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*Research Article*

# Antibacterial Activities of *Penicillium roqueforti, Penicillium camemberti* and *Geotrichum candidum* on Multi-Resistant Bacteria

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**ABSTRACT**

Hospital-acquired infections become prevalent and dangerous because they are caused by multiresistant bacteria. In this work, the resistance of some nosocomial bacteria was tested using an antibiotic resistance test; then, three moulds isolated from Roquefort and Camembert cheeses were evaluated for their antibacterial activity. The results showed that all the bacteria were resistant to several antibiotics. The three moulds isolated from the cheeses were identified as *Penicillium roqueforti*, *Penicillium camemberti* and *Geotrichum candidum*. These species demonstrated good antibacterial activity against the tested bacteria, although *P. roqueforti* performed best. *Enterobacter cloacae* was the most sensitive to the moulds, and Staphylococcus aureus was the only bacterium that was resistant to all the antibiotics and all three moulds. The *in vivo* antibacterial activity of *P. roqueforti* confirmed that this mould was able to treat *Escherichia coli* and *E. cloacae* infections in mice. Similar to the *in vitro* activity, *E. cloacae* demonstrated the highest *in vivo* sensitivity to the mould.

**Keywords:** *nosocomial infections, antibiotic resistance, cheese moulds, isolation, antibacterials*

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## INTRODUCTION

Infections acquired in hospitals are very frequent and difficult to treat. These pathologies are mainly caused by bacteria that developed resistances to many drugs (Peleg and Hooper, 2010). Gram negative bacteria are the most able to acquire resistances, in particular Enterobacteriaceae and Staphylococcus aureus. In fact, several investigations declared that the hospital-acquired infections due to Enterobacteriaceae were the most abundant and those due to *S. aureus* were the most dangerous (Sanchez *et al*., 2002; Kshetry *et al*., 2016). In order to resolve this scourge, the researchers tried to found new antibiotics that could be active on multi-resistant bacteria. But the latest advances showed that other means could be more efficient than antibiotics, like the use of some secondary metabolites or the use of antagonistic microorganisms (Khoramnia *et al*., 2013; Vallone *et al*., 2014).

This study main interest was the use of some moulds that are benign to humans in order to inhibit the growth of nosocomial bacteria. Based on the fact that biological control had a great used, particularly in agriculture, its use in the medical field became more evident (Agrawal *et al*., 2016; Wang *et al*., 2016). An ordinary antibiotic resistance test was conducted on the selected bacteria to confirm that they were multi-resistant. The moulds isolated from Roquefort and Camembert cheeses were applied to them by an in vitro test. The most active mould was tested in vivo against the bacteria which showed a good in vitro sensitivity to it. All these experiences were carried out in order to know if the cheese moulds could replace antibiotics for treating nosocomial infections.

## MATERIALS AND METHODS

**Bacteria:** Nosocomial bacteria came from the University Hospital Center of Constantine (Algeria) and the Hospital of Jijel (Algeria). Before the use, the bacterial suspensions were diluted to obtain a concentration of 106 CFU/ml.

**Antibiotic resistance testing:** Antibiotic resistance testing was carried out to amoxicillin, streptomycin, tetracycline,

imipenem and erythromycin. Bacteria were seeded on Muller Hinton Agar surface with a swab, then the antibiotic disks were placed on the agar. After suitable incubation of 18 hours at 37°C, the inhibition diameters were measured (Javadi *et al*., 2020).

**Isolation and identification of cheese moulds:** The used cheeses were Camembert President (made in Normandie, France), Bleu de Brebis Lactis (Roquefort Société, made in Rodez, France), Camembert Tassili (made in Tizi Ouzou, Algeria).

Moulds were taken from the Roquefort cavities and the surface of Camembert and seeded on Malt Extract Agar plates. After an incubation of 48 hours at 25°C, the fungal species were purified by successive seeding on the same agar medium (Botton *et al*., 1990).

The identification was based on the macroscopic and microscopic characterization of the mycelium after an incubation of 7 days on Malt Extract Agar. When the mycelium was hyaline, a drop of fuschine was added on the slide before the microscopic observation to simplify the characterization (Ropars *et al*., 2020).

