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Pathogenic Fungi and Bacteria from Homogenates and Commercial Beverages of *Auricularia polytricha*

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Abstract *Auricularia polytricha* is an important edible and medicinal fungus in Taiwan. The cultivation environment and sterilization of the mushroom cultivation bag before cultivation may affect the fungal species that occur along the fruiting body for *Mucor irregularis*, *M. fusiformis*, and *Trichoderma longibrachiatum* and the stalk, for *Hypocrea koningii*, *Rhodotorula mucilaginosa*, and *Coprinellus radians* from the stalk. The bacterial species associated with *A. polytricha* were also identified by PCR amplification, PCR-RFLP analysis, sequencing and BLASTn analysis. Bacterial species were identified as *Bacillus cereus*, *Pseudomonas tolaasii*, *Sphingomonas paucimobilis*, *Acinetobacter pittii*, and *Lysinibacillus fusiformis* in the homogenates after Pasteur sterilization. During a flavor test of 11 commercial drinks, the testers reported experiencing diarrhea. Bacterial examination of the drinks found that three samples were contaminated. Five bacterial species were identified as *Pantoea agglomerans*, *Serratia liquefaciens*, and *Pseudomonas psychrophila* in sample 275, *Cronobacter sakazakii* and *Pseudomonas azotoformans* in sample 684 and *Pseudomonas azotoformans* in sample 539, and more than one bacterial species occurred in two samples. In conclusion, pathogenic fungal and bacterial species were obtained from the fruiting bodies, stalks, homogenates, and drinks of *A. polytricha* after Pasteur sterilization. To prevent foodborne diseases from *A. polytricha*, sanitation procedures should be enforced during cultivation, processing and post-harvest storage.

Keywords: *Auricularia polytricha, bacterium, fruiting body, fungus, pathogen, stalk*

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1. Introduction

Auricularia polytricha is one of the main species of wood-ear fungi. Unlike the closely related species *A. auricula-judae*, *A. polytricha* contains a medullary layer in the fruiting body, has long velvety hairs and commonly lives on dead trees in broad-leaved forests located at lower altitudes. The stalk is often used as a fertilizer for plant growth, while its fresh and dried fruiting bodies are used for food. The polysaccharides from different medicinal fungal species differ in their physiological activity due to their different compositions of sugar types and chemicals, bonding chains, molecular weights, and branches and structures, especially their glycosidic linkages.

Recently, polysaccharides have been investigated for their functions in the activation of macrophages to secrete TNF-α and IL-6 to inhibit tumor growth [1] and to inhibit growth and promote apoptosis in adenocarcinomic human alveolar basal epithelial cells [2]. The purified (1,3)-*β*-D-glucans of enoki mushroom, *A. polytricha* and Hongxi mushroom can modulate immune responses and inhibit tumor cell growth [3,4]. The β-(1,6)(1,3)-*β*-D-glucans of *Agaricus blazei* Murill can stimulate lymphocyte T-cell subsets in mice [5]. Therefore, polysaccharides from different fungi can stimulate various immune functions.

During harvest, physical separation can cause wounds at the bottom of the stalk, and further separation can cause wounds at the top of the stalk and the bottom of the fruiting body. Given the nutrients in the fungus and its high moisture levels, when the fruiting body and stalk are kept for over 12 hours before cool storage and processing, microbes can grow on these surfaces. Occasionally, *A. polytricha*-associated food poison has been reported. Therefore, incorrect storage and sterilization can allow microbes to proliferate and cause food toxicity. However, the fungal and bacterial species living on the fruiting body and stalk have seldom been investigated.

2. Fungal Species

Foodborne fungal infections are seldom found in humans, and the identification of species involved in fungemia in humans is a long process. Previously, we reported the fungal species from the fruiting bodies and the stalks identified by PCR amplification, sequencing, and sequencing comparison. The possible fungal species differed, with *Mucor irregularis*, *M. fusiformis*, and

Trichoderma longibrachiatum from the fruiting body and *Hypocrea koningii*, *Rhodotorula mucilaginosa*, and *Coprinellus radians* from the stalk [6]. This difference in fungal species may be due to incomplete sterilization of the mushroom cultivation bag before cultivation because fungal contamination of the stalk and environmental conditions may cause fungal infection of the fruiting body.

