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Antimicrobial activity of *Wickerhamomyces anomalus* mycocins against strains of *Staphylococcus aureus* isolated from meats

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Abstract

Staphylococcus aureus is among the major pathogens involved in food poisoning, and meat contaminated with *S. aureus* coagulase positive is considered a public health risk because the bacterium is resistant to several conventional antimicrobials. Mycocins are substances produced by yeasts that secrete glycoproteins, which can also be called toxin killers, they have an inhibitory action on other microorganisms. The *Wickerhamomyces anomalus*, is one of the microorganisms capable of producing these mycocins, resulting in the action of disturbances on the cell wall of the pathogen causing deleterious effects. This work aims to evaluate the antimicrobial activity of the mycocins produced by *W. anomalus* WA45 against the 29 strains of *S. aureus* coagulase positive isolated from bovine, porcine and chicken meat and 1 standard strain. The antimicrobial action of the mycocins present on the culture supernatant of *W. anomalus* WA45 was tested by microdilution and the results were satisfactory, since 100% inhibition of strains of *S. aureus* coagulase positive. We concluded that the mycocins present in the supernatant of *W. anomalus* WA45 showed antimicrobial action, being candidates for the development of new products for the biocontrol and bioconservation of meat.

Keywords: mycocins; antimicrobial activity; meats; *Staphylococcus aureus*.

Practical Application: Control of antimicrobial activity from meats.

1 Introduction

The *Staphylococcus aureus* can cause food poisoning, and is among the bacterial pathogens most commonly involved in foodborne diseases due to widespread distribution, and can be found in grains, cereals, egg products, dairy products, fish, meat products and in the meat *in natura* (Akineden et al. 2008; Normanno et al. 2007; Simon & Sanjeev, 2007). The study of staphylococci are area interesting because, as they are considered the main contaminants of small and medium meat processing plants (Koreňová et al., 2015; Nasser, 2015). About 45% of the worldwide food poisoning are caused by bacteria of the genus *Staphylococcus*. Contamination occurs mainly during food production and storage periods, as it is during this period that temperatures are proper for the proliferation of this microorganism (Cunha et al., 2002; Neyaz et al., 2020).

Because it is a microorganism adaptable to several environments, contamination of food by *S. aureus* is associated with faults in the handling, processing, conservation, and hygienic and sanitary conditions of equipment and utensils (Baeza et al., 2009; Rode et al., 2007). Foods that require manipulation for their preparation and that remain at temperature without refrigeration for a certain period, such as meat, are considered high risk for staphylococcal food poisoning, since the meat presents favorable conditions for microbial growth due to the properties of its composition, like proteins (Ananou et al., 2005; Wallin-Carlquist et al., 2010). Meat contaminated with *S. aureus* is considered a public health risk, as this pathogen presents resistance to several antimicrobials (Presi et al., 2009).

Mycocins are substances produced by yeasts that secrete glycoproteins, which can also be called killers toxin, they have an inhibitory action on other microorganisms. This phenomenon, considered killer, was first observed by Bevan and Makover, in 1963, in strains of *Saccharomyces cerevisae*, which were isolated from brewery contaminants (Tay et al., 2014). This killer toxin are secondary metabolites (enzymes), of a protein or glycoprotein nature with antibiotic activity, as they cause disturbance in the cell wall of the pathogen and cause deleterious effects (Comitini et al., 2004). Other studies have identified the same potential in yeasts of the genera *Debaryomyces*, *Pichia*, *Kluyveromyces*, *Wickerhamomyces*, *Williopsis* and *Zygosaccharomyces* among others (Ceugniez et al., 2015; Chen et al., 2015; França et al., 2015; Hatoum et al., 2012; Passoth et al., 2011; Seddik et al., 2016).

Some strains of *Wickerhamomyces anomalus* are producers of substances called mycocins. The yeast *W. anomalus* has been investigated for its wide potential of antimicrobial activity against numerous pathogenic prokaryotes and eukaryotes (Polonelli et al., 2011). Mycocins from *W. anomalus* strains were used in food biocontrol because of their antimicrobial

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actions by hydrolyzing the β-1,3-glucan or β-1,6-glucan cell wall connections of susceptible strains of pathogenic microorganisms (Barbosa et al., 2010; Blasco et al., 2006).

Interest in mycocins as a new antimicrobial agent is increasing because of the broad spectrum of fungal and bacterial infections, as numerous pathogens show resistance to conventional antibiotics, such as *S. aureus* (Muccilli & Restuccia, 2015).

