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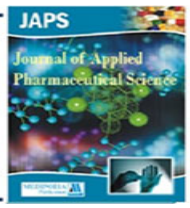

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Anti-Staphylococcal and Antifungal Substances Produced By Endospore-Forming Bacilli

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ABSTRACT

Some bacteria and fungi are related to deterioration and also transmission of foodborne diseases, emphasizing the need to search new substances that may act in the treatment and prevention of the illnesses transmitted by food. Strains from genus *Bacillus* produce a variety of substances with inhibitory activity that range from antibiotics to bacteriocins. In this work, three strains, identified as *B. pasteurii* (Pes1) and *B. insolitus* (Mam2 and Ame3) presented inhibitory action against staphylococcal strains isolated from food. Out of the 33 strains tested, 31 (94.0%) were inhibited by at least one of three main *Bacillus* producer strains, being most of them inhibited by strain Pes1, that also was able to inhibit filamentous fungi related to food spoilage. The antimicrobial substances produced by Pes1, Mam2 and Ame3 showed to be resistant to proteolytic enzymes, suggesting these substances have not an active proteinaceous compound, as typical bacteriocins. New studies are being performed to extract and characterize these antimicrobial agents to evaluate their potential application in biological control of microorganisms related to spoilage food and foodborne diseases.

Keywords: antimicrobial substances, antifungal, anti-staphylococcal, biological control application, endospore-forming bacilli.

INTRODUCTION

Fresh food products provide a favorable environment for proliferation of spoilage and pathogens microorganisms, responsible for economic loss and public health problems, respectively (Berger *et al.*, 2010; Al-Hindi *et al.*, 2011). Economic loss can affect even the world's largest exporters because fresh products are highly perishable and suffer deterioration in a few days, which difficult their marketing, especially for long distances (Brunini *et al.*, 2002; Santos *et al.*, 2008). Some pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* are of great importance to public health and are linked to outbreaks of food poisoning due to consumption of contaminated fresh products (Beuchat, 2002). Otherwise, contamination by fungi is important not only from the sensory point of view but also due to the risk of mycotoxin production (Amadi and Adeniyi, 2009; Fatima *et al.*, 2009).

Recently there has been increasing efforts to find new substances that may act in the treatment and prevention of foodborne illnesses. Biologically active compounds produced by *Bacillus* strains include well known compounds such as antibiotics, for example, butirosin, which is an aminoglycoside (Slepecky and Hemphill, 2006) and another class of substances: the bacteriocins, like thuricins and cereins (Bizani & Brandelli, 2002; Schmitt *et al*., 2007; Abriouel *et al*., 2011).

In a previous study performed by our group, ten strains isolated from fruits were submitted to Gram and spore staining and showed to be Gram-positive endospore-forming bacilli. These strains were tested for production of antimicrobial substances, using as indicators different Gram-positive bacteria. Three strains, named Ame3, Mam2 and Pes1 presented the largest spectrum of action against Gram-positive bacteria, inhibiting inclusive, different *Bacillus* and *Staphylococcus* reference strains (ATCC), suggesting that the substances produced by these bacilli may have some potential application as food biopreservative (Oliveira *et al*., 2011).

In this work, we investigated the ability of antimicrobial substances produced by Gram-positive endospore-forming bacilli strains Pes1, Mam2 and Ame3 to inhibit staphylococcal and fungal strains isolated from food samples and verify if these substances possess an active proteinaceous nature responsible for the activity, as typical bacteriocins.

MATERIALS AND METHODS

STRAINS COLLECTION

The bacilli producer strains Pes1, Ame3 and Mam2 were obtained from peach, plum and papaya samples, respectively, purchased at markets and fairs distributed in different locations around the city of Rio de Janeiro, Brazil (Oliveira *et al*., 2011). Staphylococcal strains isolated from food used as indicators were obtained in previous works performed by our group (Junqueira *et al*., 2009; Oliveira *et al*., 2009). Fungal strains were also isolated from food and belong to the collection of Laboratory of Microbiology of the Instituto Federal do Rio de Janeiro (LMIFRJ). Bacteria strains were stored on Tripticase soy broth (Micromed, Brazil) added by 40% glycerol (v/v) at -20°C and filamentous fungi were maintained on PDA (Himedia, Brazil) slants at room temperature until use.

