Identification and Characterization of the Antibacterial Activity of Fungal Metabolites on Contaminants Responsible for Foodborne Diseases

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Abstract

The emergence of new diseases due to recurrent food poisoning nowadays in the face of excessive use of conventional antibiotics leads to the search for new bioactive molecules. Thus, the objective of this work was to investigate the antibacterial activity of fungal metabolites on contaminants responsible for food poisoning. To do this, Eight (08) fungal strains belonging to the genera *Aspergillus*, *Penicillium* and *Nigrospora* were used and antibacterial tests were performed on 4 bacterial strains (*Staphylococcus aureus* ATCC 25923, *Shigella sp*, *Escherichia coli* sp and *Salmonella* sp) using the agar cylinder method (antibiotic). The results showed that seven (07) fungal isolates have high antibacterial activity with inhibition diameters ranging from 18 to 29 mm on *Staphylococcus Aureus* ATCC 25923; *Shigella sp*; *Salmonella* sp and *Escherichia coli*. Synergistic tests have shown that the combination of 4 to 5 fungal strains could increase bacterial inhibition of *Staphylococcus, Salmonella, Shigella* and *Escherichia coli* which appear resistant to the action of a single fungal strain.

Keywords: Aspergillus; Bacterial resistance; Nigrospora; Penicillium; Synergistic effect.
1. Introduction

Foodborne diseases affect nearly one in ten people worldwide, and more than 420,000 die from them [1]. These foodborne diseases are most often diarrheal diseases caused by bacteria, the most common of which in Benin are *Staphylococci*, *Shigella* sp, *Escherichia coli*, and *Salmonella* sp. At the moment, it has to be said that bacteria are becoming more and more resistant due to the abusive use of existing antibiotics and climate change. Antimicrobial resistance is responsible for 700,000 deaths annually worldwide with a high incidence in developing countries [2]. Faced with this ever-increasing increase, the need for new and effective antibacterial treatments is becoming more and more urgent [3]. Thus, researchers have embarked on the search for effective control methods by focusing on plants, algae bacteria and sometimes rarely molds. As for filamentous fungi, they are important players in the microbial world. They are involved in multitude of biological processes in the environment with the ability to synthesize a large number of very important complex substances (organic acids, alkaloids, antibiotics, terpenes and enzymes [4-5]. Given this high potential of moulds and the historical evidence of their use in antibacterial control, would it not be interesting to study the ability of certain species of the genera *Aspergillus*, *Penicillium* and *Nigrospora*, found in foods, to control gastroenteritis bacteria that develop increased antibiotic resistance?

2. Materials and methods

2.1. Microbial strains

To assess antimicrobial activity, the following fungal strains were used: *Nigrospora oryzae*, *Aspergillus ustus*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *aspergillus flavus* mutant, *Penicillium fellutanum*, *Penicillium spinulosum*, *Penicillium expansum*. These strains come from the strains Bank (CMBB) of the biochemistry and molecular biology laboratory of the Faculty of Technical Sciences of the University of Abomey-Calavi. As for the bacterial strains, *Escherichia coli*, *Shigella* sp, *Salmonella* sp, these are clinical strains derived from the stools of people suffering from diarrhoeal diseases that have been analysed at the Centre National Hospitalier et Universitaire (CNHU) in Cotonou and the reference strain *Staphylococcus aureus* ATCC 25923.

2.2. Antibacterial activity

The agar cylinder technique described by [6] was used to evaluate the action of fungal metabolites on the bacterial strains tested. For the test, 1 mL of bacterial inoculum is placed on each agar box. After a 5-minute impregnation, the excess inoculum is removed by suction. Agar cylinders of each fungal strain with a diameter of 7 mm are cut as close as possible to the center of the colony. Each cylinder is placed on the surface of the boxes pre-seeded with bacteria. The latter are dropped at 4°C in a refrigerator for 2 hours to allow a pre-diffusion of bioactive substances, then placed in an oven at 37°C for 24 to 72 hours [6]. Inhibition diameters are measured after 24 hours and after one week. Chloramphenicol (1 mg) was used as control.

