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Anti-bacterial Efficacy of Bacteriocin Produced by Marine *Bacillus subtilis* Against Clinically Important Extended Spectrum Beta-Lactamase Strains and Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Objective: *To investigate the anti-bacterial efficacy of bacteriocin produced by Bacillus subtilis SM01 (GenBank accession no: KY612347), a Gram-positive marine bacterium, against Extended Spectrum Beta-Lactamase (ESBL) producing Gram-negative pathogens Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli, and Gram-positive pathogen Methicillin-Resistant Staphylococcus aureus (MRSA).* ***Methods:*** *A marine bacterium was isolated from mangrove sediment from the Red Sea coast of Jeddah, Kingdom of Saudi Arabia, and identified based on its morphological, biochemical, and molecular characteristics. The bacteriocin production using this isolate was carried out in brain heart infusion broth (BHIB) medium. The Anti-bacterial activity of bacteriocin was evaluated against selected ESBL strains and MRSA by the well agar method. The effects of incubation time, pH, and temperature on the Anti-bacterial activity were studied.* ***Results:*** *The bacteriocin Bac-SM01 produced by B. subtilis SM01 demonstrated broad-spectrum Anti-bacterial activity against both Gram-negative and -positive bacteria. The present study is the first report that the bacteriocin Bac-SM01 inhibits the growth of ESBL producing Gram-negative strains A. baumannii, P. aeruginosa, and E. coli, and a Gram-positive MRSA strain. The optimum incubation time, pH, and temperature for the Anti-bacterial activity of Bac-SM01 was 24 h, 7, and 37°C respectively.* ***Conclusion:*** *The overall investigation can conclude that the bacteriocin Bac-SM01 from the marine isolate Bacillus subtilis SM01 could be used as an alternative Anti-bacterial agent in pharmaceutical products.*

Keywords: Marine mangrove isolate, Bacteriocin, Optimization, Anti-bacterial activity

INTRODUCTION

Bacteriocins are ribosomally synthesized, antimicrobial proteins or peptides that inhibit the growth of several human, animal, food, and plant pathogens [1-3]. Bacteriocins inhibit the growth of sensitive cells or kill them by interfering with the synthesis of the cell wall or by forming pores in the cell membrane. The first bacteriocin was discovered by Gratia in 1925, and in recent years, many other bacteriocins have been successively identified [4].

Many Gram-positive and Gram-negative bacteria are known to produce bacteriocins, and among them, lactic acid bacteria (LAB) and *Bacillus* species are the most well-known producers [5,6]. LAB and Bacilli are generally recognized as safe (GRAS) organisms [7].

Bacteriocins from *Bacillus* species offer a much broader spectrum of potential applications compared with LAB bacteriocins. Applications of *Bacillus* bacteriocins have been reported already in the fields of human health, livestock, agriculture, and food preservation [8-11].

Antimicrobial resistance (AMR) is an increasingly serious threat to public health globally. Extended Spectrum Beta-lactamase (*ESBL*) genes and Metallo Beta-Lactamase (*MBL*) genes present in the Gram-negative bacteria make them resistant to penicillin; first, second and third generation cephalosporins; and aztreonam antibiotics [12,13]. The increasing bacterial resistance to conventional antibiotics of clinical application has resulted in a growing interest in the consideration of bacteriocins as alternative antimicrobials for the treatment of human and animal infections [14]. Therefore, in the present investigation we aimed to assess the Anti-bacterial efficacy of bacteriocin from the marine isolate *Bacillus subtilis* SM01.

MATERIALS AND METHODS

Isolation and Identification of Marine Bacterium

Bacillus subtilis SM01 was isolated from mangrove sediment from the Red Sea coast of Jeddah (Latitude: 21°32′32″ N, Longitude: 39°11′52″ E), Kingdom of Saudi Arabia, by serial dilution in Brain Heart Infusion (BHI) agar and Blood agar medium. The isolate was identified based on its phenotypic culture characteristics, Gram stain reaction, motility, and biochemical characteristics. An array of biochemical tests, including catalase, oxidase, indole, methyl red, Voges-Proskauer, triple sugar iron, citrate utilization, urease, nitrate reduction, and carbohydrate fermentation tests, were performed as per the standard protocols [15].