***In vitro* antibacterial activity of moulds:** Bacteria were seeded on Nutritive Agar plates with swabs. Then disks of 6 mm agar taken from the mycelial fronts of moulds were cut and transferred into the center of Petri dishes. The incubation was done for 72 hours at 25°C. The results were expressed by inhibition diameters around disks (Rajkowska *et al*., 2012). Disks of sterile Nutritive Agar were tested as negative control and disks of Nutritive Agar containing 10 µg of amoxicillin were used as positive control.

***In vivo* antibacterial activity of the most active mould against *Enterobacter cloacae* and *Escherichia coli*:** This activity was tested only for the most active cheese mould. A suspension of this one was prepared with sterile water to obtain 107 CFU/ml. The bacterial suspension had a concentration of 106 CFU/ml.

Eight-week-old C57BL6 male mice (20 ± 2g) were used for this experience. The animals were kept in clean cages and served autoclaved food and water. The infection was caused by 0.5 ml suspension of the species injected intraperitoneally. Then after 24 hours, 0.5 ml of the mould suspension was administered orally at 1 and 3 hours after the bacterial challenge (Neu and Kamimura, 1981).

Faeces were sampled once a day directly by gently pressing the abdomen of the animals. The suspension faces was prepared (1 mg in 10 ml of distilled water) then it was plated on Drigalski agar (Chibani-Chennoufi *et al*., 2004). The incubation was occurred for 24 hours at 37°C. Yellow colonies were obtained for *Escherichia coli* and *Enterobacter cloacae*. Before counting them on Petri dishes, a biochemical identification using the API20E microgallery was used to confirm that the observed colonies were those of *Escherichia coli* or *Enterobacter cloacae*.

Groups containing five mice each were used. Two control groups were tested, the first contained infected and untreated animals, and the second contained animals which were infected and treated with intramuscular administration of 200 mg/kg of antibiotics 3 hours after the infection (imipenem for the infection caused with *Escherichia coli*, and streptomycin for the infection caused with *Enterobacter cloacae*).

**Statistical analysis:** The experiments of the antibiotic resistance testing, the in vitro and *in vivo* antibacterial activity of moulds were repeated twice. The results were expressed as mean value ± standard error of the mean (SEM).

## RESULTS

**Antibiotic resistance testing:** Antibiotic resistance testing showed that all the bacteria were multi-resistant. Specially *S. aureus*. This species was resistante to all the tested antibiotics. *Escherichia coli* and Pseudomonas aeruginosa were sensitive to only one antibiotic each and resistant to all the others. Klebsiella pneumoniae, *Enterobacter cloacae*, Citrobacter koseri and Proteus mirabilis were sensitive to two antibiotics. All the inhibition zones were very small, they were ≤ 10 mm of diameters (Table 1).

**Identification of the cheese moulds:** The mould isolated from "Bleu de Brebis Lactis" cheese had a blue-green mycelium with a white margin. After 7 d of incubation the color became gray-green and the margin disappeared. The microscopic observation of this thallus showed a septate mycelium. It possessed terverticillate penicillia branched in cylindrical metula and slightly elongated phialides. Penicillia were asymmetric and granular. Conidia were produced in dry chains and they emanate from the tips of the phialides (Plate 1A). These entire characteristics indicated that the species was *Penicillium roqueforti* (Ropars *et al*., 2020).

The isolation from "Camembert President" cheese gave a mould with fluffy white mycelium. It had conidophores with biverticillate and triverticillate penicillia. They were asymmetric and irregular (Plate1B). Metula and philaides produced conidia in short chains. This species was identified as Penicillium camemberti (Ropars *et al*., 2020).

**Table 1:**
Results of the antibiotic resistance testing (expressed in mm)

| | *Escherichia coli* | *S. aureus* | *K. pneumoniae* | *Pseudomonas aeruginosa* | *Enterobacter cloacae* | *C. koseri* | *Proteus mirabili* |
|---|---|---|---|---|---|---|---|
| **Amoxicillin (10 µg/disc)** | 0.00 | 0.00 | 10.33 | 10.00 | 0.00 | 0.00 | 0.00 |
| **Streptomycin (10 µg/disc)** | 0.00 | 0.00 | 8.33 | 0.00 | 10.00 | 0.00 | 0.00 |
| **Tetracyclin (30 µg/disc)** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 10.33 | 9.33 |
| **Imipenem (10 µg/disc)** | 9.00 | 0.00 | 0.00 | 0.00 | 0.00 | 9.00 | 7.33 |
| **Erythromycin (15 µg/disc)** | 0.00 | 0.00 | 0.00 | 0.00 | 8.00 | 0.00 | 0.00 |

