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Original Article

Pathogen killing pathogen: antimicrobial substance from *Acinetobacter* active against foodborne pathogens

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Abstract

Introduction: Antimicrobial substances (AMS) produced by bacteria may reduce or prevent the growth of pathogenic and spoilage microorganisms in food. In this study, 16 isolates of *Acinetobacter baumannii/calcoaceticus* (ABC) complex, previously obtained from reconstituted infant milk formula (IMF) samples and the preparation and distribution utensils from the nursery of a public hospital, were used to screen for AMS production.

Methodology: Antimicrobial substance production and spectrum of activity assays were performed by agar-spot assay. Optimization of growth conditions for AMS production was also evaluated.

Results: Three (17.6%) isolates, namely JE3, JE4, and JE6, produced AMS against the principal indicator strain *Salmonella enterica* subsp. *enterica* serotype Typhi ATCC 19214. JE6 was also able to inhibit strains of *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Bacillus cereus,* a Gram-positive bacteria. Remarkably, JE6 was able to inhibit all the tested resistant and multidrug-resistant (MDR) strains of the ABC complex and *Shigella dysenteriae* associated with IMF and utensils, indicating a potentially valuable application. AMS produced by JE6 does not appear to be affected by proteolytic enzymes and the producer strain showed specific immunity to its own AMS.

Conclusion: This study highlights AMS produced by *Acinetobacter* with applications against MDR spoilage and foodborne pathogens - some of them, infectious disease causing agents - which, to our knowledge, has not been previously described.

Key words: *Acinetobacter baumannii/calcoaceticus*; antimicrobial substance; foodborne pathogens; bacteriocin.

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Introduction

Most commercially available preservatives and antibiotics are produced by chemical synthesis, and their long-term consumption can cause harm to consumer health or lead to microbiota reduction in the gut. Therefore, natural foods without chemical additives have become increasingly popular owing to their health benefits [1].

The use of antimicrobial substances (AMS) with antagonistic properties has become the prime candidate in food safety and preservation research. In foods and beverages, the addition of antimicrobial compounds to processed products has become a potent weapon in food preservation.

Bacteriocins form a small subgroup within AMS and have potential uses in food preservation. Bacteriocins are proteins or peptides produced by bacteria that have antimicrobial properties [2-4]. Bacteriocins differ from most therapeutic antibiotics because they have a biologically active protein component (being rapidly digested by the proteases of the human digestive system), are ribosomally synthesized, and have a narrower activity spectrum [5,6].

Biopreservation of foods using bacteriocins can be achieved by either the addition of bacteriocinogenic cultures or the direct addition of the purified substances. Bacteriocin-producing probiotic strains can establish a microbiota balance in the digestive tract, reducing gastrointestinal diseases. Alternatively, purified bacteriocins can be added directly to foods as a natural preservative [1]; this is the most plausible option when potentially pathogenic bacteria are the producers.

Acinetobacter spp. have been studied for several years to determine possible clinical, industrial, and environmental applications (Table 1); however, according to Amorim and Nascimento [7], few studies have reported the association of *Acinetobacter* spp. with food. Some studies cite their presence as bacteria that, along with others of different genera, contribute to the

taste, odor, and texture of foods (especially dairy products), owing to their proteolytic and lipolytic [8,9]. Conversely, other researchers describe *Acinetobacter* as potential pathogens, but do not emphasize their role in food. However, studies such as those by Gurung and coworkers [10] and Dijkshoorn [11], report the isolation of *Acinetobacter* strains from dairy products and claim that these bacteria may be opportunistic pathogens associated with food.

Acinetobacter strains have been recently associated with infant milk formula (IMF) and the utensils used in its preparation and distribution [12]. Surprisingly, *Acinetobacter baumannii/calcoaceticus* (ABC) complex were the most frequently isolated bacteria (37.8%), followed by *Enterobacter cloacae* (26.7%) and other members of the *Enterobacteriaceae* family, which were expected to be the most commonly isolated microorganisms. This prevalence of *Acinetobacter* indicated that these isolates could be producers of AMS capable of inhibiting the growth of other common foodborne bacteria.