ACTION OF ANTIMICROBIAL SUBSTANCES AGAINST STAPHYLOCOCCAL AND FUNGAL STRAINS

The action of the antimicrobial substances against staphylococcal was performed as described by Giambiagi-deMarval *et al*., (1990) with some modifications. Strains were grown on plates containing TSA medium (Himedia, Brazil) at 37°C for 18 h and the cultures were inoculated in the form of spots on the surface of TSA plates. After a period of 18 h at 37°C, bacteria were killed by exposure to chloroform vapors for 30 min and, after evaporation, the plates were sprayed with the indicator strains cultures (0.3 ml of a previously grown culture in 3 ml of TSA soft agar). The plates were further incubated at 37°C for 18 h and the diameters of the inhibition zones were measured. To determinate the antimicrobial action against fungi, the indicator strains were inoculated on four equidistant points on TSA agar plates concomitantly to the producer strain, inoculated as a central spot, at 25° C for 7 days to permit the observation of the inhibition of fungal growth.

IDENTIFICATION OF ANTIMICROBIAL SUBSTANCES PRODUCER STRAINS

Producer strains were analyzed according to their morphology and biochemical tests as Voges-Proskauer, nitrate to nitrite reduction, growth under anaerobic conditions, formation of acid and gas from glucose, starch, urea and casein degradation, utilization of galactose, lactose, mannose and mannitol, utilization of citrate and malonate as carbon sources, deamination of phenylalanine and arginine, as described by Clauss & Berkeley (1986) and MacFaddin (2000).

SUSCEPTIBILITY OF THE INHIBITORY SUBSTANCES TO PROTEOLYTIC ENZYMES

The effects of the proteolytic enzymes trypsin (Sigma-Aldrich, São Paulo, Brazil), pronase XXIII (Sigma-Aldrich, São Paulo, Brazil) and proteinase K (Sigma-Aldrich, São Paulo, Brazil) on antimicrobial substances activity were determined in accordance with Giambiagi-deMarval *et al*. (1990). The enzymes (1 mg/ml) were prepared in 0.05 M Tris (pH 8.0) with 0.01 M $CaCI_2$, and 40 µl were applied around the producer growth after chloroform treatment. The plates were incubated at 37°C for a further 4 h and then sprayed with the indicator strain. After the treatment with the enzymes, the absence of inhibition zones indicates that the antimicrobial substance presents am active proteinaceous compound. The antimicrobial substances were also treated with 0.2 N NaOH to rule out the possibility that the inhibition exhibited might have been due to organic acids produced by the producer strain during its metabolism.

RESULTS AND DISCUSSION

According to FDA (2011), foods that are frequently incriminated in staphylococcal food poisoning include meat and meat products, poultry and egg products and salads, foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation. Since Pes1, Mam2 and Ame3 inhibited at least three *Staphylococcus* reference strains employed in the previous work (**Table 1**), we decided to test the inhibition ability of these three main producers against several strains of *Staphylococcus* spp. isolated from salads and meat products acquired from different commercial establishments of the city of Rio de Janeiro.

Out of the 33 strains tested, 31 (94.0%) were inhibited by at least one of three main *Bacillus* producer strains, being Pes1 able to inhibit 25 (75.7%) of staphylococcal strains tested, while Ame3 and Mam2 inhibited, respectively, 20 (60.6%) and 22 (66.7%) of strains. These results are presented in **Table 2**.

Table 1: Spectrum of action of the bacilli strains isolated in this study.

