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Antibiotic Activity of *Wickerhamomyces anomalus* Mycocins on Multidrug-Resistant *Acinetobacter baumannii*

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Abstract

To evaluate the susceptibility of multidrug-resistant *Acinetobacter baumannii* to mycocins produced by *Wickerhamomyces anomalus* and to verify the cytotoxicity of these compounds. Three culture supernatants of *W. anomalus* (WA40, WA45, and WA92), containing mycocins (WA40M1, WA45M2, and WA92M3), were tested on *A. baumannii* using broth microdilution methods, solid medium tests, and cytotoxicity tests in human erythrocytes and in *Artemia saline* Leach. *W. anomalus* was able to produce high antimicrobial mycocins, as even at high dilutions, they inhibited *A. baumannii*. In a solid medium, it was possible to observe the inhibition of *A. baumannii*, caused by the diffusion of mycocins between agar. Finally, the three supernatants were not cytotoxic when tested on human erythrocytes and *Artemia salina*. According to the evidence in this study, the mycocins of *W. anomalus* have been effective and could be used in the development of new antimicrobial substances.

Keywords Mycocins · Killer yeasts · Antibiotics · *Wickerhamomyces anomalus* · *Acinetobacter baumannii* · Multidrug resistance

Introduction

For thousands of years, yeasts have played a key role in the manufacturing of foods such as bread, wine, and beer [1]. Studying them, Bevan and Makover [2] discovered the killer phenomenon, which consists on producing glycoproteins (also called mycocins or killer toxins), with an inhibitory action on other microorganisms. The yeasts are classified as killer (mycocins producers), sensitive (suffer the killer action), and neutral (do not produce or suffer the killer action) [2].

Wickerhamomyces anomalus (formerly known as *Pichia anomala* and *Hansenula anomala*) was the first yeast producing mycocins capable of inhibiting the growth of both eukaryotic and prokaryotic organisms [3]. It is a heterothallic yeast that is widely distributed in nature and can be found in fruits, plants, cereals, vegetables, products rich in sugar, soil, insect gut, water, and marine environment [4, 5].

The mechanisms proposed to justify mycocins action on other microorganisms are inhibition of DNA replication, changing of membrane permeability, inhibition of β-1.3-glucan synthase, and β-1.3-glucan or β-1.6-glucan hydrolysis. Nevertheless, many mechanisms remain unknown and still require further study [6].

Glucanase-like mycocins are the most widely known ones, acting on the hydrolysis of β-1.3-glucan or β-1.6-glucan, which causes the loss of cytoplasmic components and the subsequent death of microorganism. As mammalian cells do not have this constituent in their membranes, this mechanism becomes highly selective to the microorganisms. Thus, mycocins are considered to be minimally toxic, with a low likelihood of resistance induction [7–10].

Acinetobacter baumannii is a Gram-negative, encapsulated, non-fermenting, aerobic, ubiquitous coccobacillus with high environmental resilience [11, 12]. It is currently considered a worrying opportunistic pathogen in intensive care units (ICU), as it may cause pneumonia (mainly associated with mechanical ventilation), septicemia, skin infections,

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endocarditis, urinary tract infection, and meningitis [13, 14]. Increased bacterial resistance poses a global public health threat, as more and more antibiotics are becoming ineffective to these pathogens, thereby raising health-related costs [15, 16].

Therefore, it is necessary to develop new substances for the control of multidrug-resistant microorganisms. Substances currently studied include mycocins, which are effective against several microorganisms in the most diverse branches, including those of clinical importance. Thus, mycocins have become attractive candidates for medical application, aiming at the promising development of new antimicrobials [17].

Thus, the purpose of this study was to evaluate the susceptibility of multidrug-resistant *Acinetobacter baumannii* strains, against mycocins present in a *Wickerhamomyces anomalus* supernatant, as well as to verify the cytotoxicity of these compounds.