Therefore, the present study aims to detect AMS production by ABC isolates from IMF and utensils used, and to determine the optimal growth conditions for the production of AMS, which may have potential application against foodborne pathogens and/or food spoilage microorganisms.

Methodology

Bacterial strains and growth conditions

Sixteen isolates of *Acinetobacter baumannii/calcoaceticus* (ABC) complex (obtained in a previous study), from reconstituted IMF samples and sanitized utensils used in preparation/distribution, from the nursery of a public hospital in Rio de Janeiro, Brazil [12], were used as potential AMS producer strains in this study (Table 2).

All microorganisms were grown in Casoy broth (Himedia, São Paulo, Brazil) at 37°C for 18 hours. When necessary, the medium was supplemented with either 15 g/L or 6 g/L agar (solid or soft agar, respectively). Stock cultures were maintained at -20°C in Tryptic Soy Broth (Himedia, São Paulo, Brazil) containing 40% (v/v) glycerol.

Antimicrobial substance production and spectrum of activity assays

The agar-spot assay was performed as described by Giambiagi-deMarval *et al*. [13] with minor modifications. Each AMS producing ABC strain was grown in 5 mL of Casoy broth for 18 hours at 37°C. Five microliters of culture (approximately 5.0×10^6 cells) were spotted onto Casoy agar plates. After 18 hours at 37°C, the bacteria were killed by exposure to chloroform vapor and the plates were sprayed with the indicator strain culture *Salmonella enterica* serotype Typhi ATCC 19214 (0.3 mL of a previously grown culture in 3 mL of Casoy soft agar). This strain was chosen according to Damaceno and coworkers [14]. Plates were further incubated at 37°C for 18 hours and the diameters (in mm) of the inhibition zones were measured. To determine the spectrum of activity, Gram-negative and Gram-positive strain (Table 2) were used as indicators. The sixteen ABC used as potential AMS producers were also used as indicators.

Determination of proteinaceous nature

The effects of the proteolytic enzymes pronase (Sigma-Aldrich, São Paulo, Brazil), proteinase K (Sigma-Aldrich, São Paulo, Brazil), and trypsin (Sigma-Aldrich, São Paulo, Brazil) on AMS activity were determined in accordance with Giambiagi-deMarval and coworkers [13], with minor modifications. The enzymes (1 mg/mL) were prepared in 0.05 M Tris (pH 8.0) with 0.01 M $CaCI_2$, and 50 µL were applied around the producer spots after chloroform treatment. Plates were incubated at 37°C for 4 hours and then sprayed with the indicator strain.

Table 1. Current usage and possible environmental and industrial applications of *Acinetobacter* spp. and their products.

Applications	Examples	References
Bioremediation of waste waters and effluents	- Phosphate removal - Crude oil degradation	[31-35]
Bioremediation of industrial pollutants	- Bioremediation of effluents contaminated with heavy metals	[32,36,37]
Stabilization of oil-water emulsions, biosorption, and bioemulsans	- For use in paper-making, incorporation in shampoos and detergents, emulsification of oil waste pollutants, and in food industry products	[32,38-41]
Other potential applications	- Production of carnitine, immune adjuvants, and glutaminase-asparaginase (for clinical use in cancer treatment) - Plant growth promoters and bio-control agents against phytopathogens - Production of cellulolytic enzymes and alkaline lipase	[31-33,41-42]

Absence of inhibition zones indicated that the AMS was of proteinaceous nature. To discard the possibility that the inhibition exhibited might have been due to acids produced by the producer strain during its metabolism, the antimicrobial substances were also treated with 0.2 N NaOH.

Influence of growth conditions on AMS production

To evaluate the effect of the culture medium on AMS production, the producer strain was grown as previously described, and 5 µL of culture were spotted onto the surface of plates containing 25 mL of the following solid media: brain heart infusion (BHI, Isofar, Rio de Janeiro, Brazil), Casoy (Isofar, Rio de Janeiro, Brazil), Müller-Hinton (Himedia, São Paulo, Brazil), and nutrient agar (Himedia, São Paulo, Brazil).

