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Aerobic fermentation prior to pasteurization produces a selective substrate for cultivation of the mushroom *Pleurotus pulmonarius*

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Description of the subject. *Pleurotus* species are cultivated on lignocellulosic substrates, in which contaminant fungi such as *Trichoderma* spp. are common. A selective substrate for *Pleurotus* provides the necessary conditions for protection against contaminants. Studies show that *Paenibacillus polymyxa* and other thermophilic bacteria benefit from the selectivity of *Pleurotus* cultivation substrate, however, little is known regarding these bacterial communities.
Objectives. To evaluate the effect of substrate inoculation with *Paenibacillus polymyxa* on the productivity of *Pleurotus pulmonarius* and its protection against *Trichoderma*.
Method. Barley straw inoculated with *P. polymyxa* and non-inoculated straw (control) was used following 0, 3 or 5 days of fermentation prior to heat treatment in order to produce the cultivation substrate. The microbiological content before and after the heat treatment, the mycelial colonization by *P. pulmonarius* and *Trichoderma viride* in competition and the yield of *P. pulmonarius* were all evaluated.
Results. We observed that inoculation with *P. polymyxa* increased the number of cultivable bacteria and changed the composition of the community. The inoculation decreased the colonization ability of *T. viride* and favored the mycelial growth, although the yield of mushrooms was affected. Higher yields of *P. pulmomarius* were obtained in the control substrate where no contamination of *Trichoderma* spp. was observed.
Conclusions. Addition of *P. polymyxa* modified the natural microbiological succession in a fermentation of barley straw for 5 days, favoring the competitiveness of *P. pulmonarius* against *T. viride*. Fermentation of barley straw for 3 days, followed by heat treatment, benefits the yield and protection of *P. pulmonarius* against *Trichoderma viride*.
Keywords. *Trichoderma viride*, *Pleurotus pulmonarius,* barley straw, antagonism.

La fermentation aérobie avant la pasteurisation produit un substrat sélectif pour la culture du champignon *Pleurotus pulmonarius*
Description du sujet. Les espèces de *Pleurotus* sont cultivées sur des substrats lignocellulosiques, où des champignons contaminants tels que *Trichoderma* spp. sont communs. Un substrat sélectif pour *Pleurotus* doit fournir les conditions nécessaires à la protection contre les contaminants. Des études ont montré que *Paenobacillus polymyxa* et d'autres bactéries thermophiles peuvent participer à la sélectivité du substrat de culture pour *Pleurotus*, mais on sait peu de choses sur les communautés bactériennes impliquées dans ces différents substrats.
Objectifs. Évaluer l'effet de l'inoculation de *P. polymyxa* dans un substrat de culture sur la productivité de *P. pulmonarius* et sur la protection contre *Trichoderma*.
Méthode. De la paille d'orge inoculée avec *P. polymyxa* et de la paille non inoculée (témoin) ont été utilisées après 0, 3 ou 5 jours de fermentation avant un traitement thermique pour produire le substrat de culture. Le contenu microbiologique avant et après traitement thermique, la colonisation mycélienne par *P. pulmonarius* et *Trichoderma viride* en compétition et le rendement de *P. pulmonarius* ont été évalués.

Résultats. Nous avons observé que l'inoculation avec *P. polymyxa* augmentait le nombre de bactéries cultivables et modifiait la composition de la communauté microbienne. Elle diminue la capacité de colonisation de *T. viride* et favorise la croissance mycélienne. Cependant, le rendement en champignons a été affecté. Les rendements plus élevés de *P. pulmonarius* ont été obtenus dans le substrat témoin où aucune contamination de *Trichoderma* spp. n'a été observée.
Conclusions. L'ajout de *P. polymyxa* a modifié la succession microbiologique naturelle lors d'une fermentation de paille d'orge pendant 5 jours, favorisant la compétitivité de *P. pulmonarius* par rapport à *T. viride*. La fermentation de la paille d'orge pendant les 3 jours qui suivent un traitement thermique est bénéfique pour le rendement et la protection de *P. pulmonarius* contre *T. viride*.
Mots-clés. *Trichoderma viride*, *Pleurotus pulmonarius*, paille d'orge, antagonisme.