Material and Methods

Wild Yeast Isolation

On Itaipu lake shores (Paraná, Brazil), 200 soil samples were collected. Approximately, 50 g of soil was homogenized in 0.9% saline solution containing 0.1% chloramphenicol and 0.05% cycloheximide and stirred at 150 rpm, 25 °C for 2 h. Each sample was diluted to the following concentrations: 10^{-3}, 10^{-4}, and 10^{-5}, plated on Sabouraud dextrose agar and incubated at 25 °C for 72 h. The yeast colonies were re-isolated, totaling 74 wild yeasts. The tests were carried out in triplicate [18].

Sorting of Mycocins-Producing Yeasts

Wild yeasts were kept at 25 °C for 48 h in modified Sabouraud agar medium (2% agar, 1% peptone, 2% glucose, 1.92% citric acid, and 3.48% phosphate of dibasic potassium, pH 4.7) before the test.

Four strains of *Candida albicans* were prepared at a 10^5 CFU mL^{-1} concentration in saline solutions homogenized to Sabouraud agar medium and modified with methylene blue test (2% agar, 1% peptone, 2% glucose, 1.92% citric acid 3.48% dibasic potassium phosphate, and 0.003% methylene blue, pH 4.7) at almost 55 °C. After solidification, wild yeasts were inoculated at equidistant points and incubated at 25 °C for 72 h.

Among the tested yeasts, only three of them were able to produce mycocins, attested by the production of an inhibition zone surrounded by blue colonies to prove that *C. albicans* strains were sensitive. And the tests were carried out in triplicate [18].

Molecular Identification of Wild Yeasts

Genomic DNAs were identified from the three studied wild yeasts, and amplified in the ITS region, which comprises the intergenic spacers ITS1 (18S rRNA) and ITS2 (5.8S rRNA). ITS1 primer oligonucleotides (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used.

The products of expansions were sequenced, and their sequences were analyzed by the BLAST program, comparing them with the sequences deposited in GenBank. The three producing mycocin wild yeasts showed 98, 99, and 99% similarity with *Wickerhamomyces anomalus* and were deposited in GenBank. So, the access numbers for *W. anomalus* WA40, WA45, and WA92 nucleotide sequences are KT580792, KT580794, and KT580796, respectively. Available at http://www.ncbi.nlm.nih.gov/BLAST.

Production of *Wickerhamomyces anomalus* Mycocins

Individual suspensions of the three mycocin-producing yeasts were adjusted to 10^8 CFU mL^{-1} and inoculated in 200 mL of modified Sabouraud broth (1% peptone, 2% glucose, 1.92% citric acid, and 3.48% dibasic potassium phosphate, pH 4.7 ± 0.2) and incubated at 25 °C for 5 days in a 180° tilted flask. After this time, the broth was centrifuged at 4500*g* for 10 min. Thus, the mycocins WA40M1, WA45M2, and WA92M3 were considered to be the supernatant of the *W. anomalus* WA40, WA45, and WA92 strains, respectively. The obtained supernatant was sterilized by 0.22 μm filter membrane and stored at 4 °C for a maximum of 15 days.

Multidrug-Resistant Strains of *Acinetobacter baumannii*

Multidrug-resistant strains of *Acinetobacter baumannii* (n = 50) were isolated from the respiratory tract (46%), urine (16%), purulent secretion (16%), blood (12%), and invasive materials (10%) from two clinical laboratories in Cascavel, PR, Brazil.

The strains were recovered in Tryptic Soy Broth (TSB), transferred (500 μL) to an Eppendorf tube with glycerin (300 μL), and stored at approximately − 20 °C. Prior to the tests, the strains were grown on nutrient agar 36 °C for 24 h.