2.3. Synergy tests

The test of the antibacterial activity of the extracts obtained consists in seeking their synergistic effects on the development of bacterial species. To do this, a sterile swab dipped in the bacterial suspension is used to
uniformly seed the entire surface of the Mueller - Hinton agar box. After drying the surface, 7 mm diameter fungal culture cylinders are placed on the surface of the boxes stocked with bacteria. The latter are deposited at 4°C in a refrigerator for 2 hours to allow a pre-diffusion of bioactive substances, then placed in an oven at 37°C for 24 to 72 hours [6].

3. Results and discussion

3.1. Antibacterial activity of each fungi

The antibiotic test performed on the bacteria showed that the eight (08) fungal strains developed antibacterial activity on at least one of the bacteria tested. Indeed, an inhibition zone with a diameter between 13.5 mm and 29 mm around the deposited agar cylinders was measured. The results of these tests are presented in Table 1 below. In this study we tested the antibacterial activity of a total of 08 strains belonging to the genera Aspergillus, Penicillium, and Nigrospora on four bacteria (Escherichia coli sp, Staphylococcus aureus ATCC 25923, Salmonella sp and Shigella sp). Inhibition of bacterial growth by mold cultures demonstrates the development and excretion by fungal isolates of antimicrobial substances that diffuse into the agar medium [7]. In Staphylococcus aureus ATCC 25923, the inhibition diameters of the strains are between 16.5 mm and 29 mm (figure 1). These diameters are all larger than that of chloramphenicol, which is 20 mm. The most active strain is Penicillium spinulosum (figure 5). In Salmonella sp and Shigella sp, the inhibition diameters of secondary metabolites secreted by fungal strains are between 13.5 mm and 22.5 mm. Figures 3 and 4 shown the effect of différents fungi tested. The most active fungal strain is Aspergillus ustus with a diameter of 22.5 mm larger than chloramphenicol which is 18 mm. In Escherichia coli, the inhibition diameters obtained are between 13.5 and 23.5. The most active strain is Penicillium expansum (figure 1; figure 2). According to LCSI (8), the inhibition diameter of chloramphenicol on strains of Staphylococcus Aureus ATTC 25923, Salmonella sp, shigella sp, Escherichia coli is 20 mm, 18 mm, 18mm and 17 mm respectively. Fungal strains with inhibition diameters greater than or equal to that of chloramphenicol are Nigrospora oryzae, Penicillium fellutanum, Penicillium spinulosum, Penicillium expansum, Aspergillus ustus, Aspergillus fumigatus, and Aspergillus flavus mutant. The most active fungal strains on the four bacteria tested belong to the genus Aspergillus and the genus Penicillium, namely Aspergillus ustus and Penicillium expansum, which have synthesized more secondary metabolites that can inhibit gram positive and gram negative bacteria. These results are in line with those of Mansour and his colleagues [9], which also showed that the genus Aspergillus has a significant antibacterial activity of up to 33 mm against Staphylococcus aureus ATCC 25923. Amadi and his colleagues [10] have demonstrated that the genus Aspergillus has antibacterial activity that would result in the secretion of mycotoxins, alkaloids and fumiclavins A and B. Nayak and Anitha [11] showed antibacterial activity of Aspergillus ustus against Escherichia coli and demonstrated that the inhibitory power of the bacterium is due to the production of silver nanoparticles (AgNPs). These results are also consistent with those of Gimenez and his colleagues [12] which go further and suggest that the antibacterial activity of Aspergillus sp and Penicillium sp on gram-negative bacteria is due to the synthesis of polyketides that inhibit cell division such as berkeleydione produced by Penicillium sp. Staphylococcus aureus ATCC 25923 was found after testing to be the most sensitive due to the large inhibition diameters obtained. These results can be explained by the fact that these two groups of bacteria differ morphologically, because Gram-negative bacteria have an outer membrane that is a polysaccharide membrane carrying lipopolysaccharide structural components, this makes the cell wall impermeable to
lipophilic compounds, unlike Gram-positive bacteria, which will be more sensitive because they only have an outer peptidoglycan layer that is not an effective permeability barrier [13].