Indicator Strains	Producer strains		
	Ame3	Mam2	Pes1
Ame1[a]	-	-	+
Ame3[a]	-	-	+
3Ba1[a]	+	-	+
3Ba5[a]	-	-	+
Mam2[a]	-	-	+
Bacillus cereus LMIFRJ	+	+	+
Bacillus circulans LMIFRJ	+	+	-
Bacillus megaterium LMIFRJ	+	+	-
Bacillus stearothermophilus NCTC 10339	+	+	+
Bacillus thuringiensis LMIFRJ	+	+	+
Micrococcus luteus LMIFRJ	+	+	+
Staphylococcus epidermidis ATCC 14990	+	+	+
Staphylococcus epidermidis ATCC 35984	+	+	-
Staphylococcus aureus ATCC 12600	+	+	+
Staphylococcus xylosus LMIFRJ	+	-	+

+, inhibition; -, no inhibition; [a], Gram-positive endospore-forming bacilli; LMIFRJ, Laboratory of Microbiology of Instituto Federal do Rio de Janeiro (Adapted from Oliveira *et al*., 2011).

Table. 2: Inhibition of *Staphylococcus* sp. strains isolated from food by the three main antimicrobial substance producer strains found in this work.

Indicator strains	Producer strains		
	Pes1	Ame3	Mam2
S1	+	-	-
S9	+	-	-
S10	-	++	++
S11	++	-	-
S14	+	+++	+++
S15	-	-	-
S17	-	-	-
S18	++	++	++
S19	+++	+++	+++
S21	++	+++	+++
S24	+	-	-
S28	++	++	+
S29	-	++	-
S30	+	-	+
S32	++	+++	+++
S34	+	-	++
S35	+	-	++
S36	+	-	-
S37	-	+++	++
S39	+++	+++	+++
S40	+	+	+++
M1	+	+++	+++
M3	-	+++	++
M6	+++	+++	+++
M11	-	+++	-
M25	+	-	-
M26	-	-	+++
M29	+	++	+
M31	++	-	-
M44	+++	+++	+
M45	++	++	+
M46	++	+++	+++
M48	++	++	+

+, Inhibition halo between 10 and 20 mm; ++, Inhibition halo between 21 and 30 mm; +++, Inhibition halo up to 30 mm; -, no inhibition. S, staphylococcal strains isolated from salads; M, staphylococcal strains isolated from meat products.

Many antimicrobial substances show potential applications in food preservation, showing broad spectra of antimicrobial activities, inhibiting Gram-positive and Gram-negative foodborne pathogens, and in some cases, even fungi, since *Bacillus* group is widely disseminated and adapted to survive under different conditions (Abriouel *et al*., 2011; Maróti *et al*., 2011). The three producer strains used in this work were submitted for identification that revealed that Pes1 belongs to *Bacillus pasteurii* (currently *Sporosarcina pasteurii*) and Mam2 and Ame3 to *Bacillus insolitus* (currently *Psychrobacillus insolitus*) species. Although the interest in studying the arsenal of antimicrobial substances produced by *Bacillus* spp. have increased in recent years, no other study linking these species to the production of antimicrobial substances was conducted to date.

Strains Pes1, Mam3 and Ame2 were tested against filamentous fungi but only the strain Pes1 showed antimicrobial activity against strains of *Aspergillus niger*, *A. flavus* and *A. parasiticus* (**Figure 1**). Strains of *A. ochraceus*, *Penicillium expansum*, *Rhizopus sp.* and *Fusarium* sp. presented slight or none inhibition.

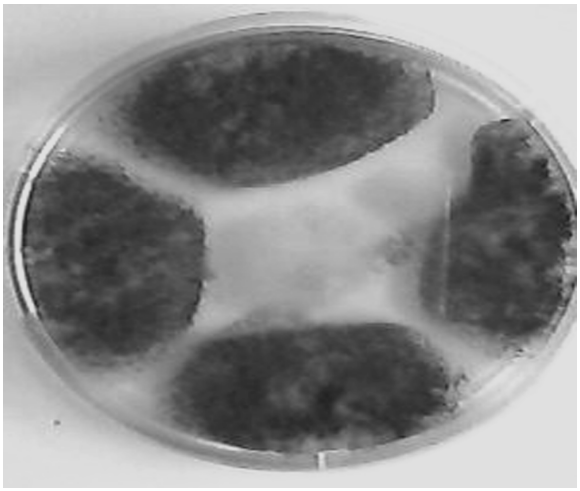

Fig. 1: Inhibition of *Aspergillus parasiticus* by the producer strain Pes1. Even after two weeks incubation, the fungus was not able to grow around the producer strain.