1. INTRODUCTION

Pleurotus species hold second place in terms of production of edible mushrooms worldwide, with 19% of the total production, exceeded only by the shiitake mushroom, *Lentinula edodes* (Berk.) Pegler (22% of total production). The popular white button mushroom, *Agaricus bisporus* (J.E. Lange) Imbach, now occupies fourth place after the *Auricularia* species (Royse et al., 2017). *Pleurotus* spp. are commonly cultivated on lignocellulosic substrates, where a recurring problem is the presence of competing molds of the genus *Trichoderma* (Sánchez et al., 2007), causing green mold disease. Following addition of water to the lignocellulosic materials, several methods are used by mushroom producers to prepare the substrate in order to eliminate any disease and insect problems that may occur during the culture, including chemical disinfection, sterilization, cooking in hot water, steam pasteurization and aerobic fermentation (Hernández et al., 2003; Sánchez et al., 2012). However, it is not clear which substrate type guarantees high biological selectivity for *Pleurotus* spp. and prevents the manifestation of competing fungi (Muez-Orobia & Pardo-Nuñez, 2001).

A substrate with biological selectivity may contain microbial communities rich in bacteria, *Actinomycetes* and thermophilic fungi as protectors; these are all non-competitors for the cultivated mushroom. In the case of *Pleurotus*, different studies show the importance of beneficial thermophilic bacteria such as *Paenibacillus polymyxa* (Prazmowski) Macé (Stolzer & Grabbe, 1991; Velázquez-Cedeño et al., 2006a; Velázquez-Cedeño et al., 2006b), which consume the sugars that could be a resource for contaminating microorganisms or antagonists during incubation. They also produce metabolites that provide selectivity for development of *Pleurotus*. As well as *P. polymyxa*, *Bacillus* spp. are involved in selectivity by inhibiting the growth of *Trichoderma harzianum* Rifai and stimulating the defenses of *Pleurotus ostreatus* (Jacq.) P. Kumm. through the induction of laccases (Nagy et al., 2012; Mwangi et al., 2017).

Bioprotection by inoculation of beneficial microorganisms is a promising way to increase selectivity in *Pleurotus* substrates. For instance, immersing paddy straw in boiling water for 30 min and inoculating it with *Pseudomonas fluorescens* Migula or *Bacillus subtilis* (Ehrenberg) Cohn isolates reduced green mold intensity and enhanced the yield of *Pleurotus sajor-caju* (Fr.) Singer, compared to non-inoculated straw (Shah & Nasreen, 2011). Aerobic fermentation of the substrate is also known to permit development of a thermophilic bacterial community. The native microorganisms of the substrate metabolize the available components and heat is generated as a consequence. The substrate itself then reaches temperatures of between 50°C and 70°C for several days. This condition provides a favorable microhabitat for thermophilic microorganisms. Thus, short composting is successfully applied in the preparation of selective substrates for the industrial production of *Pleurotus* spp. (Villa Cruz et al., 1999; Hernández et al., 2003; Castañeda-de-Leon & Leal-Lara, 2007).

Controlling the microbial community during aerobic fermentation in short composting of substrate for *Pleurotus* cultivation can optimize its development, increase production and reduce the risk of contamination by *Trichoderma* spp. (Velázquez-Cedeño et al., 2008). The aim of the present study was to evaluate the effects of a cultivation substrate preparation that combines an aerobic fermentation phase with a *P. polymyxa* strain and steam pasteurization, in terms of its efficiency at protecting against *Trichoderma viride* Pers ex S.F. Gray and on the productivity of *Pleurotus pulmonarius* (Fr.) Quél.

2. MATERIALS AND METHODS

2.1. Biological material

The CDBB35 strain of *Paenibacillus polymyxa*, provided by the Center for Research and Advanced Studies (CINVESTAV) of Zacatenco, Mexico City, was used. The strain was maintained at 4 °C on 2.4% nutrient agar (AN) (DIFCO®) with 10% glycerol. The strain IE-115 of *Pleurotus pulmonarius*, obtained from the collection of the Institute of Ecology A.C. (INECOL), Xalapa, Veracruz was studied. It was

preserved in Potato Dextrose Agar (PDA) medium at 4 °C and propagated to PDA, with incubation for 14 days at 26 °C. Sorghum seeds (*Sorghum vulgare* Pers.) were used for the preparation of primary and secondary inoculum (Guzmán et al., 2013). *Trichoderma viride* (strain IE-637), isolated from a *Pleurotus* substrate produced at INECOL in 1999 was used, and conserved lyophilized in sorghum grains at 4 °C.