3.2. Synergy test

The synergy test is a test that evaluates the extent of antibacterial activity of the different secondary metabolites secreted by combining two or more fungal strains. A first test realized with $10^6$ UFC of bacteria suspension showed a strong synergy of action between all fungal species tested which was expressed by a complete absence of bacterial development. To highlight the synergies between the compounds of the different moulds, the test was repeated with a higher bacterial load of $2.5 \times 10^6$.

The results are as follows:

- On *Staphylococcus aureus*:

There is a partial synergy between the strains *Penicillium expansum* and *Penicillium spinaulosum* on the one hand and between *Penicillium expansum* and *Aspergillus nidulans* on the other hand. This results in the appearance of an inhibition zone between pairs of fungal strains. The synergistic action between *Penicillium expansum* and *Aspergillus nidulans* may be due to an aggressive effect from the *Penicillium expansum* strain that would have activated the *Aspergillus nidulans* strain which was previously very inactive. The synergistic action between *Penicillium expansum* and *Aspergillus nidulans* may be due to an aggressive effect from the *Penicillium expansum* strain that would have activated the *Aspergillus nidulans* strain which was previously very inactive.

- On *Salmonella* sp:

There is a synergy of action between the metabolites secreted by *Nigrospora oryzae* and *Penicillium fellutanum*, which allowed the bacterial inhibition observed around the agar cylinder deposition pairs. A synergistic effect is also observed between *Nigrospora oryzae* and *Aspergillus ustus*, between *Nigrospora oryzae* and *Penicillium expansum* ; between *Penicillium expansum* and *Aspergillus nidulans* and finally between *Penicillium expansum* and *Aspergillus flavus* mutant.

- On *Escherichia coli*

The synergistic activity of the metabolites secreted by the *Nigrospora oryzae* and *Aspergillus ustus* strains inhibited bacteria around the cylinders and significantly increased inhibition diameters.

There is also a synergy between *Nigrospora oryzae* and *Penicillium expansum* and between *Aspergillus ustus* and *Penicillium expansum*. These different synergistic actions can be due either to molecular interactions (affinities) between the different secondary metabolites of the collaborative strains.

