Identification of contaminants as members of the Bacillus pumilus *group and analysis of contamination sources in the cultivation of* Pleurotus geesteranus *mushrooms*

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ORIGINAL ARTICLE

Identification of contaminants as members of the *Bacillus pumilus* group and analysis of contamination sources in the cultivation of *Pleurotus geesteranus* mushrooms

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Abstract

Oyster mushroom (*Pleurotus geesteranus* Singer) is grown widely in China withut problems, but occasionally severe bacterial contamination occurred in cultivation bags of *Pleurotus geesteranus* during high summer temperatures in some companies in the Chunan county of Zhejiang province in 2018, belonging to the so-called *Bacillus pumilus* group by morphological characteristics, 16S rRNA, GyrB and aroE genes sequencing, fatty acid analysis and MALDI analysis. The inoculation tests confirmed that *Bacillus pumilus* strains were contaminants causing mycelial disappearance of *P. geesteranus*. The dual culture tests demonstrated that several strains of *B. pumilus* were able to inhibit mycelial growth of *P. geesteranus*. In order to find the sources of contamination the, process of mushroom cultivation was investigated. A 41.0% contamination rate occurred in cultivation bags with sponge plugs, as opposed to a very low contamination rate (<4%) in cultivation bags with cotton plugs. It was also shown that a 13.0% bacterial isolation rate was obtained from autoclaved sponge pieces, as opposed to 2.0% or 0.0% from cotton pieces, respectively. Fifteen strains isolated all were identified by MALDI-Biotyper as *Bacillus* spp., belonging to four species (*Bacillus circulans*, *B. pumilus*, *B.cereus* and *B. halosaccharovorans*). Dual culture tests showed that *B. circulans*, *B. cereus* and *B. halosaccharovorans* strains had not inhibitory effect on mycelial growth of *P. geesteranus*. This study furthermore shows that sponge plugs containing *B. pumilus* contaminants were the contamination source in cultivation bags of *P. geesteranus*.

Keywords Mushroom cultivation · Contaminants · *B. pumilus* · Contamination source · Primordial induction

Introduction

Pleurotus species belonging to family *Agaricaceae* (*Basidiomycetes*), known commonly as oyster mushroom, are wood-degrading saprophytic fungi being widely distributed and cultivated throughout the world. Oyster mushrooms, such as *P. ostreatus*, *P. geesteranus, P. eryngii* and *P. pulmonarius*, indicate the qualities of *P. geesteranus* as compared to other species are the source of nutrients, and particularly proteins, minerals and vitamins B, C and D (Randive 2012). Recent findings show that exopolysaccharides produced by *P. geesteranus* Singer are considered to be a a potential natural drug for the prevention of liver damage (Song et al. 2018).

P. geesteranus is becoming popular throughout the world because of its abilities to utilize various lignocelluloses and to grow at a wide range of temperatures (from 20 to 30 °C). A large variety of lignocellulosic materials, such as hardwood sawdust and crop wastes

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(paddy straw, wheat straw, corn cobs, soybean straw, cotton stalks, and sugar cane discards and so on), have been used for this and other mushroom cultivation in China (Chen et al. 2010). *P. geesteranus* is widely cultivated in hardwood sawdust in Chunan county of the Zhejiang province, China, a mountainous area with forest resources. With the development of mushroom cultivation, mushroom production has become an important industry that provides the local peasants escapes from poverty. Meanwhile, the industrial development provides the diversification of agricultural production as well.

Oyster mushroom cultivation is usually performed during spring and autumn in Chunan county and the fresh fruiting bodies are mainly used as a food source. Due to the fact that high temperatures inhibit fruiting development of *P. geesteranus*, no product is sold in the market during high summer temperatures, resulting in a sharp rise in demand for mushroom products. To meet the needs of the market, the high temperature-tolerant *P. geesteranus* strain Nongxiu 6 (breeding of the Zhejiang Academy of Agricultural Sciences) was introduced by local research institute (Vegetable Research Institute, Hangzhou Academy of Agricultural Sciences). At the meantime, the cultivation technology was improved as well and new technique extended mushroom production and harvest period. The mushroom products from Chunan county meets the consumer's demands during high temperature summer and become popular in the market, also raising income for growers. However, contamination in cultivation bags occurred seriously in early July 2018 in Xiangyin mushroom industrial limited company (Pingmen Township), Chunan county, affecting the mushroom production with over 40% yield loss. Symptoms of contamination are similar to the wet spot or sour rot caused by *Bacillus* spp. in grain spawn jars (http://en.psilosophy.info/what_are_ common_ contaminants_of_the_mushroom_culture.html) but there is no report of contaminants in cultivation bags. In addition, few studies showed that some of *Bacillus* spp., such as *Bacillus subtilis* and *B. amyloliquefaciens*, do not inhabit hyphal growth of *Pleurotus* spp. and even can be used in the biocontrol of *Trichoderma* fungi (Constantinescu et al. 2004; Mwangi et al. 2017).

Therefore, in this study, symptoms of contamination in mushroom cultivation bags were observed and described, contaminants were isolated and identified by morphological characteristics, sequencing of 16S rRNA, GyrB and aroE genes, fatty acid analysis and MALDI analysis. In addition, effect of contaminants on hypha growth of *P. geesteranus* was determined and the contamination resources were investigated and analyzed.

Materials and methods

Observation of contamination symptom

Contaminating symptoms in the cultivation bags of *P. geesteranus* was conducted in early July 2018 in Xiangyin mushroom industrial limited company (Pingmen Township). Thirty mushroom bags with different contamination level were brought to laboratory for contaminant isolation.