The influence of the initial pH, growth temperature, and NaCl on AMS production was determined as described by Fleming and coworkers [15]. Because Casoy agar presented the best results (inhibition halos with the largest diameters), it was used in further experiments. The producer strains were spotted on Casoy agar plates and incubated at 37°C for 18 hours. The pH of the culture media was adjusted to achieve pH values of 5.0, 6.0, 7.0, and 8.0 with 1 N HCl or 1 N NaOH. The effect of the growth temperature was evaluated by incubating Casoy agar plates at room temperature (27°C), 37°C, and 42°C for 18 hours, and the influence of salt was determined by growing the producer strains on Casoy agar plates with different NaCl concentrations (0.5, 1.0, 2.0, and 3.0 g/L).

Table 2. Bacterial strains used as producers and indicators of antimicrobial substances.

Strains	Relevant characteristics	Reference/Source
Producer/Indicator strains		
Acinetobacter baumannii/calcoaceticus JE2	MDR	[12]
A. baumannii/calcoaceticus AE1	MDR	[12]
A. baumannii/calcoaceticus JE3	MDR	[12]
A. baumannii/calcoaceticus JE4	MDR	[12]
A. baumannii/calcoaceticus JE6	MDR	[12]
A. baumannii/calcoaceticus JE7	MDR	[12]
A. baumannii/calcoaceticus JR1	MDR	[12]
A. baumannii/calcoaceticus JR2	MDR	[12]
A. baumannii/calcoaceticus JR3	MDR	[12]
A. baumannii/calcoaceticus JR4	MDR	[12]
A. baumannii/calcoaceticus JR5	MDR	[12]
A. baumannii/calcoaceticus JR6	MDR	[12]
A. baumannii/calcoaceticus ME2	MDR	[12]
A. baumannii/calcoaceticus MR1	MDR	[12]
A. baumannii/calcoaceticus PR1	Non-MDR	[12]
A. baumannii/calcoaceticus PR2	Non-MDR	[12]
Indicator strains		
Bacillus cereus LMIFRJ	-	LMIFRJ
Escherichia coli ATCC25922	-	ATCC
E. coli LMIFRJ	-	LMIFRJ
Klebsiella pneumoniae ATCC4352	-	ATCC
Pseudomonas aeruginosa ATCC 27853	-	ATCC
P. fluorescens ATCC13525	-	ATCC
Proteus mirabilis LMIFRJ	-	LMIFRJ
P. vulgaris LMIFRJ	-	LMIFRJ
Salmonella enterica serotype Typhi ATCC19214	-	ATCC
Serratia marcescens LMIFRJ	-	LMIFRJ
Shigella dysenteriae BIR3	Non-MDR	[22]
S. dysenteriae ME1	MDR	[22]
S. dysenteriae ME3	MDR	[22]
S. dysenteriae ME4	MDR	[22]
S. dysenteriae ME5	MDR	[22]
Staphylococcus aureus ATCC12600	-	ATCC
S. aureus ATCC25923	-	ATCC

ATCC, American Type Culture Collection; LMIFRJ, Collection of the Laboratory of Microbiology of the Instituto Federal do Rio de Janeiro; MDR, multidrug resistant.

The effects of aeration conditions on bacteriocin activity were evaluated after incubation of the producer strains spotted on Casoy plates at 37°C, both aerobically and anaerobically. Anaerobic conditions were created using the AnaeroGen atmosphere generation system (Oxoid Ltd., Hampshire, England).

For these experiments, *S.* Typhi, *Bacillus cereus* and *Proteus vulgaris* were used as indicator strains.