Coda et al. (2011) studied the antifungal activity of the mycocins produced by *W. anomalus* on the wheat flour used in baking, and observed the extension of the life of the wheat flour bread. Aloui et al. (2015) described results for the use of *W. anomalus* agaisnt *P. digitallium* in food preservation, they observed a future potential application of these mycocins as effective and promising alternatives to synthetic antifungal agents for maintaining quality attributes and controlling green mold of oranges. Some authors conclude the mycocins can be are studied as new molecules and potential candidates to develop new antifungal due to their broad spectrum of action (Aloui et al., 2015; Paris et al., 2016). Nascimento et al., (2020) reported that mycocins have an antimicrobial action, and are minimally toxic to human cells. Junges et al. (2020) confirmed this low toxicity of mycocins, by the cytotoxicity tests in *Artemia salina* Leach.

The objective of this work is to highlight the antimicrobial activity of *W. anomalus* mycocins against the inhibition of strains of *S. aureus* coagulase positive isolated from beef, pork, and chicken.

2 Materials and methods

2.1 Strains of Staphylococcus aureus isolated from meats

The strains of *S. aureus* were isolated from bovine, porcine, and chicken samples collected from 35 butchers from April to June 2018. A total of 105 samples (± 100 g per sample) were analyzed, 35 of which were bovine (topside), 35 swine (pork shank), and 35 chicken meat (thigh), *in natura*, stored under refrigeration at 4 °C. The samples were packed in plastic bags, transported to the laboratory, in an isothermal box within a maximum of two hours.

To obtain the *S. aureus* samples in meat, 25 g of each sample was weighed and homogenized in 225 mL of 0.1% sterile peptone water and processed in Stomacher® equipment for 1 minute. After homogenization, 0.1 mL was sown on Baird-Parker Agar surface and incubated at 37 °C for 48 hours. After the incubation period, typical *S. aureus* colonies (black, small, smooth, surrounded by an opaque zone and/or a halo) and atypical (black, without halo) colonies were counted. A colony of each morphotype was spiked in Baird-Parker Agar and incubated at 37 °C for 24 hours. Additional tests were performed, such as mannitol and coagulase. After isolation and identification, strains of *S. aureus* coagulase positive were transferred to Eppendorf tubes with glycerin and Brain Heart Infusion (BHI) and stored at -20 °C. The standard ATCC strain WDCM 00032 of *S. aureus* was used in all subsequent experiments. Additional tests were performed as a catalase, DNAse, coagulase, mannitol, Voges-Proskauer test and tolerance to 7.5% NaCl. The automated method, using the Vitek 2 Compact device (Biomerieux), confirmed the identification of all strains of *S. aureus* coagulase-positive.

2.2 Mycocins obtained from Wickerhamomyces anomalus strains WA45

The yeast strain *W. anomalus* WA45 used for the production of mycocins was molecularly identified and available from GenBank (accession number: KT580794 available at National Center for Biotechnology Information, 2020).

To obtain the mycocins, a suspension of 106 CFU/mL of *W. anomalus* WA45 strain and inoculated in a Roux vial containing 200 mL of Modified Sabouraud broth (1% peptone, 2% glucose, 1.92% citric acid and 3.48% bibasic potassium phosphate, pH 4.7), incubated at 25 °C for five days in static culture. The broth was centrifuged at 6000 rpm for 10 minutes to obtain the supernatant. It was then passed through a 0.22 μm membrane filtration process and stored at 4 °C until the *in vitro* tests were performed.

2.3 Determination of β-glucanase activity

The determination of the β-glucanase activity present in the supernatant of *W. anomalus* WA45 was performed as described by Miller (1959), with some adaptations, using laminarin 1% (*Laminaria digitata*), 50 mM acetate buffer, pH 5.0 and standard curve of glucose. A solution containing 62.5 μL of the supernatant was prepared with the WA45 and 125 μL of laminarin 1% and incubated at 37 °C for 10 minutes. It removed 100 μL from the solution and added 100 μL of 3,5-dinitrosalicylic acid (DNS) to stop the reaction. The solutions were incubated in boiling water for 5 minutes with the addition of 500 μL of sterile distilled water, and the reading of the reaction product (reduced sugar) was at 550 nm in a spectrophotometer. For the blank, the same test solution was used without laminarin. An enzyme unit (U) was defined as the amount of protein required to produce 1 μmol of reducing sugar per minute. Protein quantification was based on the absorption of Coomassie Brilliant Blue G-250 reagent proposed by Bradford (1976). To prepare the reaction, 1 mL of the Bradford Reagent was mixed with 100 μL of the enzyme extract. The mixture remained at room temperature for 5 minutes, and then the spectrophotometer read at 595 nm. The standard curve was performed at each determination of the total protein concentration by the Bradford method, using bovine serum albumin standard curve (BSA), the equation of the line being used to calculate the total concentration of proteins in mg/mL. B-glucanase activity was calculated by dividing the concentration of enzyme activity by the protein concentration, resulting in U/mg.