Antimicrobial Activity by the Microdilution Method

For the microdilution tests, the M7-A6 National Committee for Clinical Laboratory Standards [19] method was used with adaptations. Microplates containing 96 flat-bottom wells, arranged in columns (numbered from 1 to 12) and rows (with letters from A to H), were used. Fifty clinical strains of multidrug-resistant *A. baumannii* and *A. baumannii* ATCC

19606 were tested, being pre-adjusted to the concentration of 10^3 CFU mL^{-1} by counting in a Neubauer chamber, homogenized in 5 mL Mueller Hinton (MH) broth, where 100 μL were withdrawn and distributed in the wells of columns, where each column corresponds to a multi-resistant *A. baumannii* test strain. The supernatants were diluted in sterile distilled water, and 100 μL were added to the wells in lines B to F, resulting in the following dilutions (MH + broth, supernatant) (neat, 1:2, 1:4, 1:8, and 1:16). In lines A and G, sterility controls (containing only sterile broth) and growth (containing MH broth and *A. baumannii*) were, respectively, carried out. After the procedure completion, the plate was sealed and incubated at 36 °C for 48 h. The last dilution where there was no turbidity was manually stirred and aliquoted at 10 μL using a calibrated loop and seeded on nutrient agar. The absence of turbidity in the well and subsequent absence of the plaque growth were interpreted as inhibition of the tested strain. All tests were carried out in triplicate.

Antimicrobial Activity on Solid Medium

Surface Inhibition Test

Two Mueller Hinton (MH) agar media, one control and one test, were prepared. The control consisted only of MH agar and the test showed homogenized MH agar with 65% pure (1:1) supernatant containing WA40M1, WA45M2, and WA92M3 mycocins. Both media—control and test—were poured into divided Petri dishes. A suspension of multidrug-resistant *A. baumannii* strain 16 was adjusted to 10^8 CFU mL^{-1} and with a 10-μL calibrated loop, seeded in the control and in the test, subsequently incubated at 37 °C for 24 h. The multidrug-resistant *A. baumannii* strain 16 was used because it showed low sensitivity to WA40M1, WA45M2, and WA92M3 mycocins present in *Wickerhamomyces anomalus* culture supernatants (WA40, WA45, and WA92 strains) among the 50 tested strains. Thus, this was chosen because it presented the worst case.

Zone of Inhibition Check

In a Petri dish, pre-forage was made with agar-agar, and nutrient agar was added after solidification. A suspension of multidrug-resistant *A. baumannii*, strain 16, was adjusted to 10^8 CFU mL^{-1} and with the aid of a swab; the strain was plated by the surface method. Holes with approximately 6 mm in diameter were made on the nutrient agar, followed by the addition of 15 μl supernatant. The plate was incubated at 36 °C for 48 h, and any clear zone around the orifices was taken as a positive result.

Hemolysis Test

The erythrocyte cytotoxicity test was carried out according to Paris et al. [18]. Blood was collected from a healthy individual in a tube containing EDTA (ethylenediaminetetraacetic acid), which was centrifuged at 2000*g* for 10 min, and the cell mass was washed three times with PBS (phosphate buffered saline) with a 7.4 pH. Subsequently, a 4% suspension of erythrocytes in PBS was tested under different supernatant concentrations of WA40M1, WA45M2, and WA92M3 (neat, 1:2, 1:4, 1:8, and 1:16) and incubated at 37 °C for 1 hour. After this period, the tubes were centrifuged at 2000*g* for 10 min, while supernatant was used to determine spectrophotometer absorption at 450 nm wavelength.

The same procedure was carried out for polymyxin B 0.16 mg mL^{-1} (tested in the following concentrations: 0.01; 0.02; 0.04; 0.08; and 0.16 mg mL^{-1}) for the control of integral erythrocytes (4% erythrocytes and PBS), and to control hemolysis (4% erythrocyte and 4% acetic acid).

Thus, the following equation was used to calculate hemolysis percentage:

$$\%\ \text{integral erythrocytes} = \left(1 - \frac{\text{A supernatant} - \text{A interal erythrocyte control}}{\text{A hemolysis control} - \text{A integral erythrocyte control}}\right) \times 100$$

$$\%\text{hemolysis} = 100 - \%\text{integral erythrocytes}$$

Where A is absorbance.