2.2. Preparation of the inoculum of *Paenibacillus polymyxa*

Paenibacillus polymyxa was first grown on 2.4% AN (DIFCO®) medium and incubated at 28 °C for two days. It was then further developed in 250 ml Erlenmeyer flasks with 80 ml liquid medium of 0.1% AN. The flasks were shaken at 200 rpm for five days and the contents were then centrifuged at 10,000 rpm for 10 min. The supernatant was removed, and the pellet was suspended in 40 ml of 10% semi-skimmed milk (LALA™). A second centrifugation was performed at 10,000 rpm for 10 min. The supernatant was removed and the precipitate with the bacteria was lyophilized (modified from Requena et al., 1996; Korsten & Cook, 1996).

2.3. Fermentation and heat treatment of the substrate

One hundred kg (wet weight, 70% ± 5) of barley straw (*Hordeum vulgare* L.) were composted for each treatment. For the experimental treatment, 500 ml of a suspension of *P. polymyxa* were added at a concentration of 1.73×10^3 units·ml^{-1} on day 0. For the control, 500 ml of water were added. In both treatments, the substrate was placed into a metal container 2 m in length x 1 m in width x 1 m in height. This was subjected to a fermentation of 5 days, with the substrate mixed on day 3 to promote aeration and a daily superficial irrigation to maintain the humidity at 70%.

On days 0, 3 and 5, 25 kg of substrate (wet weight) were taken from both the inoculated substrate and the control, and a heat treatment of steam pasteurization for 12 h at 60 °C applied to these samples, followed by a period of 24 h at 48 °C (modified from Castañeda-de-Leon & Leal-Lara, 2007; Velázquez-Cedeño et al., 2008). These substrates were used for culture, microbiological analysis and confrontation tests.

Maximum and minimum temperatures, moisture content and pH of the substrate were recorded daily. The temperature was recorded 3 times daily with a thermometer introduced into the center of the mound. The moisture content of the substrate was estimated by weight difference, for which 20 g of straw (wet weight) were taken and dried in an oven for 24 h at 50 °C. To measure the pH of the substrate, 10 g of straw were placed in a flask with 20 ml sterile distilled water (v:v 1:2) and the pH determined with a potentiometer (Hanna®). For both measurements (humidity and pH), the samples were processed in triplicate.

2.4. Analysis of bacteria groups

The presence of microbial populations in the substrate was quantitatively analyzed by counting the Colony Forming Units (CFU) of total microflora (TM), *Bacillus*, *Pseudomonas* and *Actinomycetes* using specific culture media in Petri dishes. For TM, peptone agar medium (0.5% peptone, 0.5% yeast, 1% glucose, 1.5% agar) was used at pH 7.6. For *Bacillus*, the same medium as for TM was used, but following sterilization: 0.1% of sterile ketoconase was added when the medium reached a temperature of 50 °C. For *Pseudomonas*, the medium used was 2% peptone, 0.14% $MgCl_2$, 1% K_2SO_4 and 1.36% agar, and for *Actinomycetes*, a modified medium of Pochon and Tardieux (1962) was used (1% glycerol, 0.1% L-aspargine, 0.1% K_2HPO_4, 1.5% agar and 0.1% K_2CR_2O7).

The bacterial groups were analyzed in the substrate with *P. polymyxa* and in the control on days 0, 3 and 5 of fermentation and following the heat treatment. For each sampling point, a 20 g subsample of the substrate was taken from different parts of the mound and mixed in a blender with 180 ml of 0.05% sterile Tween 80® to obtain a stock solution, which was liquefied twice for 10 seconds at 1 min intervals. Dilutions up to 10^{-5} (modified from Velázquez-Cedeño et al., 2004) were made from this solution. For *Bacillus*, the stock solution was incubated in a water bath for 10 min at 80 °C and dilutions (10^{-5}) were carried out. Fifty microliters were taken from each dilution and inoculated on specific culture media, homogeneously spread on the medium with the aid of sterile L-shaped glass rods. This was performed in triplicate for each group. The Petri dishes were incubated at 25 °C and 48 °C to favor development of the mesophilic and thermophilic microflora, respectively. A CFU count was performed at 24 h of incubation and the results were expressed as CFU per gram of dry substrate. The data were subjected to a variance analysis (ANOVA) and the difference in the means was estimated by a Tukey post hoc multiple range test ($\alpha = 0.05\%$) with the software Statistica® ver. 6.1.