2.4 Microbiological test of solid surface inhibition

The test was performed on a divided sterile plate. One part for the control test containing 10 mL of Mueller Hinton agar and the other part as a test containing 5 mL of Mueller Hinton agar was added, and 5 mL of supernatant was added. *W. anomalus* WA45 at the concentration of 0.4 U/mg β-glucanases. In both parts, strains of *S. aureus* coagulase positive were seeded in a single stria technique incubated at 37 °C for 24 hours. The test was performed in triplicate.

2.5 Microdilution test

For the microdilution tests, the M7-A6 method - National Committee for Clinical Laboratory Standards (2003) was used with some adaptations. Microplates containing 96 wells, arranged in columns (numbered from 1 to 12) and lines (alphabetically, from A to H) were used. Twenty-nine strains of *S. aureus* coagulase-positive, including the ATCC strain WDCM 00032 from *S. aureus*. The concentrations of mycocins were determined from the activity of β-glucanases, being: 0.02; 0.03; 0.06; 0.12; 0.24 U/mg. The bacteria were previously adjusted to the concentration 103 CFU/mL by counting in a Neubauer chamber, homogenized in 5 mL of Mueller Hinton broth (MH), and distributed (100 µL) in the columns, where each column corresponds to a test strain of *S. aureus* coagulase. The supernatant containing the mycocins was diluted in sterile distilled water and added to the wells of line A to F (100 µL). In the G and H lines, the sterility controls (containing sterile modified Sabouraud broth and MH broth) and growth control (containing sterile modified Sabouraud broth and *S. aureus* coagulase), respectively, were performed. After completion of the procedure, the plates were sealed and incubated at 36 °C for 24 hours. The reading was visual, observing the turbidity, and the last dilution where there was inhibition of bacterial growth was taken as a result. To confirm inhibition, 10 µL aliquots were taken from the wells and seeded on nutrient agar. The test was performed in triplicate.

3 Results and discussion

S. aureus is among the major pathogens arising from the consumption of meat responsible for foodborne infections. The high incidence of *S. aureus* in meat samples is alarming since the presence of this pathogen serves as a source of contamination for other foods, so it is essential to reduce the bacterial population in the meat (Mead, 2004; Dias et al., 2008).

Staphylococcal food poisoning occurs because the microorganism produces virulence factors such as enterotoxins and the enzyme coagulase (Lamaita et al. 2005; Peton & Le Loir, 2014). For the detection of *S. aureus* positive coagulase in meats, it is necessary to apply the coagulase test, since it is one of the requirements evidencing its enterotoxigenic property (Pereira et al., 2001).

In the results presented in our study, of the 105 meat samples analyzed, 62 samples (59%) demonstrated the presence of strains of *S. aureus*. Of these 62 *S. aureus* positive samples, 46.7% are coagulase-positive *S. aureus* strains, in 29 of 62 samples. Of the coagulase-positive strains, 45% (13 of 29 samples) were isolated from beef, 31% of chicken (9 of 29 samples), and 24% (7 of 29 samples) of pork.

According to Welker et al. (2010), microbiological analyzes of food products involved in toxinfections showed that meat contaminated with *S. aureus* coagulase positive was responsible for 36% of the outbreaks investigated. Among these products, beef (39%) was the main responsible for food contamination, followed by chicken meat (30%) and pork with fish represented (14%).

The incidence of *S. aureus* in chicken meat varies with the management and hygienic and sanitary conditions since the pathogens found in the carcasses come from the skin, the feathers of the live birds and the gastrointestinal tract Menezes et al. (2018) when analyzing the chicken carcasses, found *S. aureus* in all samples, but 23.8% of the samples were characterized as *S. aureus* coagulase positive.