Artemia salina Leach Toxicity Test

Artemia salina Leach is a marine microcrustacean widely used in toxicity testing of active compounds from plant extracts [20]. The three supernatants were tested according to the methodology described by Meyer et al. [21], with some adaptations. *Artemia salina* eggs were incubated in sterile sea water at 28 ± 2 °C for 48 h under continuous aeration and illumination. After hatching, 10 nauplii (larvae) were transferred to tubes containing 1000, 100, and 10 ppm of supernatant enough for (qs) 5 mL of seawater. The same procedure was used to test polymyxin B 0.16 mg mL^{-1}. The maximum toxicity control consisted of 1 mol L^{-1} NaOH and the non-toxic control contained only sea water. The tubes were incubated at 28 ± 2 °C for 24 h, and the test was carried out in triplicate. Subsequently, the live and dead specimens were counted with the aid of an optical microscope. Organisms that had motility were classified as living, immovable, and sedimented, as dead.

Statistical Analysis

Simple descriptive statistics were used to obtain the results, average, and standard deviation, as well as analytical statistics

to compare the averages obtained by ANOVA and Wilcoxon tests, with a significance level of 0.5% and $p < 0.05$ value.

Results

The mycocins of *Wickerhamomyces anomalus* WA40M1, WA45M2, and WA92M3 showed inhibitory activity on the fifty strains of multidrug-resistant *A. baumannii*, even at diluted concentrations. The WA45M2 mycocins obtained superior inhibitory results, followed by WA40M1 and WA92M3. In contact with the neat supernatant, 100% *A. baumannii* ($n = 50$) strains were sensitive to mycocins of the three killer yeast strains and, when diluted at 1:2 rate, it was observed that 98% of multidrug-resistant strains were inhibited by the WA40M1 and WA45M2 mycocins, and 90% by WA92M3 mycocins. At higher dilutions, 1:4 and 1:8, WA45M2 mycocins stayed at high levels of inhibition, 96% and 48%, respectively (Fig. 1 and Table 1 that can be seen in the Supplementary Material). The ATCC 19606 strain remained viable until the 1:2 dilution for WA40M1, WA45M2, and WA92M3 mycocins. The microdilution test of 50 clinical strains of *A. baumannii* made it possible to compare the killer potential of three yeast supernatants of the same species.

The solid medium surface inhibition test using the three mycocins-producing yeasts also demonstrates the inhibition of multidrug-resistant *A. baumannii* (Fig. 2a–c). Another test carried out on a solid medium is the zone of inhibition check. In it, it was possible to verify the diffusion and inhibition of mycocins of each supernatant in agar, producing a halo around the orifice where they were placed. In Fig. 3a–c, the highest inhibition of WA45M2 (Fig. 3b) multidrug-resistant *A. baumannii* by mycocins was observed on the medium, followed by WA40M1 (Fig. 3a) and WA92 (Fig. 3c).

The hemolysis test was carried out to provide knowledge of cytotoxicity of the three supernatants containing mycocins. Figure 4 shows the low hemolytic action of mycocins. This result resembles the levels of hemolysis caused by polymyxin B, the therapy of choice to treat *A. baumannii* infections. Thus, it was possible to relate the hemolysis of an antibiotic that is already traded and of substances that are still being studied. The ANOVA test showed no statistically significant difference among the results, $p = 0.584$.

The toxicity test was carried out on *Artemia salina* and the values referred to live *nauplii* after being submitted to each test. According to Fig. 5, mycocins of *W. anomalus* WA40M1, WA45M2, and WA92M3 are non-toxic, as there was no toxicity in the tested brine shrimp in up to 1000 ppm concentration (standard values for test in plants). Polymyxin B at 1000 ppm concentration was toxic to *Artemia salina* with 100% inhibition. The ANOVA test showed a statistic significant difference among the results of polymyxin B with as mycocins in the beginning and the end of the experiment $p = 0.0001$.