3.3. General discussion
In this study, the antibacterial activity of 08 strains belonging to the genera *Aspergillus*, *Penicillium*, and *Nigrospora* was tested on four bacteria (*Escherichia coli* sp, *Staphylococcus aureus*, *Salmonella* sp and *shigella* sp). Inhibition of bacterial growth by mould cultures demonstrates the development and excretion by fungal isolates of antibacterial substances that diffuse into the agar medium [7]. Thus the 08 fungal isolates showed antibacterial activity on at least one of the test bacteria. From these observations it can be deduced that the different fungal metabolites secreted by the moulds have a growth inhibitory effect on *Staphylococcus Aureus* with inhibition diameters larger than that of chloramphenicol (8) which is the control. *Staphylococcus aureus* ATCC 25923 is a clinical laboratory bacterial strain responsible for diarrhoeal infections, sensitive to antibacterial agents and lacking the β-lactamases enzymes causing the destruction of β-lactamines produced by moulds; hence the large diameters observed after the antibacterial test (Michelle Callegan). The results of this work are in line with those of Mansour and his colleagues [9], which showed that the genus *Aspergillus* has a significant antibacterial activity of up to 33 mm against *Staphylococcus aureus*. Amadi and his colleagues [10] have demonstrated that the genus *Aspergillus* sp has antibacterial activity that would result in the secretion of mycotoxins produced by endophyte molds responsible for the production of alkaloids, fumiclain A and B that can inhibit bacterial growth. Strains of the genera *Penicillium fellutanum* (*Penicillium fellutanum*, *Penicillium spinulosum*, and *Penicillium expansum*) and *Aspergillus* (*Aspergillus usus*, *Aspergillus fumigatus*, *Aspergillus flavus* mutant) were found to be the most active except for *Aspergillus nidulans*. Our results also agree with those of Abdelaziz [14], whose results showed that the fungal strains *Aspergillus* sp and *Penicillium* sp had a strong inhibitory effect on two Gram positive (+) bacteria, including *Staphylococcus aureus* with a diameter ranging from 12 mm to 30 mm. Tanaka and his colleagues [15] also demonstrated the inhibitory activity of *Nigrospora* on Gram-positive bacteria in general and highlighted some secondary metabolites of the species such as Nigrosporins A and B, which supports our results regarding its antibacterial activity. The *Penicillium spinulosum* strain is the mould with the highest antibacterial activity with an inhibition diameter of 29 mm on *Staphylococcus aureus*. For Gram-negative bacteria, fungal strains of *Nigrospora oryzae*, *Penicillium fellutanum*, *Penicillium spinulosum*, *Penicillium expansum*, *Aspergillus usus*, *Aspergillus fumigatus*, and *Aspergillus flavus* mutant had inhibition diameters on the *Salmonella* sp strain greater than or equal to that of chloramphenicol (Table 1). The *Aspergillus* nidulans strain has a smaller inhibition diameter than chloramphenicol. The most active strains belong to the genera *Aspergillus* (*species: usus, flavus, fumigatus*) and *Penicillium* (*species: spinulosum, fellutanum, expansum*) which would therefore secrete bioactive substances with high inhibitory potential. The salmonella strain from the stools of patients with diarrhoeal diseases had been found to be resistant to penicillin G, gentamicin, cotrimoxazole, erythromycin, oxacillin. Fungal metabolites that have inhibited salmonella have a stronger antibacterial potential than the antibiotics to which they are resistant. On *shigella* sp, fungal strains with inhibition diameters greater than or equal to chloramphenicol are *Penicillium spinulosum*, *Penicillium expansum*, *Aspergillus usus*, *Aspergillus fumigatus*, *Aspergillus flavus* mutant. On the other hand, *Nigrospora oryzae*, *Penicillium fellutanum* and *Aspergillus nidulans* have an inhibition diameter smaller than the chloramphenicol diameter. The most active strains of *Shigella* sp. are *Aspergillus usus* and *Penicillium expansum* (Table 1). According to Frisvald and his colleagues [16], *Penicillium expansum* secreted metabolites such as roquefortin, rubratoxin, thomitrems A and E, patulin and others, which together would have had a more pronounced inhibitory effect on penicillin G, gentamicin, cotrimoxazole, erythromycin, oxacillin, spritinamycin and spiramycin-resistant *Shigella* sp. *Aspergillus usus*
was synthesized according to Houbraken and his colleagues and Raistrick and his colleagues [17-18], the bioactive molecules with antibacterial properties include ustic acid, autocystin, austalide, and sterigmatocystin. The metabolites secreted by *Penicillium expansum*, *Penicillium fellutanum*, *Aspergillus ustus*, *Aspergillus fumigatus*, *Aspergillus flavus* mutant, *Nigrospora oryzae*, *Penicillium spinulosum* respectively inhibited *Escherichia coli* with inhibition diameters larger than chloramphenicol. The strain of *Aspergillus nidulans* was less active. Rathod and his colleagues [19] obtained similar results with regard to the antibacterial activity of *Nigrospora Oryzae* on *Escherichia coli* sp and added that this activity would be due in particular to the secondary metabolite griseofulvin produced by *Nigrospora*. Nayak and Anitha [11] showed an inhibitory activity of *Aspergillus ustus* against *Escherichia coli* and demonstrated that the inhibitory power of the bacterium is due to the production of silver nanoparticles (AgNPs). Thennarasu and his colleagues [20] demonstrated the antibacterial activity of *Penicillium spinulosum* metabolites on *Escherichia coli* with a diameter of 13mm, which further supports us since this work found a 19.5mm larger diameter. These results are consistent with those of Gimenez and his colleagues [12] who go further and hypothesize that the antibacterial activity of *Aspergillus* sp and *Penicillium* sp on gram-negative bacteria is due to the synthesis of polyketides that inhibit cell division such as berkeleydione produced by *Penicillium* sp. Aouarib and Lemsara [21] found similar results for the antibacterial activity of *Aspergillus* on *Escherichia coli* with diameters ranging from 9.6 to 25 mm. *Aspergillus ustus* and *Penicillium spinulosum* appear to be the most active strains on the four bacteria tested. *Staphylococcus aureus* ATCC 25923 was found after testing to be the most sensitive due to the large inhibition diameters obtained. These results can be explained by the fact that these two groups of bacteria differ morphologically. Metabolites of the genera *Penicillium* sp, *Aspergillus* and *Nigrospora* sp prove their inhibitory power in Gram-positive and gram-negative bacteria with variable inhibition diameters. These different fungal strains would therefore have secreted antibacterial substances (antibiotics) into the agar. *Aspergillus ustus* and *Penicillium spinulosum* appear to be the most active strains on the four bacteria tested (Figure 1). These two fungi would have synthesized a large number of bioactive metabolites belonging to either β-lactamins, cephalosporins, aminosides, phenicols, tetracyclines, macrolides, quinolones and polypeptides; this could potentially be the basis for the inhibitory effect observed on bacteria.