Collection of Pathogens

ESBL producing Gram-negative pathogens *A. baumannii*, *P. aeruginosa*, *E. coli* and Gram-positive pathogen Methicillin-Resistant *S. aureus* (MRSA) were selected for the screening of the Anti-bacterial activity. The bacterial strains were obtained from Department of Medical Laboratories, College of Science, Al Zulfi, Majmaah University, Kingdom of Saudi Arabia. Cultures were maintained in screw-capped test tubes containing nutrient agar (Hi-media) slants and stored at 4°C. All cultures were checked for viability and purity by regular plating.

Inoculum Preparation

Pure cultures of *A. baumannii*, *P. aeruginosa*, *E. coli*, and *S. aureus* were inoculated with nutrient broth (Hi-media) and incubated at 37°C for 24 hours. The inoculum size was adjusted to yield uniform suspensions containing approximately 1.5×10^8 CFU/mL (No. 0.5 McFarland turbidity standard).

Bacteriocin Production

The strain of *Bacillus subtilis* SM01 (2.0% inoculum, v/v) was grown in 500 mL of BHI medium (Hi-media,) at 37°C in a rotary shake at 110 cycles per min for 16 h. Cells were removed by centrifugation at 10,000 g for 15 min. The supernatant fluid was adjusted to pH 6.8, sterilized with 0.45 µM filter membranes and then stored at 4°C until utilization [16]. This preparation was designated as the crude bacteriocin Bac-SM01. The filtrates were used for the characterization of bacteriocin.

Anti-bacterial Assay

The Anti-bacterial activity of bacteriocin was evaluated against selected strains by the well agar method [17]. Culture plates were prepared by adding 20 ml of sterile nutrient agar medium. The surface of the plates was swabbed with indicator strains in three directions, turning the plates by 60° between each swabbing. Wells (5 mm) were then made in the medium using sterile cork borer, and 25 µl of crude bacteriocin Bac-SM01 was transferred into these wells. The plates were incubated for 24 hours at 37°C. After incubation, the diameter of the zone of inhibition was measured. All the experiments were performed in triplicate and the results were the mean of the observations. The antibiotic sensitivity test was analyzed using the standard antibiotics such as piperacillin/tazobactam (100 µg/10 µg) for Gram-negative bacteria and levofloxacin (5 µg) for the Gram-positive bacterium *S. aureus*.

Effect of Incubation Time, pH and Temperature on Anti-bacterial Activity

The effect of different incubation times (12, 24, 36, 48, 60, and 72 hours), pH (3, 5, 7, 9, and 11), and temperatures (27°C, 37°C, 47°C, 57°C and 67°C) on the Anti-bacterial activity of bacteriocin in BHI medium was studied. All the samples were tested for the Anti-bacterial activity against each pathogen. All the experiments were performed in triplicate and the results were the mean of the observations.

Molecular Characterization and Gene Sequencing Analysis

Once the bacteriocin proved to have Anti-bacterial activity, DNA was extracted using Invitrogen pure link genomic DNA kit (Catalog no: K1820-01, USA). The DNA was quantified using a Nanodrop (Thermo Scientific, USA). The PCR products were amplified in proFlex PCR system, Thermal cycler, (Applied biosytem, USA). The PCR condition for the amplification of 16S ribosomal DNA were optimized. The PCR master mix contained universal forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer: 5′ACGGCTACCTTGTTACGACTT-3′,10 mM dNTPS, 10PCR Buffer, 1 U Taq DNA polymerase, 2 mM Mgþ and (100e200 ng) template DNA. The PCR steps included initial denaturation at 95°C for 5 min, then 35 PCR cycles at 95°C for 1 min, 56°C for 2 min, 72°C for 1 min and finally at 72°C for 10 min. The PCR products were sequenced using the BigDye terminator cycle sequencing kit (V3.1 3500 series part no. 4336917 LOT: 1501371) on an ABI 3500 automatic DNA sequencer (Applied Biosystems, CA, USA). The genomic sequences were phylogenetically aligned with reference homology gene sequence of NCBI data base (Software used: SeqScapeTM Software v3.0, Thermofisher Scientific Catlog no: 4474978) and deposited in GenBank (Accession no: KY612347).

RESULTS

The marine isolate was identified as *Bacillus subtilis* SM01 (KY612347) by its phenotypic and molecular characteristics (Figures 1-3). The test results are summarized in Table 1. The results of the Anti-bacterial activity and antibiotic sensitivity test are shown in Table 2. The largest zone of inhibition of crude bacteriocin Bac-SM01 was observed against *S. aureus* (19.5 ± 1.23 mm), followed by *P. aeruginosa* and *E. coli* (11.7 ± 1.16 and 11.6 ± 0.80 mm, respectively). The smallest zone of inhibition was detected against *A. baumannii*. The bacteriocin activity was compared with standard antibiotics piperacillin/tazobactam and levofloxacin. The crude bacteriocin Bac-SM01 exhibited greater inhibitory activity against the tested pathogens when compared with standard antibiotics (Figure 4).