Discussion

Infections caused by *Acinetobacter baumannii* are considered a challenge for medicine, as this bacterium has become

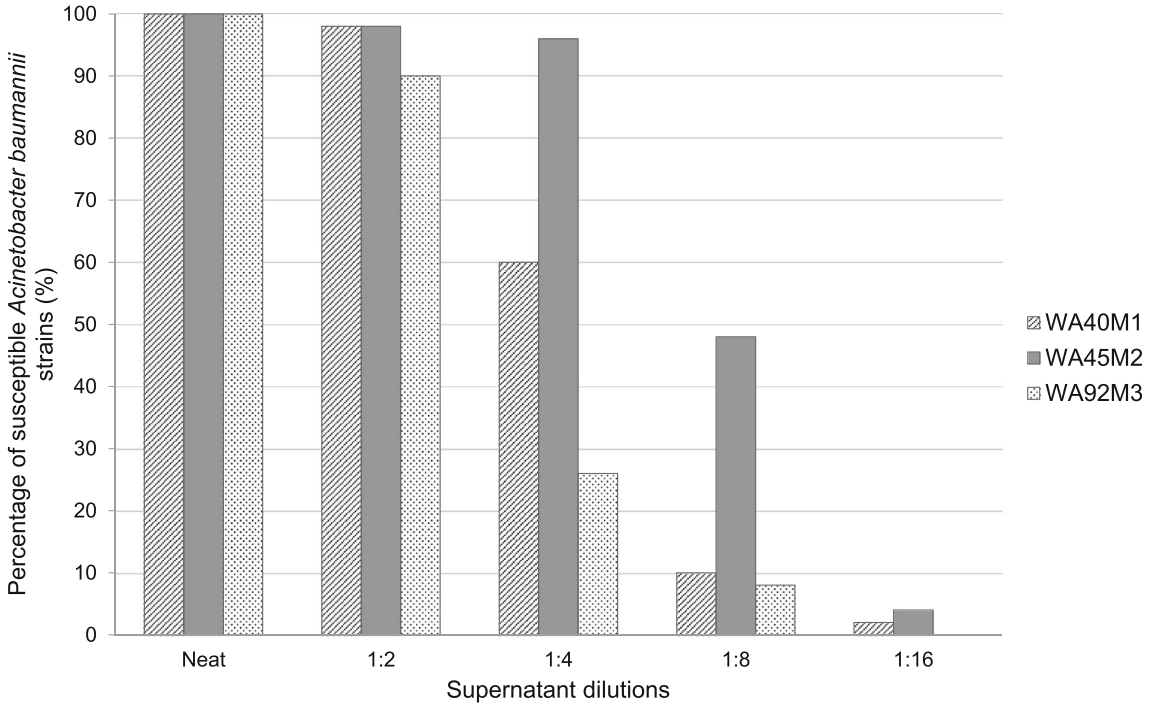

Fig. 1 Susceptibility percentage of 50 multidrug-resistant *Acinetobacter baumannii* strains according to the dilutions of WA40M1, WA45M2, and WA92M3 mycocins present in supernatants of *Wickerhamomyces anomalus* cultures (WA40, WA45, and WA92 strains)

Fig. 2 **a**–**c** Susceptibility of multidrug-resistant *Acinetobacter baumannii* according to the mycocins present in supernatants of *Wickerhamomyces anomalus* cultures (WA40, WA45, and WA92 strains). Test carried out on Petri dishes separated in (**a**), (**b**), and (**c**), where on the left side of the plates contain Mueller Hinton agar + mycocins WA40M1, WA45M2, and WA92M3, respectively; and on the right side, there was only Mueller Hinton agar. The seeding was carried out on a 10^6 CFU/mL *Acinetobacter baumannii* surface on both sides of the plate

increasingly resistant to antimicrobials. The lack of new antibiotics demands that medicine returns to the use of old drugs, such as polymyxin B, used in comparative tests in this study [22].

The high prevalence of respiratory tract infections caused by *A. baumannii* is justified by the use of mechanical ventilation, which favors rupture of the skin and mucosa, facilitating microorganism intake [23]. This justifies the high percentage (46%) of respiratory tract samples infected by *A. baumannii* in this study. Similar data were found out in Vahdani et al.'s study [24] (22%), in which the respiratory tract was the predominant site (39%) to isolate *A. baumannii*, followed by urine (22%), cerebrospinal fluid (17.5%), wound (9.5%), blood (5%), catheter (4.5%), and others (2.5%).