2.5. *Pleurotus* production

Production of *P. pulmonarius* was evaluated on cultivation substrates after the heat treatment of barley straw. The culture was carried out using substrate from the mound in fermentation on days 0, 3 and 5, both from the substrate inoculated with *P. polymyxa* and that of the control.

Samples of 2 kg of substrate were spawned with 5% barley grains colonized by *P. pulmonarius* mycelium in bags of 20 × 30 cm. The bags were incubated in the dark at 24 °C for three weeks. The samples were placed in a room with ventilation and artificial lighting (automatic system featuring 12 h of light: 12 h of darkness) at 26 °C and relative humidity between 75% and 80% (automatic nebulization) for fruiting body production. The plastic bags were removed when colonization finished. Fruiting bodies were harvested for 40 days. Biological Efficiency (BE) was calculated as the fresh weight of fruiting bodies (g) produced on 100 g (dry weight) of substrate. Production Rate (PR) was estimated as the biological efficiency per day of production.

The experimental design was completely random, consisting of six treatments: a substrate inoculated with *P. polymyxa* and a control substrate on days 0, 3 and 5. Ten replicates were conducted for each treatment. The BE and TP were subjected to an ANOVA and differences in the means identified by Tukey post hoc multiple range test ($\alpha = 0.05\%$) with Statistica® software ver. 6.1.

2.6. Mycelial confrontation of *Pleurotus pulmonarius vs Trichoderma viride*

For the reactivation of *T. viride*, the flask with freeze-dried grains was immersed in a water bath for 10 min at 25 °C, and the seeds then incubated on PDA at 25 °C for three days. Two days prior to using the strain, an implant (0.7 cm Ø) of medium colonized with *T. viride* was taken and inoculated on 1.5% agar-water medium to obtain mycelium without spores (modified from Pérez-Merlo & Mata, 2002).

Fermented substrates (with *P. polymyxa* and the control) were used after the heat treatment on days 0, 3 and 5 of fermentation. In addition, some parts of the same substrates were sterilized (121 °C for 1 h) in order to observe the effect of absence of the bacterial community on the development of *P. pulmonarius* and *T. viride*. For the confrontations, 10 g of straw from each treatment were placed in a Petri dish under sterile conditions. One barley grain of spawn of *P. pulmonarius* was placed at 1 cm from the border and, 1 cm from the edge at the opposite side, a *T. viride* implant (0.7 cm Ø) developed in agar-water was placed. Ten replicates were conducted per treatment.

For each treatment, the mycelial growth of *P. pulmonarius* and *T. viride* was measured, estimating the area occupied by each on days 3, 5 and 7 of incubation at 25 °C. The areas occupied were digitized and measured using Adobe Photoshop CS3 extended 10.0 software. The data were subjected to an ANOVA and the difference in the means identified by Tukey post hoc multiple range test ($\alpha = 0.05\%$) with Statistica® software ver. 6.1.

3. RESULTS

3.1. Physicochemical parameters during the fermentation process

The treatment with *P. polymyxa* (Pp) showed a faster increase of temperature in 24 h compared to the control. However, the maximum temperature was 55 °C on day 4 while the control reached a maximum of 62 °C on day 5. The pH was gradually alkalized in both treatments, but the inoculated substrate presented a higher pH than the control. The moisture content was maintained at between 76-83% (**Table 1**).

3.2. Development of microorganisms

The fermentation progressively increased the amount of microorganisms; however, the heat treatment promoted the development of thermophilic groups (**Figures 1** and **2**) and decreased the mesophilic groups (**Figures 3** and **4**). The inoculated substrate showed significant differences in the thermophilic *Bacillus* after heat treatment on days 1 and 5 (**Figure 2**), while no differences were observed in the control substrate (**Figure 1**).

The development of microorganisms was different in the control compared to the inoculated substrate. The highest amount of the thermophilic *Pseudomonas*

Table 1. Average of changes in pH, moisture content and maximum temperature in barley straw during fermentation with *Paenibacillus polymyxa* (Pp) and in a control — *Taux de variation du pH, de la teneur en humidité et de la température maximale de la paille d'orge pendant la fermentation avec* Paenibacillus polymyxa *(Pp) et dans un témoin*.