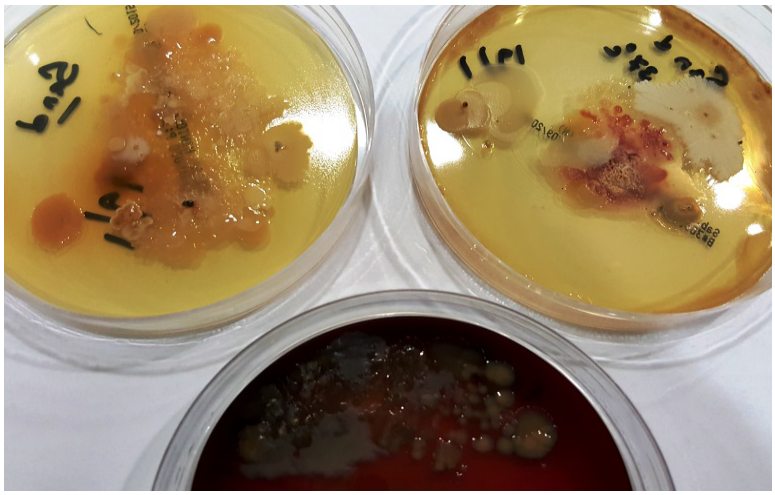


Figure 1 Primary isolation of *Bacillus subtilis* on Brain Heart Infusion and Blood agar media