Faced with the increase in microorganism resistance and the lack of new drugs available on the market, mycocins have been gaining prominence among researchers thanks to their antibiotic capacity [25]. Nevertheless, the presence of these compounds is often unrecognized, since the right experimental conditions are necessary to observe their action, as well as the selection of a test-sensitive strain [26]. Thus, mycocins production demands the control of pH, temperature, chemical composition, and cellular concentration, which may vary for each killer yeast. Therefore, optimal production of most mycocins normally occurs at pH 4.5, at 25 °C [27].

According to the results obtained in this study, the WA40M1, WA45M2, and WA92M3 mycocins present in *Wickerhamomyces anomalus* culture supernatants (WA40, WA45, and WA92 strains) have high potential to inhibit multidrug-resistant *A. baumannii* strains. Using the same *W. anomalus* strains, Paris et al. [18] demonstrated that there is high antimicrobial activity of these mycocins on strains of *Candida albicans* isolated from blood. In the same study, mycocins obtained from the cell wall of *W. anomalus* WA45M2 also obtained superior activity to the others. Comitini et al. [28] also tested different mycocin concentrations of *W. anomalus* (Pikt), which was able to inhibit *Dekkera brettanomyces* strains, yeast commonly associated to wine deterioration.

Hatoum et al. [29] isolated *W. anomalus* LMA-827 from milk and, by the transmission electron microscopy method, observed that the culture supernatant of this yeast could inhibit

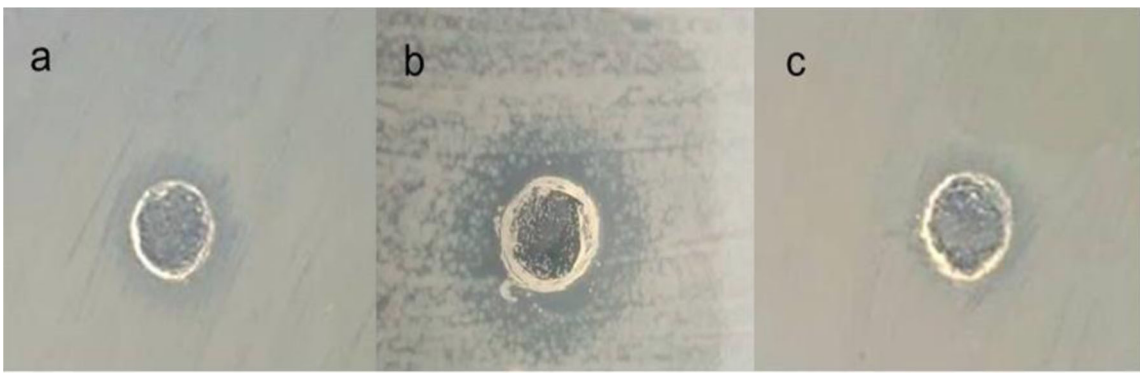

Fig. 3 **a**–**c** Inhibition zone of mycocins present in supernatants of *Wickerhamomyces anomalus* cultures (WA40, WA45, and WA92 strains). The test was carried out on nutrient agar. **a**, **b,** and **c** correspond respectively to the inhibition zone of mycocins WA40M1, WA45M2, and WA92M3 present in holes of the multidrug-resistant *Acinetobacter baumannii* strain, seeded on a surface with a swab up from a 10^8 CFU/ml suspension