Day of fermentation	pH		% Moisture		Temperature	
	Pp	Control	Pp	Control	Pp	Control
D0	8.08	7.82	76.50	77.17	27.5	25.0
D1	9.65	8.96	77.23	81.83	54.0	40.0
D2	9.85	9.76	81.00	82.17	55.0	52.0
D3	9.68	9.52	81.33	83.33	54.0	57.0
D4	9.72	9.38	82.50	83.67	55.0	60.0
D5	9.92	9.38	81.67	83.67	53.0	62.0

pH and moisture were processed in triplicate; temperature was recorded from three points into the mound — *Les mesures de pH et d'humidité ont fait l'objet de trois répétitions ; la température a été enregistrée à partir de trois points dans l'échantillon*.

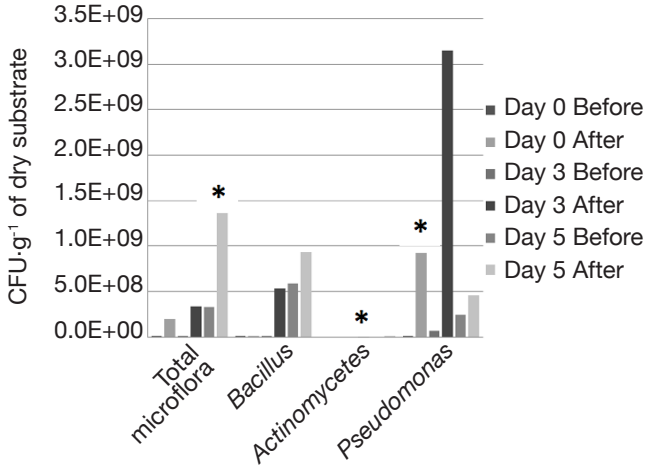

Figure 1. Groups of thermophilic microorganisms in the control substrate at three times during the fermentation process, before and after the heat treatment — *Groupes de micro-organismes thermophiles dans le substrat témoin à trois moments durant le processus de fermentation, et avant et après le traitement thermique*.

*: statistical differences within the group of microorganisms — *différences statistiques dans le groupe de micro-organismes*.

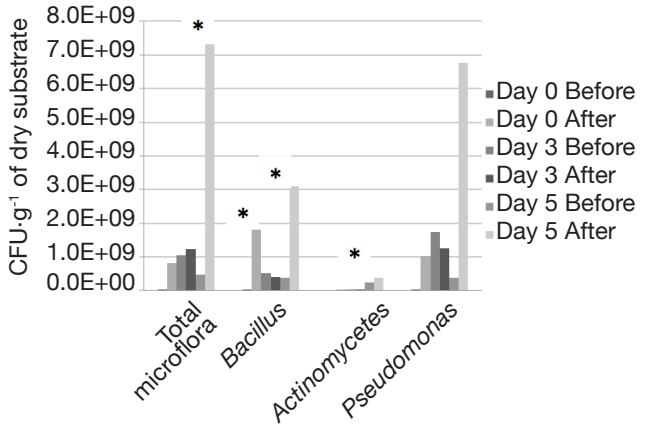

Figure 2. Groups of thermophilic microorganisms in the substrate inoculated with *Paenobacillus polymyxa* at three times of fermentation process, before and after the heat treatment — *Groupes de micro-organismes thermophiles inoculés avec* Paenobacillus polymyxa *à trois moments durant le processus de fermentation, et avant et après le traitement thermique*.

*: see **figure 1** — voir ***figure 1***.

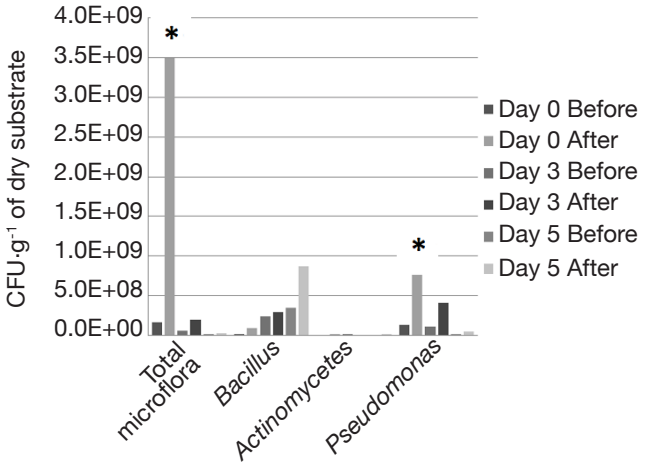

Figure 3. Groups of mesophilic microorganisms in the control sustrate at three times of fermentation process, before and after the heat treatment — *Groupes de micro-organismes mésophiles dans le substrat témoin à trois moments durant le processus de fermentation, et avant et après le traitement thermique*.