Statistical analysis

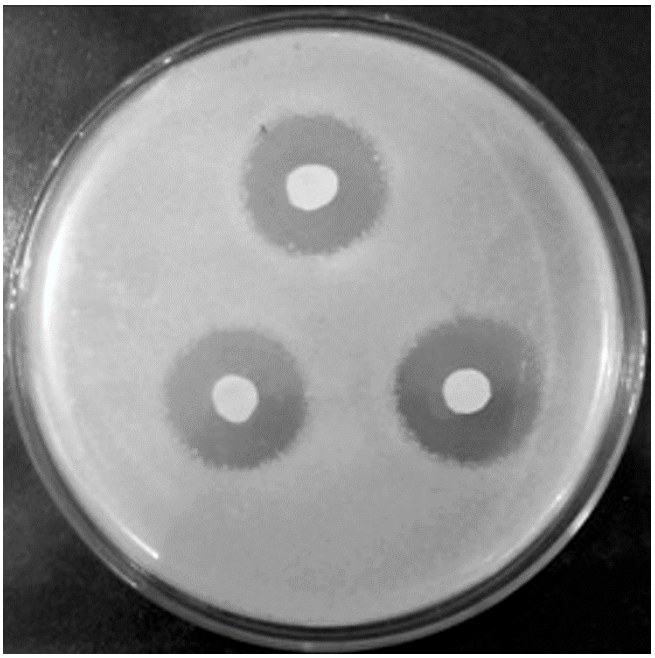
One-way ANOVA with Tukey's post-hoc test was used to assess differences in the parameters. For all significance tests, p values < 0.05 were considered statistically significant. At least three replicates of each experiment were performed.

Results

In this study, sixteen isolates of ABC complex were tested for AMS production. Three (18.7%) were isolated from IMF preparation jars, namely, JE3, JE4, and JE6, and were able to produce AMS against the indicator strain *Salmonella* Typhi, chosen as the main indicator strain due to its sensitivity to some antimicrobial substances produced by Gram-negative bacteria [14,16].

Isolate JE6 was able to inhibit *S.* Typhi, *Klebsiella pneumonia*, *Proteus vulgaris*, and remarkably, *B. cereus* (Figure 1), a Gram-positive bacterium. Therefore, JE6 was chosen for the subsequent experiments.

Figure 1. Agar-spot assay demonstrating the inhibitory activity of JE6 AMS represented by the clear zones of inhibition against the indicator strain *Bacillus cereus*.



JE6 was also able to inhibit the others 15 *ABC* isolates tested (12 were MDR), indicating the potential application of this AMS against these MDR pathogens. Assays were performed with proteolytic enzymes (protease and trypsin) to verify whether the activity of JE6 AMS is affected, however, regardless of the indication used, here was no inhibition of AMS by

Table 3. Effect of growth conditions on antimicrobial substance production by *Acinetobacter baumannii/calcoaceticus* JE6.

		Indicator strains		
		Salmonella* Typhi	***Bacillus cereus***	***Proteus vulgaris***
Growth media	Casoy agar	13.0 ± 1.8 [a]	30.4 ± 0.5 [a]	21.6 ± 1.3 [a]
	Müller-Hinton agar	- [b]	17.7 ± 1.1 [b]	- [b]
	Nutrient agar	- [b]	32.0 ± 5.0 [a]	- [b]
	BHI agar	13.3 ± 0.5 [a]	27.8 ± 0.9 [a]	15.2 ± 1.1 [c]
pH	5.0	29.0 ± 5.0 [a]	32.0 ± 3.6 [a]	19.6 ± 8.7 [a]
	6.0	21.0 ± 3.0 [b]	29.8 ± 2.1 [a]	29.3 ± 0.9 [b]
	7.0	18.0 ± 2.0 [b]	23.4 ± 0.5 [b]	26.6 ± 2.0 [b]
	8.0	16.7 ± 1.9 [b]	18.0 ± 1.0 [b]	- [c]
NaCl	control	18.0 ± 3.3 [a]	32.3 ± 3.3 [a]	15.0 ± 0.8 [a]
	1.0%	17.5 ± 1.7 [a]	25.0 ± 2.3 [b]	12.7 ± 1.8 [a]
	2.0%	20.0 ± 1.1 [a]	27.0 ± 1.1 [b]	15.5 ± 3.5 [a]
	3.0%	19.0 ± 4.3 [a]	26.5 ± 2.0 [b]	13.3 ± 2.6 [a]
Temperature	28°C	- [a]	26.6 ± 1.7 [a]	17.7 ± 2.4 [a]
	37°C	20.7 ± 2.5 [b]	34.3 ± 1.8 [b]	23.5 ± 2.4 [b]
	42°C	- [a]	28.2 ± 4.8 [a]	- [c]
Atmosphere	Aerobic conditions	17.2 ± 2.4 [a]	24.0 ± 1.6 [a]	32.8 ± 4.4 [a]
	Anaerobic conditions	- [b]	9.7 ± 3.34 [b]	19.3 ± 2.6 [b]