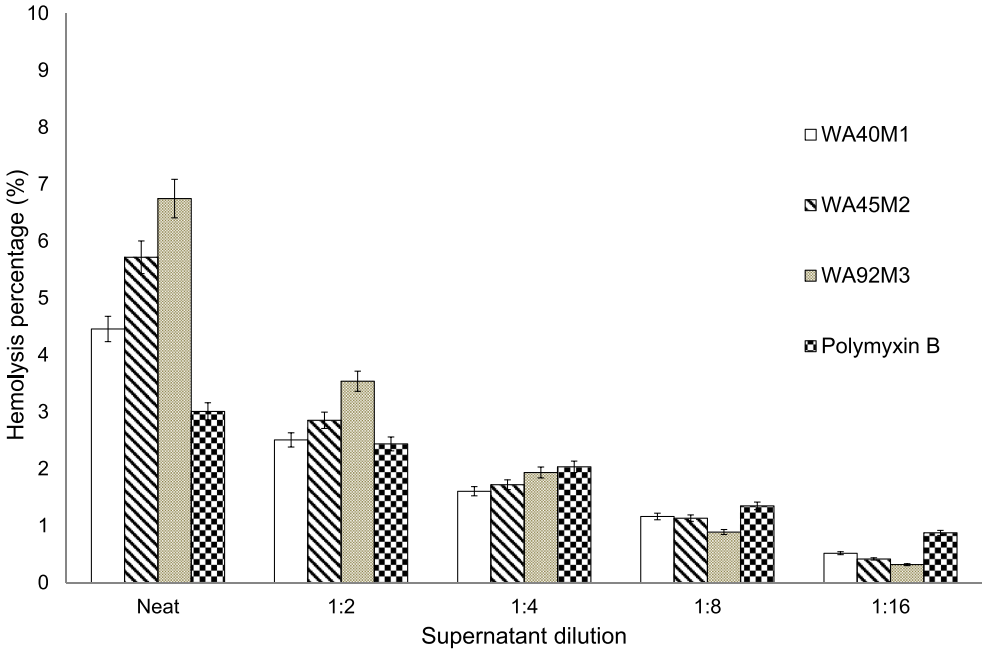

Fig. 4 Haemolytic activity of WA40M1, WA45M2, and WA92M3 mycocins present in supernatants of *Wickerhamomyces anomalus* cultures (WA40, WA45, and WA92 strains) when compared with polymyxin B. The ANOVA test showed no statistic significant difference among the results, $p = 0.584$

Listeria monocytogenes LMA-1045, a pathogenic bacterium able of causing meningitis in humans.

In an assay conducted with strains of enterobacteria grown on wheat grains and inoculated with *W. anomalus*, it was found out 60 days after storage of the cereal that yeast significantly inhibited all enterobacterial species [30].

Isolated yeasts from grape must and wine were tested for their susceptibility to killer yeasts. *W. anomalus* CBS 1982 and *W. anomalus* NCYC 434, and when tested at pH 4.5, presented 54% and 80%, respectively, of yeasts sensitive to them [31].

In addition to the inhibition of bacteria, it is already known that mycocins produced by *W. anomalus* have killer activity on other kinds of organisms, such as dermatophytes, yeasts, parasites, and viruses [18, 32–34].

In a similar methodology, Wang et al. [35] observed inhibition zones by testing *Williopsis saturnus* WC91-2 on *Metschnikowia bicuspidata*, *Saccharomyces sp.*, *Candida albicans*, *Candida tropicalis*, *Cryptococcus aureus*, *Yarrowia lipolytica*, and *Lodderomyces elongisporus*. Guo et al. [36] observed an inhibition zone of *M. bicuspidate* yeast around the hole containing *W. anomalus* YF07b mycocin. A