Isolation of contaminants

A small piece of cultivation substrate with mycelia was cut from the margin of a spot in a bag and diluted serially ($10–10^5$) in 1.0 ml of sterile water with pH 7.2 from each sample and the single bacterial colony was obtained by the streak-plating method on Lysogeny broth (LB) agar (yeast extract 5 g, tryptone 10 g, NaCl 10 g, agar 15 g, pH 7.2–7.5) at 28 °C for 24 h. The different colonies were chosen on the bases of colony charecteristiccs such as size, colour, shape (convex, flat), surface (rough, smooth surface) and margin (irregular margin, fine or regular margin) structures. Subsequently, each colony was purified at least three times and stored in 20% glycerol at −20 °C.

Identification of bacterial strains

To identify the bacterial strains, they were cultured on LB agar at 30 °C for 20–24 h and characterized using the growth pattern, morphology and gram staining as described by Awais et al. (2007).

To analyze the 16S ribosomal RNA (rRNA) genes and housekeeping genes, the isolates were cultured on LB agar or NA (trypton 10 g, NaCl 5 g, beef extract 3 g, agar 20 g, pH 7.0–7.5). The 16S rRNA genes were amplified by using primers 27F and 1492R (Zhang et al. 2018) and two housekeeping genes were amplified using primer pairs aroEF/ aroER for 3-phosphoshikimate 1-carboxyvinyltransferase (aroE) gene and gyrBF/gyrBR for gyrase B subunit (GyrB) gene (Liu et al. 2013a). The amplified products were submitted to the Sangon Biotech Company Limited (Shanghai, China) for sequencing of two strands. The resulting sequence was edited with the Bioedit 7.19, aligned with the Clustalx1.83 and then compared with homologous sequences in the GenBank database using BLAST for searching the most similar sequences. The representative strains were chosen, their sequences were submitted to GenBank database and accession numbers were obtained. Phylogenetic trees were generated using the neighbor-joining method in the MEGA 7.0 program (Kumar et al. 2016). The strengths of the internal

branches of the resulting trees were statistically evaluated by bootstrap analysis with 1000 bootstrap replications. *B. cereus* ATCC 14579^T was used as the outgroup.

Gas chromatographic analyses of whole-cell fatty acid methyl esters (FAME) were performed for identical strains grown on TSA plates at 28 °C for 20 h as described by Jeon et al. (2003). Methanolic NaOH solution were added to the bacterial cells for heat treatment and after cooling, fatty acids were extracted with hexane after adding hydrochloric acid. The fatty acid composition was analyzed with the Sherlock system following the protocol of the Microbial Identification System with the TSBA6 6.0 library. Bacterial identification was generated for each sample and ranked by similarity indices.

Inoculation tests of contaminants

The three representive strains were cultured in LB in a WY-211B rotary shaker at 150 rpm at 30 °C for 20 h. Two mL of cell suspension of each strain (approx. 10^8 cells mL^{-1}) was inoculated into the mycelium-covered substrate from the mouth of a bag inoculated with mushroom spawing before two weeks. Each strain suspension was inoculated in three bags, respectively. The mushroom spawing was cultured in the bottles containing sterile wheat grains inoculated with mycelia of *P. geesteranus* strain Nongxiu 6 at 25 °C for one month. After the bags were sealed with the same plugs, they were incubated at 28–30 °C. Three bags inoculated with LB were used as control and each treatment was performed with three replicates. The bags were observed daily once symptoms of contamination appeared. Contaminants were re-isolated as described above.

Inhibitory activity of bacterial strains on hypha growth of *P. geesteranus*

For testing inhibitory effect of bacteria strains on hypha growth of *P. geesteranus*, three representive strains were determined by using a disc diffusion method (Bauer et al. 1966). A disc containing mycelia (8 mm in diameter) was taken from the colony edge of *P. geesteranus* strain Nongxiu 6 on PDA at 25 °C for 4 days and placed onto the center of each PDA plate (90 mm diameter) with pH 7.2. A sterile filter paper disc (5 mm in diameter) containing cell suspension of approx. 10^8 cells ml^{-1} of each test strain cultured in LB in a rotary shaker under the same condition above was placed 2.5 mm from tested fungus. The mycelial growth was measured as a diameter (mm) at 28 °C three days after incubation. A sterile filter paper disc with sterile water was used as control and each treatment was performed with three replicates.

Investigation of contamination source

Investigation of contamination source was conducted in late August 2018 in Yinong mushroom professional cooperative (Linqi town), Xiangyin mushroom industrial limited company (Pingmen Township) and Jinnyinong mushroom professional cooperative (Pingmen Township), Chunan county, Zhejiang province, China. The process of mushroom cultivation was surveyed from spawning to harvesting. The contaminated mushroom bags were recorded and contamination index was assessed for each bag on a 0 to 4 rating scale (0 = no contaminants, 1 = 1 to 20% coverage by the contaminants, 2 = 21 to 40% coverage by the contaminants, 3 = 41 to 60% coverage by the contaminants, 4 = 60 to 80% coverage by the contaminants, 5 = 80 to 100% coverage by the contaminants). Contamination index = Σ(contaminant rating × number of contaminated mushroom bags) /(maximum rating × total number of contaminated mushroom bags) × 100.

Isolation and characterization of contaminants from sterilized materials

Based on the sites of contamination occurrence, possible materials causing contamination were analyzed and they were collected in the cultivation bags, respectively, from thee mushroom cultivation sites: Yinong mushroom professional cooperative (Linqi town), Xiangyin mushroom industrial limited company (Pingmen Township) and Jinnyinong mushroom professional cooperative (Pingmen Township). For simulating sterilization conditions, the samples for each site were sealed in the cultivation bags and sterilized with *P. geesteranus* substrate cultivation bags together in a autoclave (60M^3, Xiangyin Energy Saving Technology Co., Ltd.) used for mushroom production at 103 °C (20 hPa) for 2 h (as daily sterilization conditions) in the Xiangyin mushroom industrial limited company, and then 100 pieces of cotton or sponge from each site were moved onto the plates containing LB agar. After the bacterial strains were isolated and purified as described above, they were identified by using MALDI-TOF MS Biotype system, a MicroFlex™ mass spectrometer (Bruker Daltonics, Bremen, Germany) in the Center of Analysis and Measurement of Zhejiang University.