4. Conclusion

Food-borne diseases are the cause of many illnesses and deaths in developing countries. But poor management of antibiotic treatments and the rapid evolution of microorganisms have led to the rapid evolution of many multi-drug resistant strains. The collective and individual consequences of bacterial resistance are serious as it is an increasing cause of treatment failure [22]. This work opens up possibilities for the development of new broad-spectrum anti-bacterials from moulds. Testing for the antibacterial activity of species *Penicillium expansum*, *Penicillium fellutanum*, *Aspergillus ustus*, *Aspergillus fumigatus*, *Aspergillus flavus* mutant, *Nigrospora oryzae*, *Penicillium spinulosum* and *Aspergillus nidulans* has revealed that they have variable inhibitory activity against the development of the bacteria responsible for the gastroenteritises (*Staphylococcus aureus* ATCC 25923, *Salmonella* sp, *Shigella* sp and *Escherichia coli*). It would be of interest to isolate and characterize the bioactive molecules from the secondary metabolites of these fungi to reduce the risk of toxification.
### Table 1: Inhibition diameter of fungal strains on different bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Diameters</th>
<th>Staphylococcus aureus ATCC 25923</th>
<th>Salmonella sp</th>
<th>Shigella sp</th>
<th>Escherichia coli</th>
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</thead>
<tbody>
<tr>
<td>Fungal strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NiO)Nigrospora Oryzae</td>
<td>25±00</td>
<td>18±00</td>
<td>17.5±0.70</td>
<td>21.5±2.12</td>
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<tr>
<td>(P1) Penicillium fellutanum</td>
<td>28.5±2.12</td>
<td>19.5±0.70</td>
<td>17.5±0.70</td>
<td>22.5±3.53</td>
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</tr>
<tr>
<td>(P2) Penicillium spinulosum</td>
<td>29±4.24</td>
<td>21.5±2.12</td>
<td>19±1.41</td>
<td>19.5±2.12</td>
<td></td>
</tr>
<tr>
<td>(P3) Penicillium expansum</td>
<td>26±5.65</td>
<td>19±2.82</td>
<td>20.5±4.94</td>
<td>23.5±3.53</td>
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<tr>
<td>(A1) Aspergillus ustus</td>
<td>25.5±2.12</td>
<td>22.5±0.70</td>
<td>22.5±2.12</td>
<td>22±1.41</td>
<td></td>
</tr>
<tr>
<td>(A2) Aspergillus fumigatus</td>
<td>28±4.24</td>
<td>20.5±0.70</td>
<td>18±00</td>
<td>21.5±0.70</td>
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<tr>
<td>(A3) Aspergillus nidulans</td>
<td>16.5±2.12</td>
<td>13.5±2.12</td>
<td>13.5±2.12</td>
<td>13.5±2.12</td>
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<tr>
<td>(A4) Aspergillus flavus Mutant</td>
<td>25±00</td>
<td>22.5±3.53</td>
<td>18±00</td>
<td>20.5±0.70</td>
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<tr>
<td>Chloramphenicol (50µL)</td>
<td>20±00</td>
<td>18±00</td>
<td>18±00</td>
<td>17±00</td>
<td></td>
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</tbody>
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**References**