Fast-growing *Mucorales* can cause fungemia in humans. Several methods have been used to identify species such as *Mucor irregularis* by ITS sequence analysis and to perform genotyping by MLST and AFLP analysis [7]. However, sequence variations in the ITS region limit the application of these techniques to differentiate the following species: *Mucor circinelloides*, *M. flavus*, *M. piriformis*, and *Zygorhynchus moelleri* [8]. The evolution of parasitic *Trichoderma* occurs through horizontal gene transfer from fungi in *Ascomycota* such that they become generalist fungi capable of causing degradation of plants [10] and fungemia in humans [11]. Among *Trichoderma* species, *T. longibrachiatum* (26%), *T. citrinoviride* (18%), the *Hypocrea lixii/T. harzianum* complex (15%), *T. bissettii* (12%) and *T. orientale* (11%) are the main species infecting the respiratory tract (40%), followed by deep tissue (30%) and superficial tissues (26%) [9]. On the other hand, *T. ovalisporum* and *T. koningiopsis* can be used in the biocontrol of plant fungal infections, and *T. reesei* has been as a model species for industrial cellulase production [12]. The marine fungus *Hypocrea koningii* PF04 from *Phakellia fusca* can synthesize the furan derivatives hypofurans A and B, the cyclopentenone derivatives hypocrenones A-C and 7 compounds with known antibacterial and antioxidant abilities (DPPH radical scavenging capacity) [13]. Clinical *Rhodotorula* spp. strains are resistant to caspofungin and voriconazole, while antibodies against chitin have been developed to diagnose and cure *R. mucilaginosa* infection [14]. Furthermore, phytic acid (PA) in combination with *R. mucilaginosa* enhances the control of *Penicillium expansum* infection, inhibits patulin production from wounded apples and prevents apple decay [15].

3. Bacterial Species

3.1. Identification of Bacterial Species

Bacterial identification was first performed with Gram staining, and then PCR was performed with the U1 primer CCAGCAGCCGCGGTA ATACG and U2 primer ATCGGYTACCTTGTTACGACTTC to amplify the 16S rDNA region. PCR conditions included predenaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 1 min; and final extension at 72°C for 1 min. Further, the PCR products were digested with restriction enzymes *Alu*I, *Eco*RI, and *Sau*3AI to differentiate the isolates. The purified PCR products were sequenced, and then the sequences were compared to those in the NCBI databases using BLASTn software to determine the possible bacterial species.

3.2. Bacterial Species from *Auricularia polytricha* Homogenates after Pasteur Sterilization

PCR products were approximately 1,000 bp in size and PCR-RFLP analysis separated six strains into five genotypes (Figure 1). Sequencing and BLASTn analysis determined five bacterial species, including *Bacillus cereus*, *Pseudomonas tolaasii*, *Sphingomonas paucimobilis*, *Acinetobacter pittii*, and *Lysinibacillus fusiformis* (Table 1). These data indicate that Pasteur sterilization may be not enough to eliminate microbial contamination.