For MALDI-TOF MS sample preparation, bacterial culture of approx. 10^8 cells mL^{-1} in 1.0 ml sterile double distilled water was centrifuged (using an Eppendorf centrifuge 5417R, Hamburg, Germany) at 13,000 rpm for 2 min and inactivated in 1.0 mL of 75% ethanol HPLC grade, and bacterial protein was extracted, as previously described (Barreiro et al. 2010). MALDI-TOF MS analyses were performed and raw spectra data were processed using MALDI BioTyper 1.1 software (Bruker Daltonics, Bremen, Germany) with default settings. The results of pattern matching were expressed as numerical score ranging from 0 to 3.00. The result was

provided by means of a log score with a maximum value of 3.0. Score values higher than 1.7 were considered reliable for genus identification, and scores higher than 2.0 were considered probable for species identification (Barreiro et al. 2010).

According to the results of identification, 15 different species strains were chosen and their inhibitory activity on hypha growth of *P. geesteranus* was determined by using a disc diffusion method as described above. To observe bacteria forming endospores, different species strains were cultured in LB broth at 37 °C with shaking at 150 rpm for 24 h. All cells samples were stained with malachite green/safranin (Kanetsuna 1964; Solari et al. 1968) and visualized using a Zeiss Axiophot 2 microscopy with Axiocam CCD camera and Axiovision digital imaging software (AxioVision Software Release 3.1., v.3–2002; Carl Zeiss Vision Imaging Systems).

Results

Contamination symptoms

Contamination occurred seriously in cultivation bags. The initial symptoms appeared at the mouth area of a mushroom cultivation bag (Fig. 1a). The yellowish-brown spots were produced in white mycelia-covered substrate and some spots coalesced together into larger one. Subsequently, symptoms of contamination extended and fungal mycelia disappeared, resulting in exposure of brown substrate (Fig. 1b). When fungal hyphae disappeared completely, the upper substrate of a bag was the mucus-like brownish slime and presented excessively wet symptom (Fig. 1c), which was similar with that of wet spot in grain spawn jars (http://en.psilosophy.info/what_are_ common_ contaminants_of_the_mushroom_culture.html). If opening a contaminated bag, the substrate gave off a strong but foul odor. Before the end of fungal vegetable phase, contamination level in different mushroom bags was different (Fig. 1e). Occasionally, it was found that the substrate in a discarded mushroom bag had been almost completely contaminated but primordial development seemed to not be inhibited (Fig. 1d).

Identification of contaminants

Based on observation of symptoms, it was suspected that contamination could be caused by bacteria. However, characteristics of colonies of isolates on LB agar plates were almost similar from different samples. Subsequently, thirty strains of bacteria were obtained after they were isolated, chosen and purified, and characteristics of their colonies were observed. They all were opaque and off-white, roughly circular, but with an irregular margin and 1.8–2.0 mm in diameter after 36 h growth. Gram reaction showed that they all were gram-positive rod bacteria.

Analysis of the 16S rRNA genes showed that the sequences of 30 strains were completely identical. The result of BLAST search showed that all the strains had 99.93–100% identities to sequences of *Bacillus pumilus, B. safensis* and *B. altitudinis* (GenBank accession Nos. MK521063, MK414964 and MK521068). Based on morphological characteristics and sequences of 16S rRNA genes, the 30 strains were considered in the same species and only three representative strains (XZG 01, XZG 09 and XZG 015) isolated from mushroom bags with different contamination level were submitted to GenBank (MN704736, MN704737 and MN704738, respectively) and their sequences were used to construct the phylogenetic tree. Phylogenetic analyses showed that three studied strains firstly were separated into one group with *B. pumilus*, *B. safensis*, *B. stratosphericus*, *B. altitudinis* and *B. aerophilus* (Fig. 2), being well-separated from the other species of *Bacillus*. In this group, three studied strains clustered together with *B. pumilus* HT16 and NR 043242 as a clade with a bootstrap value of 83. Furthermore, three studied strains (XZG 01, XZG 09 and XZG 015) were analyzed further using two housekeeping genes (*gyrB* and *aroE*) and their sequences were submitted to GenBank under accession numbers MT682381, MT 682382 and MT682383 for *gyrB* gene and MT682378, MT682379 and MT682380 for *aroE* gene. The phylogenetic tree of *gyrB* generated by using the neighbour-joining method with 13 taxa, indicated that XZG 01, XZG 09 and XZG 015 clustered with three reference strains *Bacillus pumilus* GBSW19, AUEC29 and BP-hd-4 as a clad with a bootstrap value of 100 (Fig. 3). Similarly, the phylogenetic tree of *aroE* showed that XZG 01, XZG 09 and XZG 015 clustered with the reference strain *Bacillus pumilus* MCCC1A06996 and PDSLzg-1 as a clad with a bootstrap value of 100 (Fig. 4). They formed the sister clades with the taxa including *Bacillus safensis* with a bootstrap value of 99.

Analysis of cellular fatty acid profiles of the three stains (XZG 01, XZG 09 and XZG 015) were shown in Table 1. In three strains, cellular fatty acid profiles all revealed that the iso-C15:0 (55.36%), anteiso-C15:0 (21.31), C16:0(2.62%), 17:1 iso w10c (2.88%), iso-C176:0 (6.73%) and anteiso-C17:0 (4.47%) were predominant, and it was identified as *B. pumilus* with a strong match with the MIDI database (with similarity index 0.789–0.809). Combining phylogenetic analysis, XZG 01, XZG 09 and XZG 015 all were identified as *B. pumilus* group.