According to Fosse et al. (2008), *S. aureus* is important in the pork chain because it is a commensal microorganism in pigs and humans, capable of causing food toxinfections due to the production of enterotoxins and, as an important carrier of antimicrobial resistance genes for other microorganisms.

The presence of resistant strains of *S. aureus* may lead to the contamination of meat and meat products intended for human consumption. Studies have already isolated methicillin-resistant *S. aureus* (MRSA) from animal foods, including pork, beef, and chicken (Boer et al., 2009).

3.1 Determination of β-glucanase activity

B-glucanases are enzymes that hydrolyze glycosidic bonds of type β-1,3 and β-1,6-glucans, releasing glucose as the main product (Bauermeister et al., 2010). According to Tay et al. (2014), in isolating and identifying by mass spectrophotometry, the mycocins of *W. anomalus*, resulted in the presence of β-1,3-glucanases. These enzymes are secreted by yeast-producing mycocins and have the action of destroying the cell wall of bacteria and fungi (Bauermeister et al., 2010; Fleuri & Sato, 2008).

Studies by Marco & Felix (2007) used laminarin 1% to determine the activity of β-glucanases produced by *Trichoderma harzianum* and obtained a concentration of 0.3 U/mL. Lima et al. (2013) evaluated the production of β-glucanases from strains of *W. anomalus*, which defined the enzymatic action of 0.071 U/mg. In this study, the enzymatic activity for the β-glucanases present in the supernatant of the *W. anomalus* WA45 culture of our experiment obtained a concentration of 0.4 U/mg and may be related to the production conditions of *the W. anomalus* strain.

3.2 Microbiological test of solid surface inhibition

W. anomalus can develop in several habitats and is associated with the deterioration or processing of food and grain products, such as beer, bread, and dairy products, as well as a biocontrol agent against pathogenic microorganisms (Passoth et al., 2006).

The first indications of action of mycocins produced by *W. anomalus* (*Hansenula anomala*) strains demonstrating activity against pathogenic microorganisms were described by Polonelli et al. (1986), where it found the antimicrobial activity of these strains against *S. aureus* bacteria.

To evaluate the antimicrobial activity of *W. anomalus* WA45 myocardial microorganisms against *S. aureus* coagulase positive strains, the microbiological test of solid surface inhibition was carried out. In Figure 1, the total inhibition of the bacterium where the mycocins was added is observed (Side A).

3.3 Microdilution test and antimicrobial potential of mycocins

Yeast *W. anomalus* WA45 demonstrated the production of β-glucanases (mycocins) and antimicrobial activity. Mycocins, which have little or no toxicity to human erythrocytes,

and are low in resistance, are being studied as potential candidates for antimicrobial development (Izgu et al., 2011; Paris et al., 2016).

In this study, the *W. anomalus* WA45 mycocins demonstrated antimicrobial potential for all strains of *S. aureus* coagulase positive isolated from beef, pork, and chicken. The best results were for the concentrations of 0.1; 0.2 and 0.4 U/mg, where 100% of the bacterial inoculum were inhibited. However, inhibition of coagulase-positive *S. aureus* was observed until the concentration of 0.02 U/mg, as shown in Figure 2.

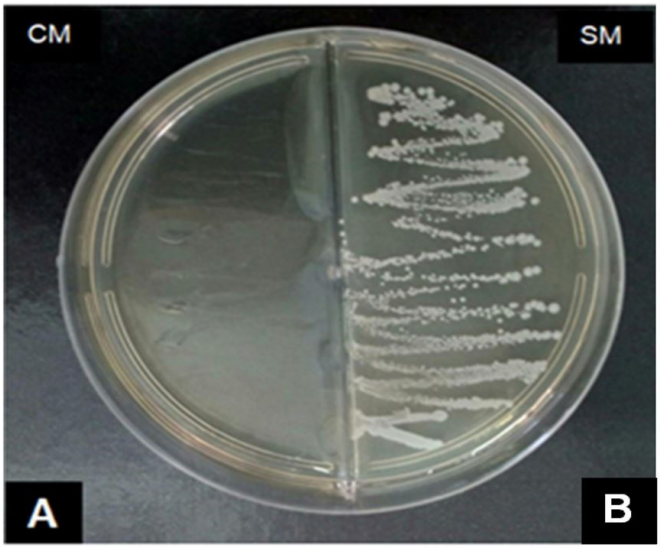

Figure 1. Solid media test to evaluate the antimicrobial activity of *W. anomalus* WA45 mycocins. Side (A) CM (test) containing *W. anomalus* WA45 mycocins additive in Mueller Hinton agar and inoculated strain *S. aureus* coagulase positive ATCC WDCM 0032. Side (B) SM (Control) containing Mueller Hinton agar and inoculated the *S. aureus* strain ATCC WDCM 0032 by surface method.