*: see **figure 1** — voir ***figure 1***.

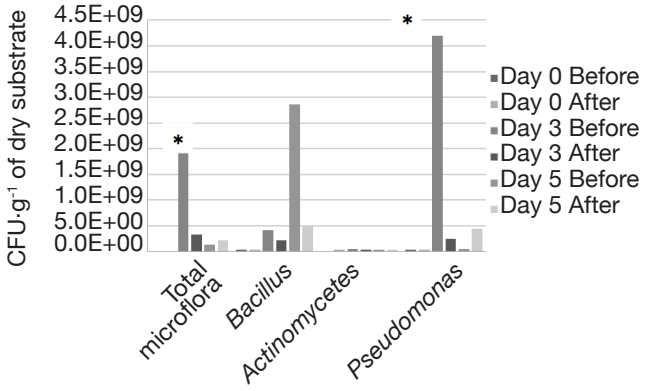

Figure 4. Groups of mesophilic microorganisms in the substrate inoculated with *Paenobacillus polymyxa* at three times of fermentation process, before and after the heat treatment — *Groupes de micro-organismes mésophiles dans le substrat inoculé avec* Paenobacillus polymyxa *à trois moments durant le processus de fermentation, et avant et après le traitement thermique*.

*: see **figure 1** — voir ***figure 1***.

was presented on day 5 in the inoculated substrate, but with no statistical differences (**Figure 2**). The control substrate showed an increase in this group after heat treatment, and significant differences were observed on day 3 (**Figure 1**).

Mesophilic microorganisms also increased during the fermentation process. The inoculated substrate showed a significant increase on day 3 for total microflora, *Actinomycetes* and *Pseudomonas*, compared with the control substrate. However, after

heat treatment, these groups decreased. On the other hand, in the control substrate, the heat treatment led to a significant increase of the total microflora and *Pseudomonas* on day 1 (**Figures 3** and **4**).

Thermophilic microflora increased clearly (**Figures 1** and **2**), with significant differences on day 5 after the heat treatment compared to other days in both the inoculated and the control substrates. Thermophilic *Actinomycetes* increased on day 5 after heat treatment in the inoculated substrate.

3.3. Production of *Pleurotus pulmonarius*

The highest Biological Efficiency (BE) value was 169% in the control (**Table 2**) at 3 days of fermentation (169%), while the lowest was 50% in the treatment with Pp at 5 days of fermentation. Both presented significant differences from the rest of the treatments ($p < 0.05$). The BE of day 0 and 5 of the control substrate was statistically similar to day 3 of the inoculated substrate. The highest Production Rate (PR) was 3.32 in the control substrate at 3 days of fermentation and the lowest was 0.86 in the treatment with Pp at 5 days of fermentation. Days 0 and 3 in the treatment with Pp substrate and days 0 and 5 of the control did not differ statistically.

The shortest time for appearance of primordia (16 days) was in the inoculated substrate on day 0 of fermentation. The days of occurrence of primordia increased in line with the days of fermentation, where the longest time for primordia appearance (28 days) was observed in the treatment inoculated with *P. polymyxa* at 5 days of fermentation (**Table 2**).

3.4. Confrontation of *P. pulmonarius* and *T. viride*

Pleurotus pulmonarius in the presence of *T. viride* showed the highest surface colonization (34.78 cm^2) on the substrate with Pp on day 5, followed by day 3 (29.37 cm^2) and day 0 (20.9 cm^2), with significant differences (**Table 3**). No statistical differences were observed in the other treatments (the control and sterile control substrates). However, the average mycelial extension of *P. pulmonarius* in the presence of *T. viride* on the control substrate was similar to the treatment where *P. pulmonarius* was cultivated alone. The lowest mycelial growth of *P. pulmonarius* in the presence of *T. viride* was recorded in the sterilized control.

Table 2. Biological efficiency, production rate and days to the appearance of primordia of *Pleurotus pulmonarius* in a heat treated substrate at days 0, 3 and 5 of fermentation — *Efficacité biologique, taux de production et jours d'apparition de primordia de* Pleurotus pulmonarius *dans un substrat traité thermiquement après 0, 3 et 5 jours de fermentation*.