The numbers represent the means and standard deviations of the diameters of inhibition zones (in mm) from three independent experiments; -, absence of inhibition halo or less than 2 mm; [a, b, c], different letters indicate statistically significant difference among the parameters; * *Salmonella enterica* serotype Typhi ATCC19214.

protease and trypsin enzymes. With regards to NaOH, the inhibitory activity produced by the JE6 isolate was also unaffected, indicating that the antimicrobial action is not due to acid production by the producing bacteria.

To determine suitable conditions for the maximum production of AMS, JE6 the producer strain was inoculated in four different media (Table 3). Müller-Hinton and nutrient agar media did not allow the production of AMS by strain JE6 when using indicators *S. enterica* and *P. vulgaris*. However, there was inhibition of the *B. cereus* indicator when the JE6 strain was grown in these two culture media. No significant difference ($p < 0.05$) in the diameters of the inhibition halos was detected between BHI and Casoy media, however, visibly, the JE6 strain produced larger and more limpid halos in Casoy medium, which was then used in subsequent experiments.

The AMS of JE6 was produced at room temperature (27°C), 37°C, and 42°C; AMS production was slightly higher at 37°C when *B. cereus* was the indicator. However, when using *S. enterica* and *P. vulgaris* as indicators, AMS production was only detected at 37°C, providing further evidence that more than one type of BLIS is produced by the JE6 strain.

Apparently, AMS that act against *B. cereus* are capable of being produced under less demanding nutrient conditions (Muller-Hinton agar and nutrient agar) and under different temperatures, whereas AMS that inhibit *S. enterica* and *P. vulgaris* require growth of the JE6 strain in a richer media and at specific temperatures.

Growth in Casoy medium, with initial pH ranging from 5.0 to 8.0, did not affect the production of AMS by the JE6 isolate, although it was significantly higher at pH 5.0 for *B. cereus* and *S. enterica*, and under pH 6.0 for *P. vulgaris*. Varying the NaCl concentrations (up to 3%) in Casoy agar had little effect on JE6 AMS production; the only significant difference observed was in relation to inhibition of *B. cereus*, which was slightly higher without the addition of NaCl.

Production of AMS JE6 was also evaluated after the aerobic and anaerobic growth of the producer strain. Inhibition zones for the three indicator strains were significantly reduced under anaerobic growth, suggesting that aerobic conditions are needed for AMS JE6 production.

Thus, in general, the optimal conditions for the production of AMS by JE6 may be represented by growth in Casoy agar at 37°C with an initial pH of 5.0, in the absence of NaCl, and under aerobic conditions.

Discussion

AMS-producing microorganisms have a competitive advantage in a particular ecological niche. Consequently, the biotechnology industry is beginning to show an interest in the potential application of these microorganisms and prospecting of such strains has begun. However, Gram-negative enteropathogenic bacteria such as *Escherichia*, *Salmonella*, *Enterobacter*, and *Klebsiella* are rarely inhibited by Gram-positive AMS, such as bacteriocins [16,17]. In contrast, Gram-negative bacteriocins, including microcin C7, colicins E1 and Ib, and the bacteriocin produced by *E. coli* strain Nissle 1917, have already shown *in vitro* and *in vivo* inhibition of several Gram-negative pathogens [17-19].

In our previous study, four representatives of the ABC group were able to inhibit indicator strains *E. coli* ATCC25922 and *S. enterica* ATCC19214 [14]. These two species of bacteria are among the major causes of foodborne diseases. As far as we are aware, the study by Damaceno and coworkers was the first research reporting the production of an AMS by *Acinetobacter* strains.

In general, antimicrobial substances, such as bacteriocin-like inhibitory substances (BLIS) and bacteriocins produced by Gram-negative bacteria, have a narrow antimicrobial activity spectrum, conferring a disadvantage that limits their industrial-scale application [2,20]. However, in this work, isolate JE6 was able to inhibit *S.* Typhi, *Klebsiella pneumonia*, *Proteus vulgaris*, and remarkably, *B. cereus* (Figure 1), a Gram-positive bacterium. Therefore, JE6 was chosen for the subsequent experiments.