In recent years research has suggested the antimicrobial activity of different strains of *W. anomalus* present in food against different microorganisms. Comitini et al. (2004) have shown that mycocins secreted by *W. anomalus* inhibit yeasts *Dekkera anomala* and *Brettanomyces bruxellensis* that cause unpleasant odors in wine during fermentation, aging, and storage. The results of Haïssam (2011) using 50 µL of 107 CFU/mL suspension of *W. anomalus* were able to inhibit the pathogens *Botrytis cinerea, Penicillium expansum, Gloeosporioides* that infect and develop rot in fruits such as apples and pears. Mohamed & Saad (2009) have electronically scanned the antagonistic effects of *Pichia anomala* cell microscopy interacting with the *Botryodiplodia theobromae* fungus, which causes pathogenesis in guavas, showing that the hyphae of *B. theobromae* were totally penetrated and destroyed by yeast cells.

In the process of bioconservation of meats using yeasts presented by Prez-Nevado et al. (2006) for the Huelva Ham, a product that requires a 4-year cure, and the climatic conditions of the place were exposed, the present yeasts had an antimicrobial and conservation action of this meat.

According to Muccilli & Restuccia (2015), yeasts *W. anomalus* can be used in the process of bioconservation, due to its antimicrobial potential. Several microorganisms and other biological agents have been considered crucial in the bioconservation of food, indirectly altering the pH or osmotic pressure, or directly producing antimicrobial components.

Virgili et al. (2012) studied yeast producing mycocin found on the surface of cured Italian hams, using them as growth biocontrol for the fungus *Penicillium nordicum* and to inhibit Ochratoxin A.

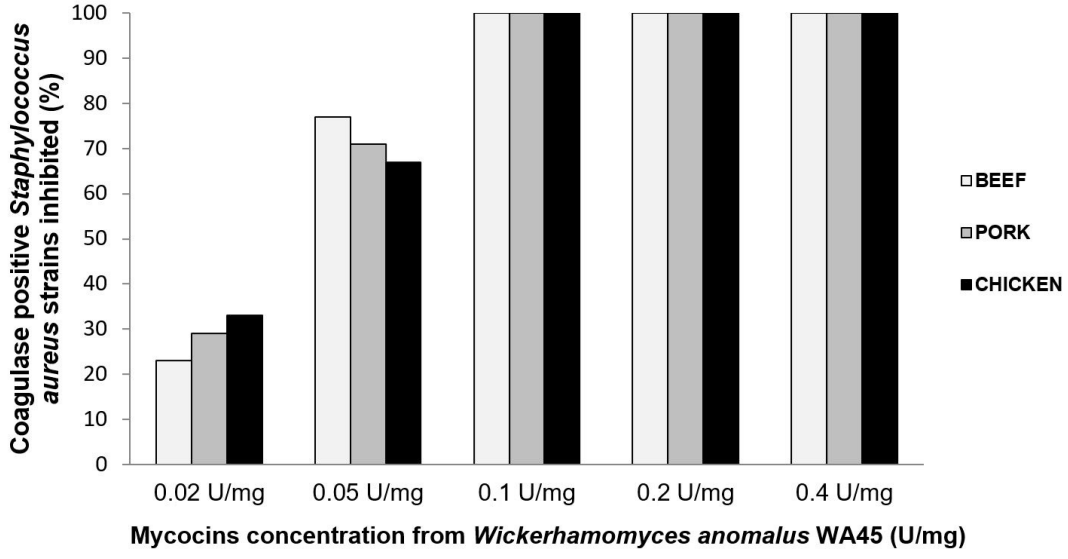

Figure 2. Antimicrobial action of *W. anomalus* WA45 mycocins in microdilution test against strains of *S. aureus* isolated from bovine, porcine and chicken meat.

4 Conclusion

We concluded that the mycocins in the culture supernatant of *W. anomalus* WA45 demonstrated antimicrobial action for strains of *Staphylococcus aureus* coagulase positive isolated from beef, pork, and chicken, being candidates for the development of new products for the biocontrol and bioconservation of meat.

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