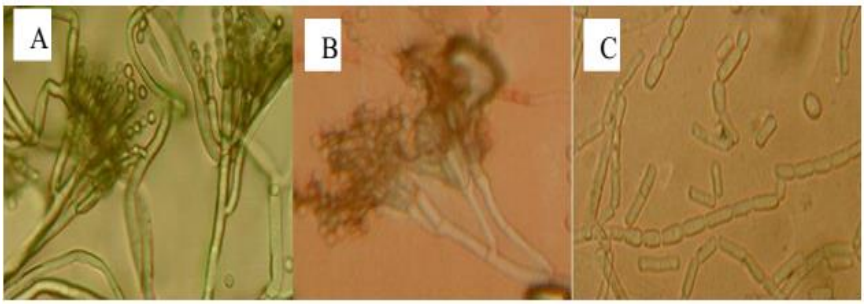


**Plate 1:**
Microscopic observation of the isolated moulds. A) *Penicillium roqueforti*; B) *Penicillium camemberti* with a drop of fuschine; C) *Geotrichum candidum* with a drop of fuschine.

**Table 2:**
Results of the moulds antibacterial activity (expressed in mm)

| | ***Escherichia coli*** | ***S. aureus*** | ***K. pneumoniae*** | ***Pseudomonas aeruginosa*** | ***Enterobacter cloacae*** | ***C. koseri*** | ***Proteus mirabilis*** |
|---|---|---|---|---|---|---|---|
| *Penicillium roqueforti* | 18.33 | 0.00 | 14.66 | 15.33 | 19.33 | 15.66 | 16.33 |
| *Penicillium camemberti* | 0.00 | 0.00 | 14.33 | 15.33 | 15.66 | 12.33 | 13.33 |
| *Geotrichum candidum* | 0.00 | 0.00 | 12.66 | 14.66 | 16.33 | 14.33 | 14.66 |
| Negative control | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Positive control | 0.00 | 0.00 | 10.33 | 10.66 | 0.00 | 0.00 | 0.00 |

The last mould was isolated from "Camembert Tassili" cheese. It had a flattened white mycelium. The aspect on the Petri dishes was smooth and pasty. The microscopic observation indicated that the thallus was septate and fragmented into cylindrical thallospores (Plate 1C).

The observed characteristics were those of Geotrichum candidum (Botton *et al*., 1990)

***In vitro* antibacterial activity of moulds:** The moulds isolated from cheeses had a good antibacterial activity against *Enterobacter cloacae*, Klebsiella pneumoniae, Pseudomonas aeruginosa, C. koseri and Proteus mirabilis. *Penicillium roqueforti* was the most active species against these bacteria. Penicillium camemberti and *G. candidum* gave fairly close inhibition zone diameters, but they were less active than *Penicillium roqueforti* (Table 2).

Among the tested bacteria, *S. aureus* was the most resistant species, followed by *Escherichia coli*. In fact, the first was resistant to the three moulds (Plate 2A), and the second was only sensitive to *Penicillium roqueforti* (Plate 2B). On the other hand, *Enterobacter cloacae* was the most sensitive to the three moulds (Table 2).