Treatment		Biological efficiency	Production rate	Days to the appearance of primordia
Paenibacillus polymyxa	Day0	95.96^{c} (23.0)	2.00^{b} (0.48)	16
Paenibacillus polymyxa	Day3	116.55^{bc} (25.6)	2.16^{b} (0.47)	22
Paenibacillus polymyxa	Day5	50.04^{d} (22.5)	0.86^{c} (0.38)	28
Control	Day0	111.09^{bc} (28.6)	2.22^{b} (0.57)	17
Control	Day3	169.29^{a} (31.5)	3.32^{a} (0.61)	19
Control	Day5	131.89^{b} (19.1)	2.44^{b} (0.35)	23

Different letters in the columns indicate significant statistical differences (Tukey, $\alpha = 0.05$) — *Des lettres différentes dans les colonnes indiquent des différences statistiques significatives (Tukey, $\alpha = 0,05$)*; The standard deviation is given into brackets — *L'écart-type figure entre parenthèses*.

Table 3. Mycelia confrontation assays. Growth of *Pleurotus pulmonarius* on barley straw after six days in the presence of *Trichoderma viride — Tests de confrontation de mycelia. Croissance de* Pleurotus pulmonarius *sur la paille d'orge après six jours en présence de* Trichoderma viride.

Day of fermentation	*Pleurotus pulmonarius*	*Pleurotus pulmonarius* in presence of *Trichoderma viride*		
	Control substrate	Substrate with Pp	Control substrate	Sterile control substrate
Day0	25.5*^{a} (2.0)	20.90^{c} (3.88)	23.57^{a} (5.57)	19.87^{a} (10.40)
Day3	28.7^{a} (3.0)	29.37^{b} (4.69)	25.00^{a} (6.64)	21.77^{a} (7.13)
Day5	27.5^{a} (3.9)	34.78^{a} (3.60)	27.73^{a} (6.44)	19.03^{a} (6.57)

*Surface colonized by the mycelium in cm^2 — *surface colonisée par le mycelium en cm^2*; Means of 10 replicates (standard deviation) — *moyenne de 10 répétitions (écart-type)*; The sterile treatment was autoclaved (1 h at 121 °C) — *Le traitement stérile a été autoclavé (1 h à 121 °C)*; Pp: *Paenobacillus polymyxa*; Different letters in the columns indicate significant statistical differences (Tukey, $\alpha = 0.05$) — *Des lettres différentes dans les colonnes indiquent des différences statistiques significatives (Tukey, $\alpha = 0,05$)*.

Mycelial growth of *T. viride* showed greater inhibition in the substrate with Pp and control than in the sterile substrates (**Table 4**). Specifically, days 3 and 5 of fermentation presented the highest inhibition in both substrates, which differed statistically from that of day 0 of fermentation and the sterile substrate.

4. DISCUSSION

In this study, we obtained a substrate that confers competiveness to *P. pulmonarius* in the presence of *T. viride*. The inoculation of *P. polymyxa* allowed the development of a microbial community of thermophilic *Pseudomonas* and *Actinomycetes* (**Figure 2**). This is an unexpected effect that serves to improve the competitiveness of *P. pulmonarius* against of *T. viride*. Mycelial growth of *Pleurotus* was higher on day 5 of fermentation in the substrate with Pp compared to that of days 0 and 3. On day 5, we observed the highest quantity of thermophilic microorganisms, including total microflora and *Pseudomonas*. A similar result has been reported previously by Kim et al. (2008), in which a strain of *Pseudomonas* significantly increased the mycelial growth of *Pleurotus eryngii*. Kang & Cho. (2014) subsequently revealed that auxin produced by *Pseudomonas* was the main compound stimulating the growth of the mycelia. In the present study, we observed a high quantity of *Pseudomonas* on day 5; this could have stimulated the mycelial growth compared to days 0 and 3, but slowed the formation of primordia. The quantity of *Pseudomonas* is an important factor for faster mycelial development in commercial production, but has not shown any benefit in terms of primordia formation.

It should be noted that fermentation of the barley straw before the heat treatment limited colonization by *T. viride* in treatments both with and without inoculation of *P. polymyxa*. The relationship between the decrease in mycelial growth and the presence of any group of bacteria was not determined. However, several studies have reported the benefits of bacterial community in terms of limiting the growth of *Trichoderma* compared to a sterile substrate (Stolzer & Grabbe, 1991; Muez-Orobia & Pardo-Nuñez, 2001; Velázquez-Cedeño et al., 2004; Velázquez-Cedeño et al., 2006a; Velázquez-Cedeño et al., 2006b; Colavolpe et al., 2013). The importance of microorganisms (including *P. polymyxa*) in selectivity mechanisms has been demonstrated *in vitro*, showing a strong inhibition against *Trichoderma* (Velázquez-Cedeño et al., 2004; Gbolagade, 2006; Velázquez-Cedeño et al., 2008).