Inoculation tests

The results of inoculation tests showed that symptoms of the yellowish-brown spots were observed in mushroom cultivation bags 15–17 days after inoculation with strains XZG 01, XZG 09 and XZG 015. Twenty-five days after inoculation, typical contamination symptoms appeared on bags, just as Fig. 1b, being similar to that in the greenhouse. LB-

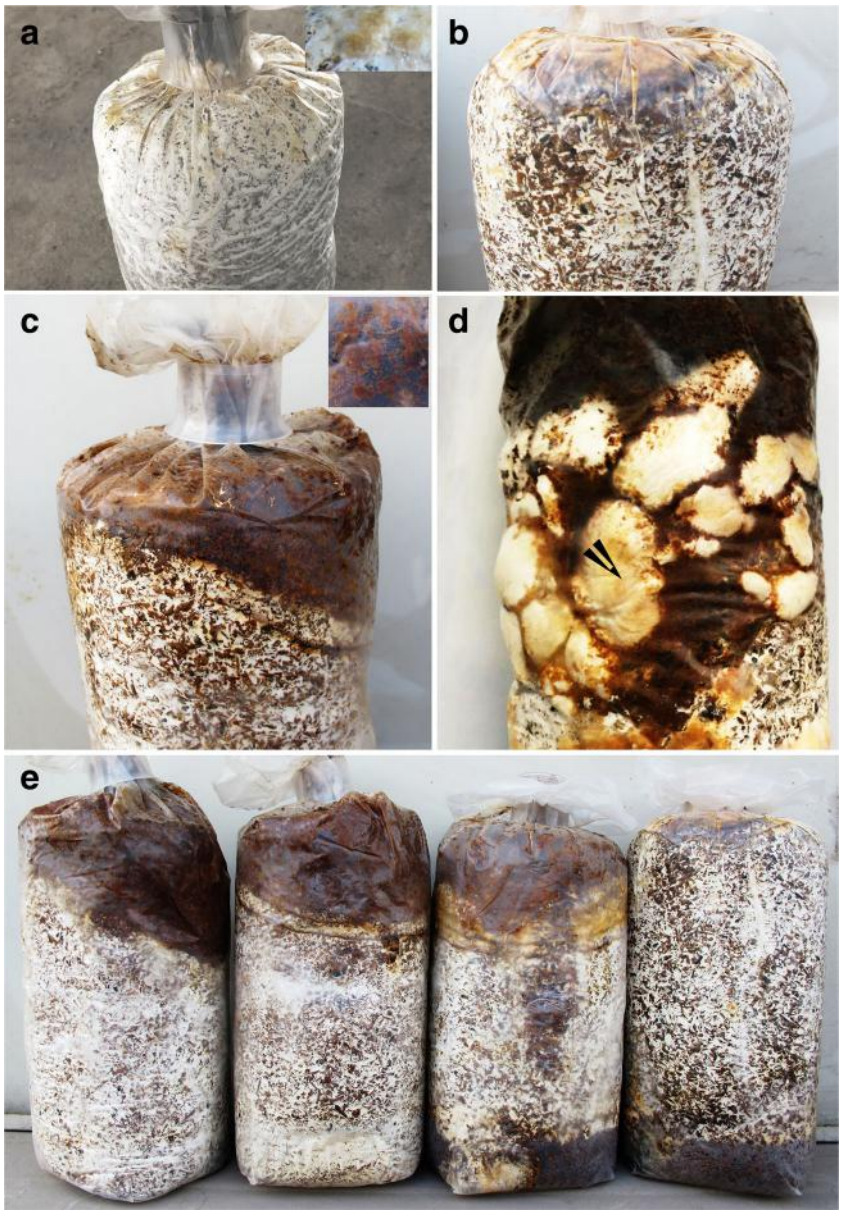

Fig. 1 Symptoms of contamination in cultivation *Pleurotus geesteranus* bags. **a** Symptom of initial contamination. **b** Contamination extending inferiorly. **c** Excessively wet symptom after fungal hyphae disappearance. **d** Primordial development (arrowhead) in a seriously contaminated mushroom bag. **e** Mushroom bags with different degree of contamination

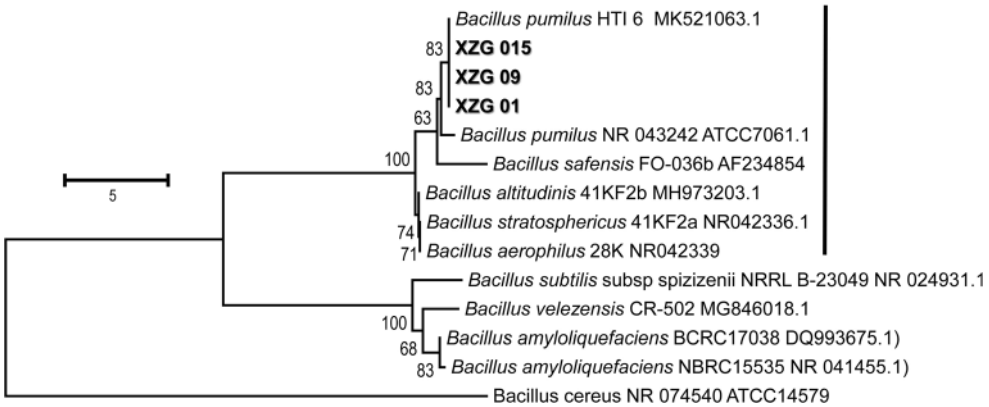

Fig. 2 Phylogenetic tree generated from neighbor-joining method from 16S gene sequences of 14 taxa of *Bacillus*. Bootstrap values supporting the branches are shown at nodes and branch lengths are proportional to divergence. Strains isolated from *P. geesteranus* cultivation bags are shown in bold