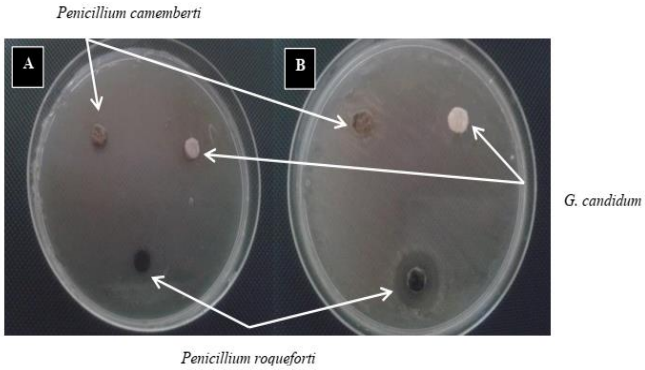


**Plate 2:**
Examples of the moulds antibacterial activity. A) on Staphylococcus aureus; B) on *Escherichia coli*

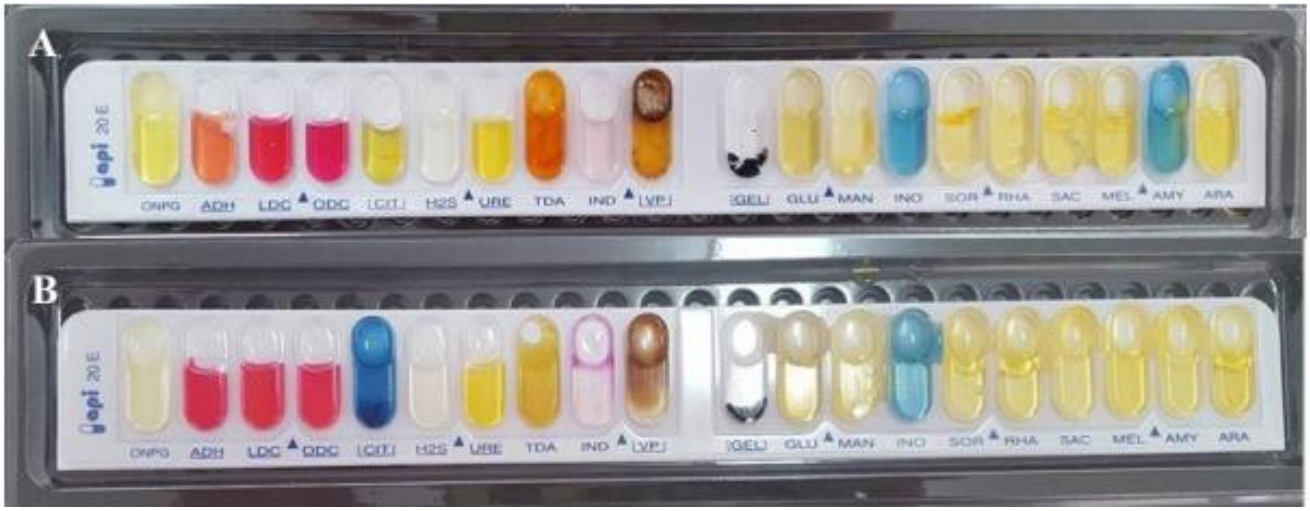


**Plate 3:**
The biochemical identification of *Escherichia coli and Enterobacter cloacae* using the API20E microgallery. A) *Escherichia coli*; B) *Enterobacter cloacae.*

**In *vivo* antibacterial activity of the most active mould against *Enterobacter cloacae* and *Escherichia coli*:** *Enterobacter cloacae* and *Escherichia coli* were chosen because they gave the biggest inhibition zones to *Penicillium roqueforti* in the in vitro activity. The results of the in vivo antibacterial activity showed that *Penicillium roqueforti* treated the infections caused by *Escherichia coli* or *Enterobacter cloacae* at the third and the second day respectively. In fact, for the infection caused by the first species, the faces gave no colonies on the plates three days after the administration of the mould suspension. And the same results were observed two days after the treatment for the infection caused by *Enterobacter cloacae* (Table 3).

The biochemical identification confirmed that the counted colonies were those of *Escherichia coli* and *Enterobacter cloacae* (Plate 3).

**Table 3:**
Results of the *in vivo* antibacterial activity of *Penicillium roqueforti* (expressed in colonies / petri dish)

| | Day 1 | Day 2 | Day 3 |
|---|---|---|---|
| *Escherichia coli* | 25.33 | 13.00 | 0.00 |
| *Enterobacter cloacae* | 18.33 | 0.00 | 0.00 |
| Untreated animals | 50.00 | 60.66 | 72.33 |
| *Animals treated with antibiotics* | 25.00 | 15.33 | 0.00 |

## DISCUSSION

Antibiotic resistance testing indicated that all the used bacteria were multi-resistant. In fact, the majority of antibiotics were inefficient against them. According to some works, the bacteria resistance was due to gene mutations in contact with the antibiotic or to the transmission of resistance genes from one bacterium to another (Davies and Davies, 2010). Kshetry *et al*. (2016) declared that *S. aureus* was able to develop resistance to vancomycin and meticillin only, but our results showed that this species could be resistant to many other antibiotics like amoxicillin, streptomycin, tetracycline, imipenem and erythromycin.