Some antimicrobial substances produced by other species of *Bacillus* group have had this antifungal activity described, as the bacteriocin produced by *Bacillus amyloliquefaciens* LBM 5006, that possess activity against different phytopatogenic fungi; the antimicrobial substance BRF1, produced by *Paenibacillus polymyxa*, able to inhibit pathogenic fungi related to foods; and the rhizocticins, phosphonate-containing oligopeptide antibiotics produced by *B. subtilis* (Benitez *et al.*, 2010; Borisova *et al*., 2010 ; Chen *et al.*, 2010). This is a promising field of research, since bacteriocins produced by lactic acid bacteria, the main antimicrobial substances studied, are limited in relation to their ability to inhibit fungi.

As the antimicrobial substances produced by Ame3, Mam2 and Pes1 were able to inhibit different genus of Gram-positive bacteria and even fungi strains, in case of Pes1, they seem not to be typical bacteriocins. To evaluate if these substances possess an active proteinaceous compound, their sensitivity to proteolytic enzymes trypsin, proteinase K and pronase XXIII was verified, however, there was no loss of ability to inhibit the indicator strain, indicating the substances are resistant to these enzymes (**Figure 2**). This fact suggests that they are not typical bacteriocins and maybe, not proteinaceous substances. The substances were also resistant to NaOH, ruling out the possibility that the inhibition of the indicator is being done by acids released by the producer strains.

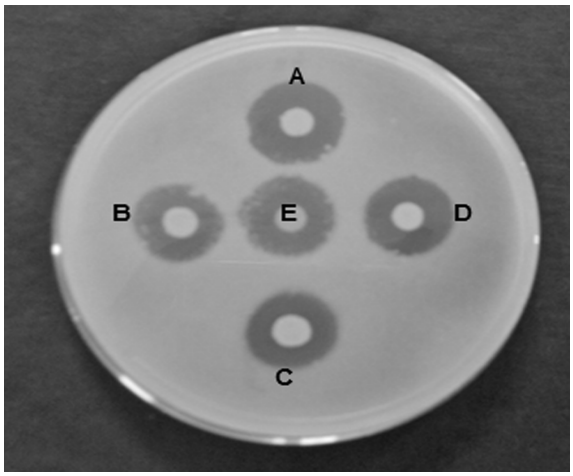

Fig. 2: Action of proteolytic enzymes and NaOH on the SAM produced by the strain Pes1. Spots from the producer strain were done by application of 10 µl of the bacterial culture onto surface of the plate. *A, B, C* and *D* correspond to the treatment with proteinase K, pronase XXIII, trypsin and NaOH, respectively. *E* corresponds to control, without treatments. The indicator strain used in these experiments was *B. thuringiensis* ATCC33679. The activity of SAM Pes1 was not affected by the proteolytic enzymes or NaOH. Substances produced by Ame3 and Mam2 presented the same results.

The best known contribution of the genus *Bacillus* for the food industry is the production of a wide variety of enzymes such as phytase, xylanase, ciclodextrinase, keratinase and amylase (Schallmey *et al*., 2004; Muhammad *et al*., 2009). However, as observed in our study, this genus may produce antimicrobial substances that are active against micro-organisms involved in spoilage and foodborne diseases as fungi and staphylococci.

CONCLUSION

The detection of these antagonistic substances revealed interesting properties that justify its importance and its study on the potential application in biological control of pathogenic microorganisms and spoilage food. For these reasons, the biochemical nature and the best conditions to production of the substances studied in this work are being investigated to further purification experiments.

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