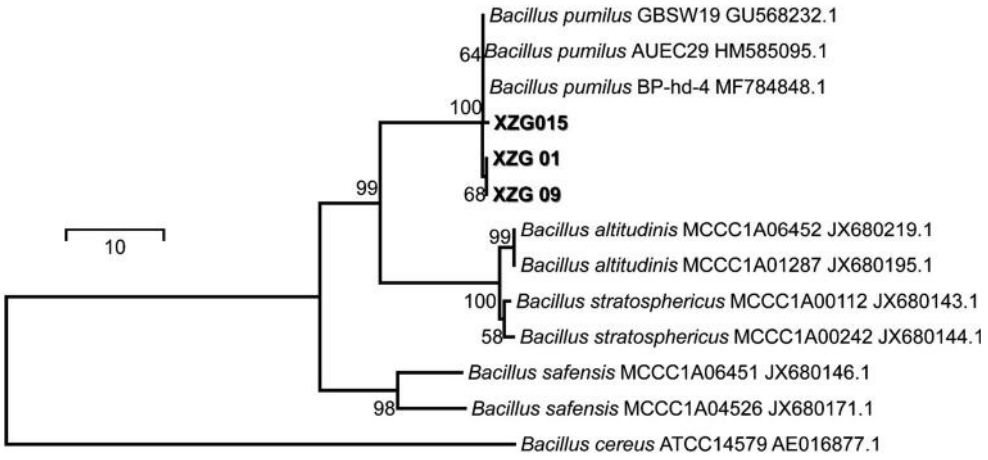

Fig. 3 Phylogenetic tree generated from neighbor-joining method from *gyrB* gene sequences of 13 taxa of *Bacillus*. Bootstrap values supporting the branches are shown at nodes and branch lengths are proportional to divergence. Strains isolated from *P. geesteranus* cultivation bags are shown in bold

inoculated controls showed no symptoms. Bacteria were re-isolated from the spots and their characteristics were confirmed to be the same as those described above.

Inhibitory activity of *B. pumilus* strains against hypha growth of *P. geesteranus*

The inhibitory activity of three strains (XZG 01, XZG 09 and XZG 015) against hyphal growth of *P. geesteranus* were evaluated using the disc diffusion assay. All three strains showed significantly inhibitory effect against hyphal growth, as showed in Fig. 5. In the test assessing growth inhibition of fungal mycelia, strain XZG 01 showed the highest percentage inhibition of radial growth (59.2%) three days after inoculation ($P < 0.05$) but there was not significant difference among the three strains (Table 2).

Investigation of contamination source

Based on our investigation, contamination occurred at the latter vegetative phase in the *P. geesteranus* cultivation. In the area of Zhejiang province, patterns of the *P. geesteranus* cultivation was similar in cultivation substrate and cultivation process among different companies. Cultivation substrate usually consisted of the mixture containing the chips ground from pruned mulberry tree twigs (30%), various hardwood sawdust (32%), cottonseed hull (20%), wheat bran (12%), corn flour (3%) and lime (3%). Their change depended on different sources. When the mixture was transferred to a polypropylene (PE) bags of 17 by 38 cm long, its opening was plugged by cotton or sponge materials and then a plastic ring wrapped around the neck to seal the PE bag. The bags filled with the mixture were placed in an autoclave ($60M^3$, Xiangyin Energy Saving Technology Co., Ltd.) and sterilized at 103 °C and 20 hPa pressure for 2 h. After cooled to room temperature, the bags were inoculated with *P. geesteranus* Nongxiu 6 spawn.

In process of cultivation, *P. geesteranus* was cultivated for more than three months, divided into several phases (Fig. 6). The first was the vegetative phase, which allowed development of the *P. geesteranus* mycelia. This phase was divided into two stages: the spawning stage, consisting of seeding the substrate with the *P. geesteranus* mycelium and the incubation stage of about thirty days at 25 °C; followed by a inoculation with spawning, in which the cultivation bags were then marked and stored on the shelves in plastic greenhouse with

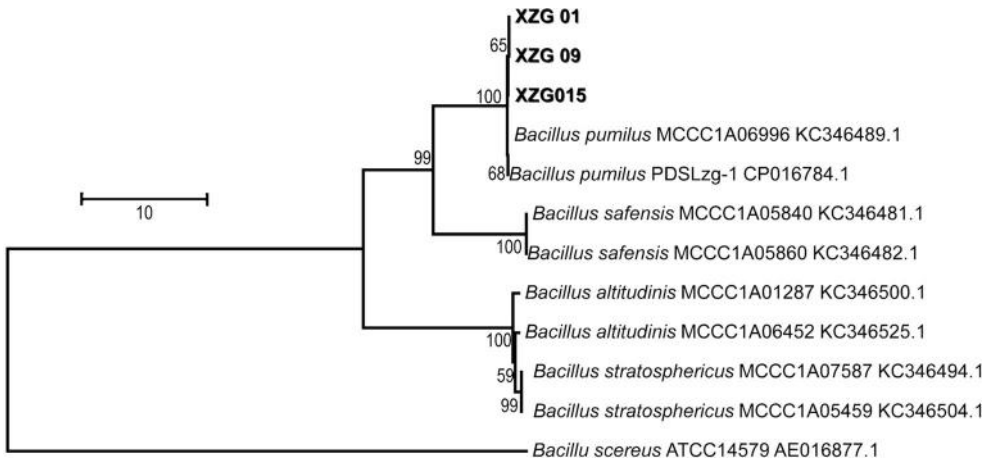

Fig. 4 Phylogenetic tree generated from neighbor-joining method from *aroE* gene sequences of 12 taxa of *Bacillus*. Bootstrap values supporting the branches are shown at nodes and branch lengths are proportional to divergence. Strains isolated from *P. geesteranus* cultivation bags are shown in bold