Figure 2 Purified colonies of *B. subtilis* SM01 on Chocolate agar media

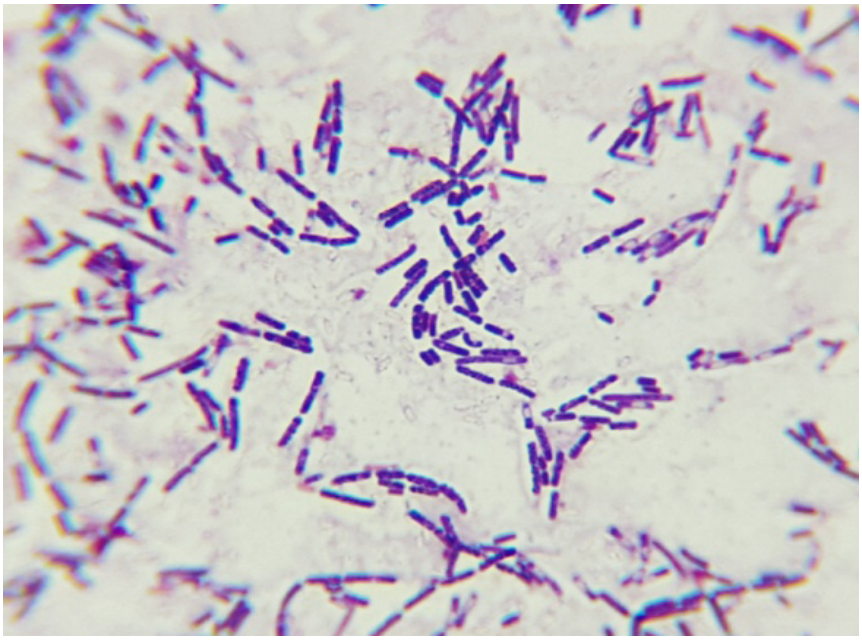


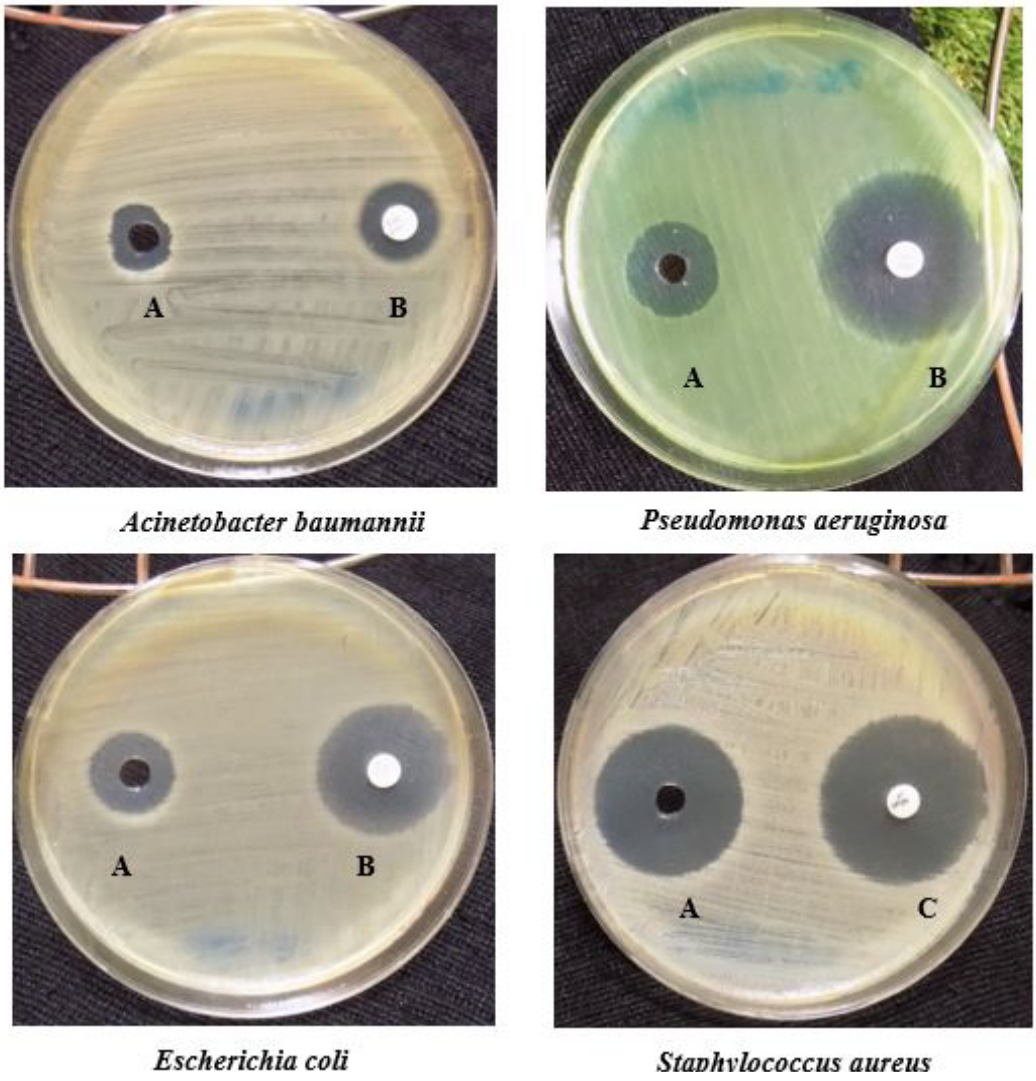
Figure 3 A gram-stained sample of B. subtilis SM01

Table 1 Phenotypic characteristics of a marine isolate

Tests	Observations
Gram staining	Positive, rod
Motility	Motile
Indole Test	Negative
MR Test	Negative
VP Test	Positive
Catalase Test	Positive
Oxidase Test	Negative
Citrate Test	Positive
Nitrate reduction test	Positive
TSI Test	Gas production & acid butt
Urease Test	Negative
Carbohydrate fermentation test	Positive
MR-Methyl Red; VP-Voges-Proskauer; TSI-Triple Sugar Iron	

Table 2 Anti-bacterial activity of bacteriocin produced by *B. subtilis* SM01

S. No.	Indicator strains	Zone of inhibition (Diameter in mm)	Standard antibiotics (Concentration)	Zone of inhibition (Diameter in mm)
1	*A. baumannii*	9.3 ± 1.11	Piperacillin/Tazobactam (100 µg/10 µg)	10.6 ± 0.67
2	*P. aeruginosa*	11.7 ± 1.16	Piperacillin/Tazobactam (100 µg/10 µg)	25.9 ± 1.27
3	*E. coli*	11.6 ± 0.80	Piperacillin/Tazobactam (100 µg/10 µg)	22.4 ± 1.31
4	MRSA	19.5 ± 1.23	Levofloxacin (5 µg)	26.4 ± 1.71
Results expressed as Mean ± Standard Deviation (n-3)				



A - Crude bacteriocin; B - Standard antibiotics (Piperacillin/Tazobactam); C- Standard antibiotic (Levofloxacin)

Figure 4 Anti-bacterial activity of crude bacteriocin Bac-SM01 produced by *Bacillus subtilis* SM01 and standard antibiotics

Incubation time plays a vital role in anti-bacterial activity. Samples were therefore incubated for a range of times, from 12 to 72 hrs. Maximum activity was achieved at 24 hours, and then as the time increased, activity decreased (Table 3). No Anti-bacterial activity was observed after 72 hours incubation.