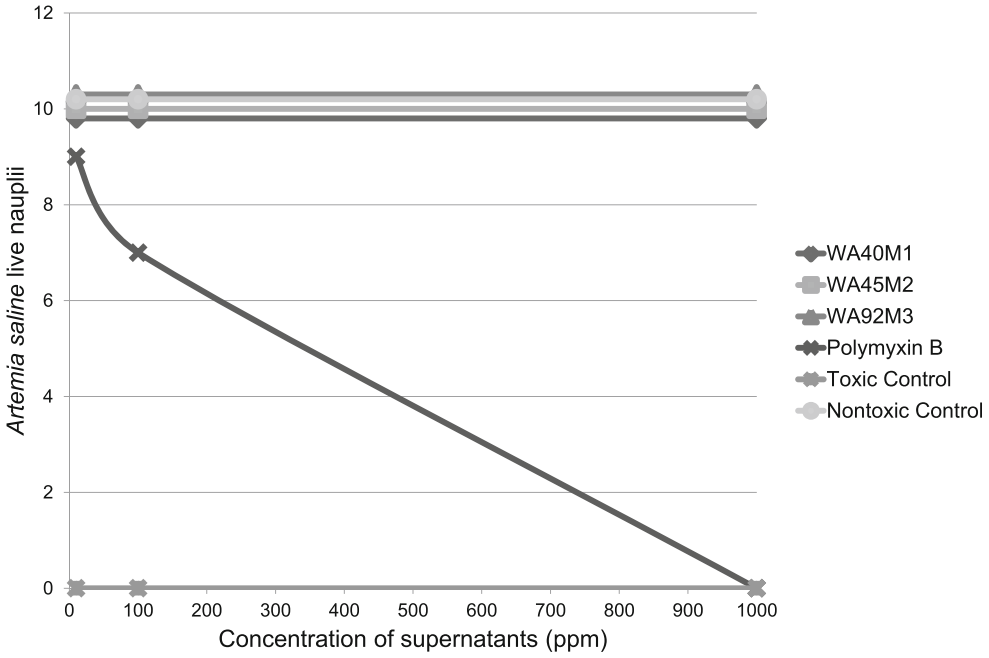

Fig. 5 Toxicity test of WA40M1, WA45M2, and WA92M3 mycocins present on supernatants of *Wickerhamomyces anomalus* cultures (WA40, WA45, and WA92 strains) in *Artemia salina* Leach. Toxic control, 1 mol L^{-1} NaOH; non-toxic control, sea water. ANOVA test showed a statistic significant difference among the results when compared with polymyxin B and mycocins, in the beginning and in the end of the experiment, $p = 0.0001$

study carried out by Ahmed Sheikh [37] registered some attractive results when testing cultures of fungi isolated from soil that, when mixed with human saliva, acted on methicillin-resistant *Staphylococcus aureus*, also by the inhibition zone method. Another study that also observed an inhibition zone by mycocins was carried out by Comitini and Ciani [38], who compared the mycocin size formed by *Kluyveromyces wickerhamii* DBVPG 6077 (Kwkt) on *Brettanomyces bruxellensis* DBVPG 6706 before and after purification, obtaining a 50% post-purification increase.

The results of this study evidenced the low toxicity of mycocins shown by the cytotoxicity tests in *Artemia salina* Leach and hemolysis test in human erythrocytes. Seddik et al. [39] showed the inhibitory activity of *Candida albicans* P51L1, isolated from children's feces on strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. As a toxicity test, the strains were found out to be non-hemolytic.

When studying yeast isolated from cheese, Ceugniez et al. [40] reported that *Kluyveromyces lactis* and *Kluyveromyces marxianus* were able to inhibit the growth of pathogenic microorganisms, such as *Candida albicans* and *Listeria monocytogenes*. In the same study, in a hemolysis test on a solid medium supplemented with horse blood, it was found out that they were not hemolytic. Paris et al. [18] tested mycocins extracted from *W. anomalus* wall on human erythrocytes and obtained similar results in relation to the ones obtained in this study, that is, low cytotoxicity. They also showed that mycocins were less toxic when compared with the amphotericin B antifungal.

In this study, the toxicity model for Artemia was used due to its advantages: ease of handling, rapidity, low cost, and the potential to replace animal use in toxicological tests [41]. No other publication involving mycocins toxicity testing on Artemia has been recorded yet.

In conclusion, multidrug-resistant *Acinetobacter baumannii* was sensitive to *Wickerhamomyces anomalus* mycocins in the liquid and solid medium, in which the WA45M2 mycocins stood out in relation to the others. Furthermore, mycocins were not considered toxic when compared with polymyxin B. Thus, *W. anomalus* WA40M1, WA45M2, and WA92M3 mycocins are attractive candidates for the development of new antibiotics, which can inhibit multidrug-resistant microorganisms.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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