the two was further investigated by comparing the composition of the fungal community structures of Tv and Ts. The alpha diversity analysis is used to reflect the richness and diversity of the microbial community (Satya et al., 2014). The analytical indices mainly include the Ace index, Simpson index, Chao index, Shannon Wiener index and Coverage index; these indices can evaluate the richness of microbial colonies (Coombs et al., 2018; Hu and Bidochka, 2021; Möhlmann et al., 2017). According to the alpha diversity analysis of the fungal communities of Tv and Ts, the richness of the microbial community of Tv was higher than that of Ts, indicating that the fungal species in Tv were more numerous in the diversity.

Venn diagrams are commonly used to count the number of common and unique species in multiple samples. They can intuitively show the similarity and overlap of species composition in environmental samples. We can clearly see that more than half of the fungal species in Tv and Ts are coincident, but there are also certain differences in the composition of their fungal communities (Fig. 4). The results showed that the dominant fungi in Tv and Ts were mainly *Cladophialophora*, unclassified *Hyaloscyphaceae*, unclassified *Ascomycota*, *Tremella* and unclassified *Cystobasidiomycetes*. Among them, it was discovered that the metabolites of the genus unclassified *Hyaloscyphaceae* have been confirmed to have high nematicidal activity, resistance to pathogenic microorganisms and cytotoxin activities (Hosoya, 2021). The secondary metabolites of the genus *Tremella* have anticancer, hypoglycaemic and immunoregulatory effects. Therefore, these lichen species have the potential to be of medicinal value (Reshetnikov et al., 2000). As mentioned earlier, the substances that exert antibacterial activity in lichen are mainly the secondary metabolites such as phenolic acids, and these compounds are mostly water-insoluble and are produced by symbiotic fungi and usually deposited on the surface of the mycelium. These secondary metabolites are synthesized in the mycelium and finally aggregate on the surface of the mycelium in the form of crystals. These symbiotic fungi and their hosts have formed a close and complex ecological relationship in the process of long-term co-evolution, and the material basis for maintaining this relationship is the secondary metabolites synthesized by endophytic fungi (Thi et al., 2014; Ye et al., 2020). These substances have been confirmed to have important application potential in the pharmaceutical and agricultural industries (Devashree et al., 2021; Padhi and Tayung, 2021).

As mentioned above, about 50% of lichen species have antibacterial activity (Ranković and Kosanić, 2015), and they have remarkable inhibitory activity against multiple important gram-positive bacteria, though their inhibitory effect on gram-negative bacteria is relatively poor. This study also confirmed the above point of view. Comprehensive analysis showed that Tv has more extensive antibacterial activity and a better overall effect than Ts, especially against gram-positive bacteria. The results of the analysis of fungal community structure diversity suggest that there are obvious differences in the antibacterial activities of Tv and Ts, which may be related to the distribution of dominant and functional microbiota in lichen samples. In addition, both methanol extracts had inhibitory effects on MRSA, so this study provides new ideas for overcoming the problem of antibiotic resistance (Cheng et al., 2021).

Through the above test results, it was found that the best way to quickly distinguish between Tv and Ts is the ultraviolet fluorescence method. When the chemical chromogenic reaction method is used to distinguish Ts and Tv, *p*-phenylenediamine ethanol solution is preferred. Compared to the above methods, thin-layer chromatography is slightly more cumbersome but can clarify the chemical composition through the partition data. These three methods can successfully distinguish Ts and Tv, and researchers can choose the ideal method based on the experimental conditions and their own needs. There were some differences in the chemical composition and fungal community composition of Ts and Tv, so their antibacterial activities *in vitro* were also quite different. It is believed that there are also some differences in other biological activities. Therefore, attention should be paid to distinguishing them. Although lichen resources are various, their application is limited due to their extremely slow growth in nature and difficulties in artificial cultivation. In addition, excessive accumulation of lichen will also negatively impact the protection of lichen. Studying lichen endophytic fungi not only protects lichen resources, but also provides a new research avenue for novel antibiotics. Therefore, it is important for researchers to isolate, culture and purify the endophytic fungi in Ts and Tv to obtain endophytic fungal strains, and screen the antibacterial activity of the products obtained after fermentation. According to the screening results, the target strain may be amplified by liquid fermentation, and then the active strain can be isolated and purified by chromatographic analysis. The structure of purified compounds can then be determined by modern spectral analysis technology to provide a theoretical basis for the development of new antibacterial drugs.

Author contributions

Haiyan Ding conceived the project, sourced the funding, provided the resources, and supervised the research. Jing Wang performed the experiments, curated the data, conducted data analysis and drafted the original manuscript. Haojun Zhao and Qixin Guo provided the resources and supervised the research, and gave further inputs to the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

Data availability

No data was used for the research described in the article.

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