Table 1 The fatty acid compositions of three strains

Fatty acid	Fatty acid content (%)		
	XZG 01	XZG 09	XZG 015
Saturated straight-chain fatty acid			
14:0	0.47	1.47	0.56
16:0	1.70	1.80	1.80
Saturated terminally branched fatty acid			
13:0 iso	0.29	1.30	0.67
14:0 iso	1.19	1.56	1.25
15:0 iso	55.36	53.37	52.38
16:0 iso	2.62	2.78	2.32
17:0 iso	6.73	5.79	6.32
15:0 anteiso	21.31	20.35	21.98
17:0 anteiso	4.47	4.99	3.97
Monounsaturated fatty acid			
16:1 w11c	0.98	1.68	2.98
ISO17:1ω10c	2.88	2.56	1.85

relative humidity level of 70–90% and temperatures of 25–30 °C (depending on temperature variation of outer circumstance), the incubation stage of two months. The second was the fructification phase, which allowed openings of the bags for mushroom growth, and after five to seven days, mushroom was harvested. The third was the recovery phase, which made vegetable growth to be vigorous for 10–15 days after the substrate sites producing fruiting bodies were scraped off with an iron scoop and sprayed with water. The four was the second harvest phase. Subsequently, mushroom growth entered the second phase and cycle was repeated for five to six times. This pattern of the *P. geesteranus* cultivation was used widely in spring and autumn and contamination was usually ignored due to very low contamination ratio.

However, during high summer temperature, the second (fructification) phase, needed to be induced by low temperature using mechanical cooling (10 °C for 12 h) after fungal vegetable growth ended (Fig. 6), whereafter cooling was stopped, greenhouse temperatures were kept at about 22 °C for first, 30 °C for second and 34 °C for third day. Subsequently, the bags, as well as side windows and roof vents of the greenhouse were opened to allow maximum mushroom growth, and mushroom are harvested after five to seven days. After the recovery phase ended, new cycle was repeated as described above. During the latter vegetative phase prior to low temperature treatment, symptoms of contamination usually occurred (Fig. 6).

The results of contamination investigation showed that contamination level of mushroom bags was different among different companies (Table 1). Severe contamination occurred in mushroom bags with 41.0% contamination rate and 24.8% contamination index in Yinshi mushroom industrial limited company (Pingmen Township), while very low contamination with 2.3% and 1.8% in the Yinong mushroom professional cooperative and 3.4% and 2.6% in the Jinnyinong mushroom professional cooperative (Pingmen Township), respectively. By comparation of contamination sites, it was found that high contamination rate was associated with sponge materials plugging the opening of a bag (Table 3).

Table 2 Inhibitory activity of three strains of *Bacillus pumilus* against *P. geesteranus*

Bacterial strains	PIRG (%)*	Fungal colony diameters (mm)
XZG 01	59.2 a	21.1 ± 0.3 b
XZG 09	55.3 a	20.0 ± 0.1 b
XZG 015	53.2 a	19.2 ± 0.2 b
control	–	40.7 ± 0.3 a

Isolation and characterization of contaminants

Isolation For confirming if the plugs of bags were the contaminated source, contaminants were isolated on LB from sterilized cotton and sponge materials in an autoclave using mushroom production. A total 15 bacterial strains were obtained. Among them, 13 strains were isolated from 11 pieces of sponge and two strains from pieces of cotton. The results of isolation showed that colonies from pieces of cotton or sponge all were bacteria but isolation frequencies were different in different samples from different origins (Table 4). The highest isolation frequency (13.0%) was obtained from pieces of sponge, while very low isolation frequency (2%) or no bacteria was obtained from pieces of cotton (Fig. 7).

MALDI-TOF MS analysis Among 15 strains submitted for MALDI-TOF-MS analysis, all strains were identified as *Bacillus* spp. (Table 5). Among them, 13 strains from sponge pieces were identified as *Bacillus circulans* (nine strains), *B. pumilus* (two strains), *B. cereus* (one strain) and *halosaccharovorans* (one strain), respectively, while two strains from cotton pieces as *B. cereus* (two strains). This reveals that *Bacillus* spp. have higher survival rates in sponge than cotton materials under conditions of incomplete sterilization.

Inhibitory tests To order to determine inhibitory activity of these strains against hypha growth of *P. geesteranus*, stains of different species were chosen. The results of inhibitory tests showed that two *B. pumilus* strains had significant inhibitory activity against hypha growth of *P. geesteranus*, as showed in Fig. 3, but the other 13 strains divided into three species (*B. circulans*, *B. cereus* and *B. halosaccharovorans*) had not significantly inhibitory activity (Fig. 5). The results of endospore staining showed that strains of four species (*B. pumilus*,

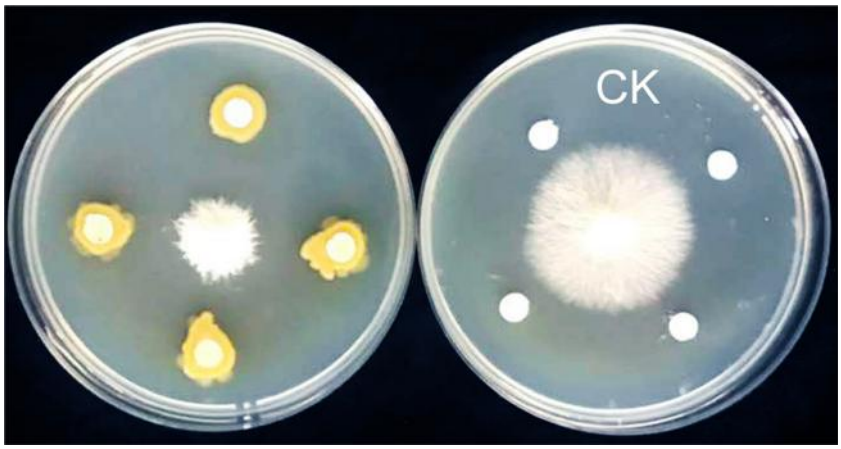

Fig. 5 Inhibitory activity of *Bacillus pumilus* XZG 01 (left) against hypha growth of *P. geesteranus* (center) using disc diffusion assay. CK: control