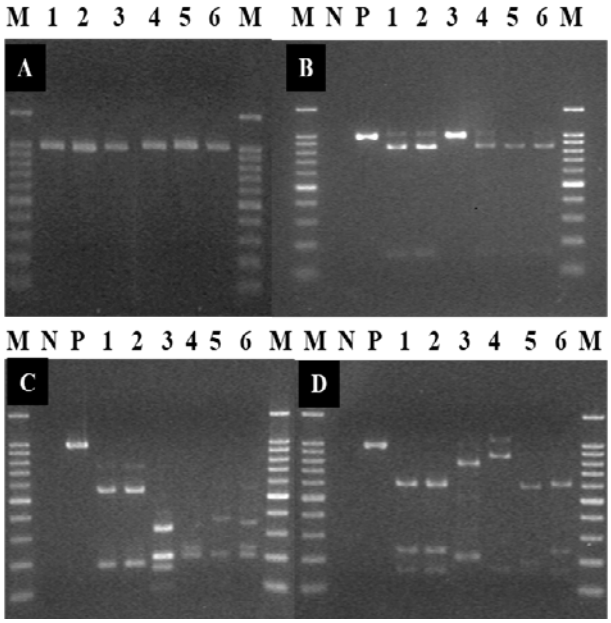

Figure 1. Gel electrophoresis of PCR products amplified from *Auricularia polytricha* by primer set U1/U2 (A), *Eco*RI digested PCR products (B); *Alu*I digested PCR products (C), and *Sau*3AI digested PCR products. M: 100-bp size markers, N: negative control, P:positive control, 1: sample DP-1, 2: sample DP-2, 3: sample DP-3, 4: sample DP-4, 5: sample DP-5, and 6: sample DP-6

Table 1. PCR-RFLP genotyping and bacterial species from the *Auricularia polytricha* after breaching

Strains	*EcoRI* (bp)		*AluI* (bo)					*Sau3AI* (bp)					Genotype	Species
	I	II	I	II	III	IV	V	I	II	III	IV	V		
	1000	170, 830	200, 550	120,180, 210,350	210, 230,	210, 360	210, 230, 350	180, 220, 600	200, 800	180, 850	200, 600	150, 250, 600		
DP-1		+	+					+					1	*Bacillus cereus*
DP-2		+	+					+						
DP-3	+			+					+				2	*Pseudomonas tolaasii*
DP-4		+			+					+			3	*Sphingomonas paucimobilis*
DP-5		+				+					+		4	*Acinetobacter pittii*
DP-6		+					+	+				+	5	*Lysinibacillus fusiformis*

Some of the identifies strains are related to human foodborne pathogens. The foodborne pathogen *B. cereus* survives in environmental soil and food to cause diarrhea in humans. *B. cereus* was not only found in milk [16] but also caused posttraumatic- and keratic-related endophthalmitis in humans [17]. Brown blotch disease (BBD) is the most devastating disease of *Pleurotus* spp. worldwide. *Pseudomonas tolaasii* can secrete the small peptide toxin, tolassin, which can be a target for species identification [18], to cause BBD of cultivatable mushrooms and chlorotic symptoms and growth arrest of *Arabidopsis thaliana* cotyledons [19]. Therefore, *P. tolaasii* without BBD were selected from *P. pulmonarius* and *P. cf. floridanus* by inoculation of the pathogen on the mushroom pileus (IMP) and on the spawned substrate (IMSS) [20].

Nonfermenting Gram-negative *S. paucimobilis* can cause pseudobacteraemia, including septic arthritis and osteomyelitis through contaminated distilled water, hemodialysis fluids and drug solutions [21]. The carbapenem-resistant *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex isolates are important pathogens in hospital-acquired infections. The carbapenem-resistant *A. pittii* carried either carbapenem resistance genes, including $bla_{\text{OXA-23}}$, $bla_{\text{OXA-58}}$, and/or $bla_{\text{IMP-14a,}}$ or showed overexpression of *adeE* [22]. *A. pittii* can be rapidly identified by the gene $bla_{\text{OXA-272/213-like}}$ [23]. *L. fusiformis* has been used for the bioregeneration of high-protein brewery spent diatomite (BSD1) with rice protein as the sole nitrogen source. Under the best medium conditions with an inoculum of 5%, glucose as the carbon source and an initial pH value of 7.0 and incubation at 30°C for 48 h, the protein concentration decreased from 156.8 mg/L to 19.2 mg/L with a protein degradation efficiency of 88%, and the content of free amino acids Phe, Tyr, Pro, Ala, Lys, Thr and His increased during the process [24]. The *L. fusiformis* strain C250R, under optimal conditions (gruel, wheat bran, yeast extract, and FeSO4), had a protease yield of 3100 U/mL, which was 4.5-fold higher than that under the initial conditions (680 U/mL). A new extracellular 51 kDa protease, SAPLF, of the serine protease family has potential application in the laundry detergent industry [25].