Another explanation could be the accumulation of secondary metabolites, such as iturin, produced by *Bacillus subtilis*. This bacterium showed strong antimycotic activity (Ohno et al., 1993; Ohno et al., 1996). This genus and other thermophilic microorganisms obtained by the heat treatment (12 h at 58 °C) and conditioning (24 h at 48 °C), favored inhibition of the growth of *T. viride*.

Interestingly, on day 5 of fermentation, the pH was at its most alkaline, at 9.9. This value is higher than optimal pH reported for mycelial growth of *Pleurotus*, which is from 6.5 to 7.0 (Kalmis et al., 2008), and Yadav & Chandra (2014) determined that a pH of between 7-8 increases mycelial development in cultivated *Pleurotus* species. Moreover, Téllez-Téllez et al. (2008) showed that substrate fermentation helps to regulate an optimum pH for the development of *Pleurotus*. However, under certain conditions (high number of microorganisms), the pH probably negatively affects the yield of *Pleurotus* but not the mycelial growth.

The yield of *P. pulmonarius* showed no increase in the inoculated substrate relative to the control. This is probably because *P. polymyxa* negatively affected the yield of *Pleurotus*. A similar report by Velázquez-Cedeño et al. (2008) showed no significant difference in the yield of *P. ostreatus* following inoculation of *P. polymyxa* and *Actinomycetes* in the substrate.

Table 4. Mycelia confrontation assays. Growth of *Trichoderma viride* on barley straw after four days in the presence of *Pleurotus pulmonarius — Tests de confrontation de mycelia. Croissance de* Trichoderma viride *sur la paille d'orge après quatre jours en présence de* Pleurotus pulmonarius.

Day of fermentation	Substrate with Pp	Control substrate	Sterile control substrate
Day0	2.57^{*Aa} (1.13)	2.58^{Aa} (1.25)	14.19^{Ab} (7.43)
Day3	0.98^{Ba} (0.88)	1.09^{Ba} (0.69)	6.19^{Bb} (1.36)
Day5	0.59^{Ba} (0.15)	0.55^{Ba} (0.14)	18.28^{Ab} (8.78)

*Surface colonized by the mycelium in cm^2 — *surface colonisée par le mycelium en cm^2*; Means of 10 replicates (standard deviation) — *moyenne de 10 répétitions (écart-type)*; The sterile treatment was autoclaved (1 h at 121 °C) — *Le traitement stérile a été autoclavé (1 h à 121 °C)*; Pp: *Paenobacillus polymyxa*; Different capital letters in the columns indicate significant statistical differences (Tukey, $\alpha = 0.05$). Different small letters in the raw indicate significant statistical differences (Tukey, $\alpha = 0.05$) — *Des majuscules différentes dans les colonnes indiquent des différences statistiques significatives (Tukey, $\alpha = 0,05$). Des minuscules différentes dans la ligne indiquent des différences statistiques significatives (Tukey, $\alpha = 0,05$).*

Beyond the use of *P. polymyxa*, the fermentation and heat treatment significantly promote the development of thermophilic microorganisms. Both of these substrates presented the best BE of *P. pulmonarius* on day 3 of fermentation, with a subsequent decrease by day 5. This means that the fermentation and proposed heat treatment offer an advantage for cultivation of *Pleurotus* in short fermentation processes. This agrees with the findings of Castañeda-de-Leon & Leal-Lara (2007), in which a short fermentation (3 days) for the substrate is recommended, followed by heat treatment to obtain a high performance in *Pleurotus*. The fermentation and heat treatment conditions used in this study represent an alternative to obtain a selective substrate for *Pleurotus* cultivation.

5. CONCLUSIONS

In conclusion, the addition of *P. polymyxa* modified the natural microbiological secession in a five-day fermentation with barley straw. It favored the competitiveness of *Pleurotus pulmonarius* against *Trichoderma viride* on day 5 compared to a control substrate, although it did not favor the yield. The fermentation and heat treatment are therefore important components in the production of a selective substrate for *Pleurotus*.

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