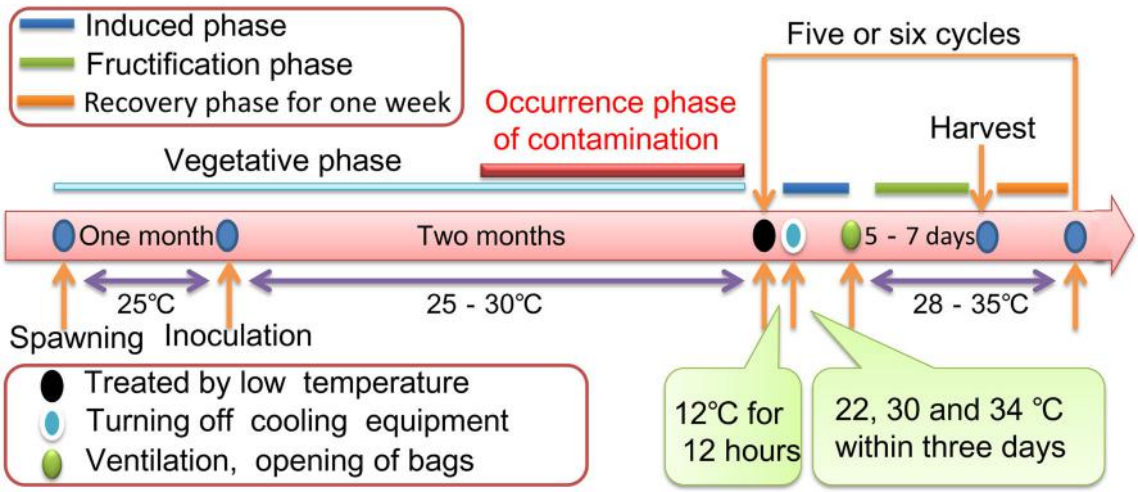

Fig. 6 The cycle of *Pleurotus geesteranus* cultivation and occurrence phase of contamination. The cooling equipment is used only for 12 h for inducing the primordial production, and other methods, such as sunshade nets and spraying water on a plastic film, are used for lowering the temperature at other times of the day

B. circulans, *B. cereus* and *B. halosaccharovorans*) all contained the endospore, confirming that they all were the endospore-forming bacteria (Supplementary Fig. 1).

Discussion

Oyster mushrooms are grown widely using popular edible mushrooms of the *Pleurotus* species, such as *P. geesteranus*, *P. ostreatus* and *P. eryngii*. Their development may be divided into a vegetative phase (including spawning and mycelial growth), a fructification phase, a recovery phase and a harvest phase (Fig. 6). However, mushroom growers are frequently challenged by mushroom diseases of bacteria. These different phases may be affected by differently bacterial contaminants and pathogens (Bellettini et al. 2018; Lim et al. 2008), which cause production losses up to 40%.

In process of spawning, strains of *Enterobacter amnigenus*, *Staphylococcus epidermidis* and *Bacillus cereus* evidenced inhibitory effects on the growth of mycelia in the substrate

Table 3 The results of contamination investigation in the cultivation of *P. geesteranus*

Origin	Plugged materials	Contamination rate (%)	Contamination index (%)
[a]YMPC	Cotton	2.3%	1.8%
[b]YMILC	sponge	41.0%	24. 8%
[c]JYMPC	Cotton	3.4%	2.6%

[a] Yinong mushroom professional cooperative (linqi town); [b] Yinshi mushroom industrial limited company (Bingmen Township); [c] Jinnyinong mushroom professional cooperative (Bingmen Township)

Table 4 The results of bacteria isolation from sterilized cotton and sponge materials

Isolation materials	Isolation frequency (%)	Origin
Pieces of cotton	0%	[a]YMPC
Pieces of sponge	13.0%	[b]YMILC
Pieces of cotton	2.0%	[c]JYMPC

[a] Yinong mushroom professional cooperative (linqi town); [b] Xiangyin mushroom industrial limited company (Bingmen Township); [c] Jinnyinong mushroom professional cooperative (Bingmen Township)

Table 5 The results of MALDI-Biotyper identification

Origin	Species	No.	Score value
[a]YMILC	*Bacillus circulans*	9	2.078–2.242
	B. pumilus	2	2.101–2.214
	B. cereus	1	2.239
	B. halosaccharovorans	1	2.101
[b]YMPC	*B. cereus*	2	2.239
[c]JYMPC	–	–	–

[a] Xiangyin mushroom industrial limited company (Bingmen Township); [b] Yinong mushroom professional cooperative (linqi town); [c] Jinnyinong mushroom professional cooperative (Bingmen Township)

bottle (Lim et al. 2008), and *Bacillus* spp. sometimes survives the sterilization process as in grain spawn jars. In fructification phase, brown blotch disease usually occurs. It was first reported in 1915 in artificial mushroom cultivation (Tolaas 1915), and *Pseudomonas tolaasii* was identified as the pathogen responsible for the disease (Mu et al. 2015). *P. tolaasii* is consistently associated with brown-reddish blotches on *P. ostreatus* sporocarps (Cantore and Iacobellis 2014). Symptoms of the disease include blotch formation and discoloration on the surface of mushroom caps. *Pantoea* spp. has been reported as a causal agent of soft rot disease with symptoms of water-soaked lesions on the stipes and pileus of *P. eryngii* (Kim et al. 2007; Liu et al. 2013b). However, during mycelial growth of vegetative phase, there is almost no research report about problem of cultivation substrate contamination. In this study, this is the first record of contamination of *P. geesteranus* cultivation bags caused by *B. pumilus*.