3.3. Bacterial Species from Commercial Beverages of *Auricularia polytricha*

Among the 11 commercial drinks of *A. polytricha* tested for flavor and examined for microbial contamination, some of the testers drank samples 539, 684, and 275 and reported diarrhea the next day. Microbial amounts differed among these three samples (Table 2). Three strains were collected from each sample: CD257-1, CD257-2, and CD257-3 from sample 275; CD684-1, CD684-3, and CD684-4 from sample 684; and CD539-1, CD539-2, and CD539-3 from sample 539. PCR-RFLP analysis separated five genotypes with three genotypes in sample 275, 2 genotypes in sample 684, and one genotype in sample 539. The microbial species were *Pantoea agglomerans*, *Serratia liquefaciens*, and *Pseudomonas psychrophila* in sample 275, *Cronobacter sakazakii* and *Pseudomonas azotoformans* in sample 684 and *Pseudomonas azotoformans* in sample 539. These results demonstrated that multiple microbial species are involved in contamination and sterilization processes are important to avoid microbial contamination.

The plant-associated bacterium *P. agglomerans* is an opportunistic pathogen that infects plants during agricultural and gardening practices, as well as infecting children via play, and in general causes human infection through contaminated medical equipment or fluids causing clinical septic arthritis, synovitis, endophthalmitis, periostitis, endocarditis and osteomyelitis in hospitalized and immunodeficient patients. Furthermore, *P. agglomerans* can infect vertebrate animals to cause equine abortion and placentitis and a hemorrhagic disease in dolphin fish (*Coryphaena hippurus*) in addition to causing diseases in many cultivable plants, such as cotton, sweet onion, rice, maize, sorghum, bamboo, walnut, an ornamental plant called Chinese taro (*Alocasia cucullata*), and a grass called onion couch (*Arrhenatherum elatius*). Additionally, closely related species of *P. agglomerans* were reported to cause bacterial blight disease in edible *Pleurotus eryngii* mushrooms cultivated in China. On the other hand, *P. agglomerans* can produce herbicolin, pantocins, microcin, agglomerins, andrimid, phenazine, and other compounds that prevent and/or treat human and animal diseases, combat plant pathogens, promote plant growth and bioremediate the environment [26]. Among its beneficial effects, *P. agglomerans* can be an antagonist of many plant pathogenic bacteria and fungi as a biocontrol agent to decrease pesticide doses. The diverse mechanisms by which this organism promotes plant growth include nitrogen fixation, phytohormone production, phytate degradation and phosphate solubilization in soil. Furthermore, *P. agglomerans* can form biofilms to prevent the penetration of harmful industrial contaminants into deeper soil layers and produces hydrogen from waste. Therefore, *Pantoea* spp. are potentially used in many biotechnological areas [27].

Table 2. The microbial contamination for commercially available beverage of *Auricularia polytricha*

Code	Total Aerobic Microbial Count	Total Yeasts and Molds Count	Code	Total Aerobic Microbial Count	Total Yeasts and Molds Count
941	0	0	517	0	0
386	0	0	842	0	0
425	0	0	539	$5.8*10^5$	$1.9*10^6$
463	0	0	684	TNTC	$5.3*10^5$
796	0	0	275	TNTC	TNTC
616	0	0			

TNTC: Too small to count.