Gram-negative pathogens pose serious threats to global public health as treatment options are limited owing to the spread of antibiotic resistance among Gram-negative bacteria [21]. These resistant bacteria are increasingly found in foods of different origins.

Recently, resistant and multi drug-resistant (MDR) strains of *Shigella* sp. were isolated from dairy products and associated food preparation utensils [22,23]. JE6 was able to inhibit all five of the *S. dysenteriae* strains isolated from lactary utensils, four of which presented a typical MDR profile. *Shigella dysenteriae* has been associated with shigellosis, an acute enteric infection that poses an important public health problem in developing and underdeveloped countries, and may have potentially devastating consequences for children and newborns [22]. According to our results, an AMS, such as that produced by JE6, may have potential for application as a potent inhibitor of foodborne pathogens, including, those resistant to antibiotics. To our knowledge, this is the first study reporting

inhibition of this MDR species by AMS produced by an ABC isolate.

Curiously, JE6 is also an MDR strain, but when it was tested as an indicator and producer, no inhibition was observed, suggesting that this strain has a specific immunity mechanism to its own AMS, that is a characteristic of bacteriocins [1]. This suggests that the JE6 AMS is not a typical bacteriocin. Resistance of AMS to proteolytic enzymes is not uncommon. Studies performed with AMS produced by *Bacillus* species have demonstrated that these are insensitive to enzymes such as proteinase K and trypsin. The presence of proteolytic enzymes may occur due to the unusual amino acids present in the structure of the N-terminal or C-terminal protected bacterial or cyclic peptides [24-26].

Production of AMS can be influenced by growth [27,28] , therefore determining the optimal conditions for the maximum production of AMS is very important for further studies on purification and cost efficacy.

The production of AMS by strain JE6 was detected in the four culture media used in this sudy, when the indicator strains were *S. enterica* and *P. vulgaris*. However, when the indicator strain was *B. cereus,* production of AMS JE6 was not detected in Müller-Hinton and nutrient agar. Our results suggest that *B. cereus* is more resistant to the AMS in these 2 media or that JE6 produces more than one AMS. Production of one or more AMS by a single strain has already been reported in lactic acid bacteria isolated from malted barley, where *Lactobacillus sakei* was able to produce two new bacteriocins called sakacin 5X and sakacin 5T. The inhibitory spectrum of each purified bacteriocin was analyzed and sakacin 5X was shown to inhibit a larger variety of microorganisms responsible for beer degradation than sakacin 5T [29].

Optimal conditions for the production of AMS by JE6 were similar to that found for the production of bacteriocin EC2, produced by *E. coli*, with inhibitory activity against other strains of *E. coli* and *S. enterica* [30]. The optimal conditions for EC2 production in Casoy medium, were growth at 37°C, with an initial pH of 6.0, without the addition of NaCl. Studies with antimicrobial substrates produced by *Klebsiella ozaenae* K and *Raoultella terrigena* L, demonstrated that the best conditions of AMS production by these strains were also in Casoy agar, at 37°C, with an initial pH of 6.0, and with NaCl concentrations ranging from 0.5 to 3.0% [15].

Under these conditions, by replacing Casoy agar with Casoy broth, experiments were performed to verify whether JE6 AMS could be obtained from the supernatant of the producing strain. The initial results were very promising and suggested that antimicrobial activity against the three main indicator strains - *S.* Typhi, *B. cereus* and *P. vulgaris* - could be detected (data not shown). Subsequent experiments will assess their activity spectrum using preparations of reconstituted IMF as a food matrix artificially contaminated with the inhibited pathogens.

Conclusion

The present study elucidates a potential novel and important application for ABC, as the AMS from isolate JE6. The antimicrobial substance reported in this work exhibited efficacy in controlling potentially pathogenic and food spoilage micro-organisms, including those with MDR characteristics, conferring an interesting approach for food safety. Additional studies are required to elucidate the nature, the mechanisms and genetics of the inhibition through this substance.

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