In process of mushroom cultivation, symptoms of contamination usually appear before cultivation substrate is full of fungal hyphae. Inoculation tests also showed that symptoms appeared at least two weeks after inoculation. Obviously, cultivation substrate is not suitable for growth and reproduction of *B. pumilus*. However, *B. pumilus* probably colonizes and disintegrates fungal mycelia for vegetative growth, leading to contamination symptom. Although the excessively wet symptom in cultivation bags is similar to that in grain spawn jars, it

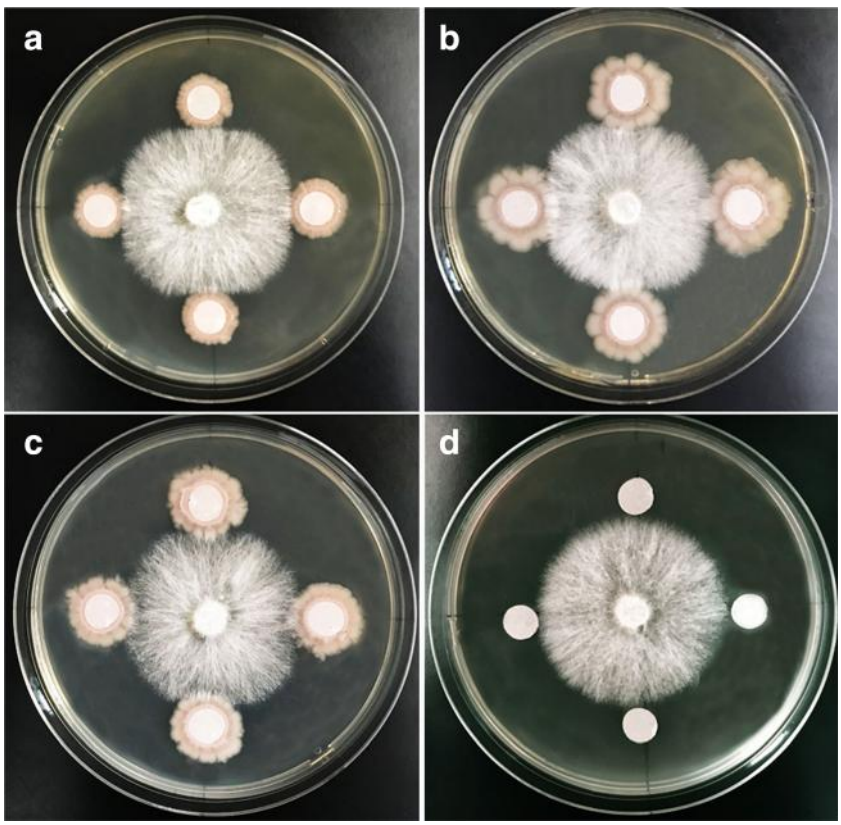

Fig. 7 Effect of three species strains against hypha growth of *P. geesteranus* S15. **a** *B. circulans*. **b** *B. cereus* S7. **c** *B. halosaccharovorans* S5. **d** Control

is produced after mycelia of *P. geesteranus* are completely degraded by *B. pumilus* as showed in Fig. 1c.

Although the endospore-forming *B. pumilus* is relatively sensitive to heat and may be completely killed under normal sterilization (Janštová and Lukášová 2001) but it can survive in incompletely sterilized materials at 103 °C (20 hPa) for 2 h in this study. This possibly is related to the large sample quantity and possible uneven temperature distribution in an autoclave, resulting in incomplete sterilization. In addition, although the results of investigation for contamination source showed that *Bacillus* spp. had higher survival rates in sponge than cotton materials under condition of the same sterilization, it was unclear if sponge material contained the more contaminants than cotton that in general. This needs to be confirmed in future. However, not all *Bacillus* spp. can cause contamination. Some studies show that *B. subtilis* and *B. macerans* have growth-promoting activity against *Pleurotus* spp. but no inhibitory activity (Cho et al. 2003). The endospore-forming *P. polymyxa* and *B. subtilis* are used for biological control of green mould (*Trichoderma viride*) in *P. ostreatus* cultivation (Constantinescu et al. 2004; Velázquez-Cedeño et al. 2008). Dual culture tests, in this study, also demonstrate that *B. circulans*, *B. cereus* and *B. halosaccharovorans* have not inhibitory activity against hypha growth of *P. geesteranus*.

In conclusion, this study describes the process of *P. geesteranus* cultivation, symptoms of bacterial contamination and time period of its occurrence, identify the contaminants as belonging to the so-called *Bacillus pumilus* group, and shows that incompletely sterilized sponge material is main contamination source. In practice, the incompletely sterilized conditions used in the mushroom industry are usually performed during spring and autumn, problem of contamination is often overlooked, but during high summer temperature, contamination in cultivation bags occurred seriously under the same sterilization condition, obviously being related to rapid growth and reproduction of bacterial pathogens. In this study, although our results show that only endospore-forming bacteria can survive under present sterilization condition, the non-spore forming contaminants are not excluded completely, as reported by Lim et al. (2008). Our findings will provide a theoretical basis for the development of ways to prevent bacterial contamination in oyster mushroom production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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