Table 3. PCR-RFLP genotyping and bacterial species from the market beverage of the *Auricularia polytricha*

Strains	*Eco*RI			*Alu*I					*Sau*3AI		Genotype	Bacterial species
	I	II	III	I	II	III	IV	V	I	II		
	1000	150,850	150,350, 500	250,350	220,350, 370	150,220, 500	220,350, 400	100,280, 350	150,200, 600	220,800		
CD257-1		+		+					+		1	*Pantoea agglomerans*
CD257-2			+		+				+		2	*Serratia liquefaciens*
CD257-3	+					+				+	3	*Pseudomonas psychrophila*
CD684-1		+					+		+		4	*Cronobacter sakazakii*
CD684-3	+							+		+	5	*Pseudomonas azotoformans*
CD684-4	+							+		+		
CD539-1	+							+		+		
CD539-2	+							+		+		
CD539-3	+							+		+		

Although *Serratia* species are common environmental organisms, *S. marcescens* and *S. liquefaciens* have been reported to be responsible for numerous outbreaks and opportunistic nosocomial infections and are usually well identified in the clinical laboratory, with 16S rRNA gene sequencing often being employed for less common *Serratia* species [28]. *S. liquefaciens* CL-1 could increase tuber dry weight by 46% and reduce metal uptake of potato tubers in metal-polluted soils [29]. *Cronobacter* spp. (*Enterobacter sakazakii*) are regarded as opportunistic pathogens linked with life-threatening infections in neonates with symptoms of necrotizing enterocolitis, bacteremia, and meningitis with fatality rates of 50-80%. *Cronobacter* spp. may survive in macrophage cells and efficiently attach to and invade epithelial cell lines. Their exopolysaccharides may contribute to the formation of biofilms, and their active efflux pumps promote resistance to bile salts and disinfectants [30]. Furthermore, the opportunistic foodborne pathogen *C. sakazakii* survives in extremely arid environments, such as powdered infant formula as a reservoir to infect humans. Therefore, stress response mechanisms and virulence factors can reveal the ability of these organisms to cause human disease [31].

Pseudomonas gessardii, *P. psychrophila*, *P. psychrophila* and *P. fluorescens* are the dominant spoilage bacteria isolated from spoiled chicken meat [32]. A facultative psychrophile *P. psychrophila* MTCC12324 isolated from the Ny-Alesund in the Arctic consisted of enzymes involved in polyunsaturated fatty acid biosynthesis, mRNA chaperones, and other cold-inducible proteins that enhanced its survival through cold adaptation. In particular, the amino acid residues of CmIDH and PpIDH may be involved in the thermal properties of this organism [33]. A drought-resistant *P. azotoformans* strain ASS1 significantly improved the accumulation of the total removal of Cu, Zn and Ni metals, potentially protecting plants against abiotic stresses and promoting plant growth and survival in semiarid ecosystems and accelerating the phytoremediation process in metal-polluted soils [34]. The cyhalofop-butyl (CyB)-that degrades *P. azotoformans* QDZ-1 could degrade 84.5% of 100 mg/L (CyB) with CyB as a carbon source for growth. The catalytic efficiency of different AOPP herbicides was in the order quizalofop-P-ethyl ≈ fenoxaprop-P-ethyl > CyB ≈ fluazifop-P-butyl > diclofop-methyl ≈ haloxyfop-P-methyl [35]. The combination of *Bacillus flexus* and *P. azotoformans (B1)* could degrade the polymers to hydrophilic compounds and achieved maximum degradation (22.7%) of UV-treated polypropylene (PP) [36].

4. Conclusion

In the process of purifying the polysaccharides from the fruiting bodies and stalks of *A. polytricha*, we identified different fungal species, and some of these fungal species can infect humans. Furthermore, five bacterial species were identified in homogenates of *A. polytricha* after Pasteur sterilization. Furthermore, three of eleven commercial beverages made with *A. polytricha* caused diarrhea and were contaminated with five bacterial species, and two samples harbored more than one bacterial species. Therefore, sterilization precautions against microbial infection should be considered during storage, processing and eating to prevent possible food toxicity.

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Statement of Competing Interests

Authors declared no conflict of interest.

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