*Escherichia coli* isolated from hospitals and veterinary clinics demonstrated that it was able to develop resistance to β-lactams, sulfonamides, clavulanic acid, tetracycline, spectinomycin, chloramphenicol and gentamicin (Sanchez *et al*., 2002). Our study could add to these antibiotics streptomycin and erythromycin. Some investigations reported that *Klebisella pneumoniae* and *Pseudomonas aeruginosa* could become resistant to β-lactams, cephalosporins, monobactams and carbapenems. Also, the possibility that they could develop resistance to all the antibiotics should not be excluded (Souli *et al*., 2008). This was in accordance with our results.

*Penicillium roqueforti* was isolated from the Roquefort cheese, and Penicillium camemberti and *G. candidum* were obtained from the Camembert chesses. This was explained by the use of these species in the fabrication and the maturing of the two types of cheese (Ismaiel *et al*., 2014; Galli *et al*., 2016; Ropars *et al*., 2020).

The results obtained for the in vitro antibacterial activity of moulds were superior to those obtained by the antibiotic resistance testing. This indicated that the moulds were more active on the multi-resistant bacteria. *Penicillium roqueforti* gave the biggest inhibition zones. It was more active than *Penicillium camemberti* and *G. candidum*. The in vivo antibacterial activity of *Penicillium roqueforti* confirmed that this mould was able to treat *Escherichia coli* and *Enterobacter cloacae* infections on mice.

Some data reported that the antibacterial activity of *Penicillium roqueforti* was due to its production of roquefortine C. This mycotoxin acted on bacteria by inhibiting the activity of cytochromes P450. This species produced also a PR toxin and mycophenolic acids that had a big antibacterial activity in presence of antagonist microorganisms (Vallone *et al*., 2014). On the other hand, our results showed that *Penicillium roqueforti* was inefficient against *S. aureus*. In contradiction with this, some works reported that this species produced fumiquinozoline F which was active on *S. aureus* (Silva *et al*., 2004). The difference between these results could be explained by the used of different strains of the mould. In fact, the produced secondary metabolites varied from one strain to another.

Penicillium camemberti had a good activity against the tested bacteria, especially against Pseudomonas aeruginosa. This could be explicated by its production of acetaldehyde, benzaldehyde, 3-methylbutanal and 1-octen-3-ol (Larsen and Knochel, 1997). These molecules are known for acting on the membrane and cytoplasm of bacteria, and in some cases they entirely changed the morphology of bacterial cells (Nazzaro *et al*., 2013).

*G. candidum* inhibited all the tested bacteria, except *Escherichia coli* and *S. aureus*. Some previous investigations explained the antibacterial activity of this mould by its ability to produce fatty acids containing lauric acid in their structure (Khoramnia *et al*., 2013).

*Enterobacter cloacae* was the most sensitive species to *Penicillium roqueforti*, *P. camemberti* and *G. candidum*. The in vivo antibacterial activity of *Penicillium roqueforti* confirmed this. In fact, *Enterobacter cloacae* was most sensitive to the mould than *Escherichia coli*. This encouraged the use of the cheese moulds for treating infections caused by this bacterium. On the other hand, *S. aureus* was resistant to all the antibiotics and to the three cheese moulds. Several works have already reported the big ability of this species to develop resistances. Many mechanisms have been investigated for the resistance of the bacterium. The most important ones were gene cassettes, the enzymatic inactivation of antimicrobial agents and the decreased of their affinity for sites of action (Lowy, 2003; Pantosti *et al*., 2007). Spontaneous mutation and horizontal transfer of genetic material have also been reported for *S. aureus* (Pantosti *et al*., 2007).

In conclusion, the study revealed that all the nosocomial bacteria were resistant to several antibiotics; they were so considered as multi-resistant. On the other hand, they were sensitive to *Penicillium roqueforti*, Penicillium camemberti and *G. candidum*. The three moulds had a great antibacterial activity, but *Penicillium roqueforti* gave the best values. This indicated that these moulds could be used for treating infections due to the bacteria. The in vivo antibacterial activity

of *Penicillium roqueforti* tested on mice confirmed this assumption because the mould was able to treat infections due to *Escherichia coli* and *Enterobacter cloacae*. The results showed that *Enterobacter cloacae* was the most sensitive species to the moulds and *S. aureus* was the most resistant one. In fact, it was resistant to all the antibiotics and moulds.

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