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Figures legends

Figure 1.

Metabolites of fungi of genre *Aspergillus*, *Penicillium* and *Nigrospora* have been tested on *Staphylococcus aureus*, *Salmonella sp*, *Shigella sp* and *E. coli* and effects compared to chloramphenicol.

Figure 2.

*Aspergillus fumigatus* (A2); *Aspergillus ustus* (A1); *Aspergillus nidulans* (A3); *Aspergillus flavus* mutant (A4); *Nigrospora oryzae* (NiO); *Penicillium expansum* (P3); *Penicillium spinulosum* (P2); *Pénicillium fellutanum* (P1)

Figure 3.

*Aspergillus fumigatus* (A2); *Aspergillus ustus* (A1); *Aspergillus nidulans* (A3); *Aspergillus flavus* mutant (A4); *Nigrospora oryzae* (NiO); *Penicillium expansum* (P3); *Penicillium spinulosum* (P2); *Pénicillium fellutanum* (P1)

Figure 4.

*Aspergillus ustus* (A1), *Aspergillus fumigatus* (A2); *Aspergillus nidulans* (A3); *Aspergillus flavus* mutant (A4); *Nigrospora oryzae* (NiO); *Penicillium expansum* (P3); *Penicillium spinulosum* (P2); *Pénicillium fellutanum* (P1)

Figure 5.

*Aspergillus fumigatus* (A2); *Aspergillus ustus* (A1); *Aspergillus nidulans* (A3); (A4) *Aspergillus flavus* mutant; *Nigrospora oryzae* (NiO); *Penicillium expansum* (P3); *Penicillium spinulosum* (P2); *Pénicillium fellutanum* (P1)
Figures

Figure 1: Effect of fungal strains on différents bacteria
Figure 2: Effects of fungi on Escherichia coli
Aspergillus fumigatus (A2); Aspergillus ustus (A1); Aspergillus nidulans (A3); Aspergillus flavus mutant (A4); Nigrospora oryzae (NiO); Penicillium expansum (P3); Penicillium spinulosum (P2); Pénicillium fellutanum (P1)
Figure 3: Effect of fungi on Shigella sp.
Aspergillus fumigatus (A2); Aspergillus ustus (A1); Aspergillus nidulans (A3); Aspergillus flavus mutant (A4); Nigrospora oryzae (NiO); Penicillium expansum (P3); Penicillium spinulosum (P2); Pénicillium fellutanum (P1)
**Figure 4:** Effect of fungi on Salmonella sp (cylinder test)

Aspergillus ustus (A1), Aspergillus fumigatus (A2); Aspergillus nidulans (A3); Aspergillus flavus mutant (A4);
Nigrospora oryzae (NiO); Penicillium expansum (P3); Penicillium spinulosum (P2); Pénicillium fellutanum (P1)
Figure 5: Effect of fungi on Staphylococcus aureus ATCC 25923
Aspergillus fumigatus(A2) ; Aspergillus ustus(A1) ; Aspergillus nidulans(A3) ; (A4) Aspergillus flavus mutant ; Nigrospora oryzae(NiO) ; Penicillium expansum(P3) ; Penicillium spinulosum(P2) ; Pénicillium fellutanum(P1)