Table 3 Effect of incubation time on Anti-bacterial activity of bacteriocin produced by *B. subtilis* SM01

S. No.	Incubation time (Hours)	Zone of inhibition (Diameter in mm)			
		A. baumannii	*P. aeruginosa*	*E. coli*	*S. aureus*
1	12	7.8 ± 0.75	8.2 ± 0.65	8.6 ± 0.67	8.9 ± 1.11
2	24	9.1 ± 1.00	10.4 ± 0.98	10.6 ± 1.16	13.5 ± 1.35
3	36	8.5 ± 1.20	9.1 ± 0.4	9.8 ± 1.03	10.7 ± 1.31
4	48	7.9 ± 1.40	8.3 ± 0.70	8.5 ± 0.85	8.7 ± 0.71
5	60	-	-	8.3 ± 0.76	7.9 ± 0.95
6	72	-	-	-	-
Results expressed as Mean ± Standard Deviation (n-3), (-) – No activity					

Temperature and pH also play an essential role in the anti-bacterial activity of Bac-SM01 by *B. subtilis* SM01. The

maximum activity was achieved at pH 7 (Table 4). The bacteriocin activity decreased in alkaline conditions. The maximum anti-bacterial activity was at 37°C. No anti-bacterial activity was observed at 67°C (Table 5).

Table 4 Effect of pH on Anti-bacterial activity of bacteriocin produced by *B. subtilis* SM01

S. No.	pH	Zone of inhibition (Diameter in mm)			
		A. baumannii	***P. aeruginosa***	***E. coli***	***S. aureus***
1	3	8.5 ± 1.05	8.7 ± 0.78	9.7 ± 0.36	10.3 ± 1.31
2	5	8.8 ± 0.70	9.4 ± 0.76	10.3 ± 0.56	10.7 ± 0.86
3	7	10.2 ± 1.01	11.5 ± 0.95	13.5 ± 0.74	14.6 ± 0.76
4	9	8.5 ± 0.82	8.6 ± 0.70	9.4 ± 0.62	9.5 ± 0.91
5	11	7.8 ± 1.03	7.8 ± 0.38	9 ± 0.85	8.4 ± 0.79
Results expressed as Mean ± Standard Deviation (n-3)					

Table 5 Effect of temperature on Anti-bacterial activity of bacteriocin produced by *B. subtilis* SM01

S. No.	Temperature (°C)	Zone of inhibition (Diameter in mm)			
		A. baumannii	***P. aeruginosa***	***E. coli***	***S. aureus***
1	27	10.4 ± 0.55	9.8 ± 0.72	10.2 ± 0.56	11.5 ± 1.00
2	37	10.9 ± 0.51	10.7 ± 1.10	12.7 ± 0.97	14.2 ± 1.31
3	47	9.1 ± 0.75	8.9 ± 1.35	10.3 ± 0.45	11.6 ± 0.85
4	57	8.1 ± 0.55	7.8 ± 0.98	8.6 ± 0.75	9.1±0.40
5	67	-	-	-	-
Results expressed as Mean ± Standard Deviation (n-3), (-) – No activity					

DISCUSSION

In this study, a potential bacteriocin producing marine isolate was identified as *B. subtilis* SM01 (KY612347) by its phenotypic and molecular characteristics. The bacteriocin produced by this strain (Bac-SM01) exhibited significant Anti-bacterial activity against *ESBL* producing Gram-negative pathogens *A. baumannii*, *P. aeruginosa*, *E. coli* and Gram-positive pathogen MRSA. Our results are in accordance with the findings of Chopra, et al. [18], who reported that a bacteriocin, Sonorensin, from *Bacillus sonorensis*, exhibited broad-spectrum activity against both Gram-positive and Gram-negative bacteria. The present finding is inconsistent with other findings from Torkar and Matijasic [19], Gray, et al. [20] and Scholz, et al. [21], who stated that Gram-positive bacteria are mostly inhibitory to Gram-positive bacteria and are less effective against Gram-negative bacteria. Previously, bacteriocins from *Bacillus thuringiensis* subsp. kurstaki Bn1 [22], *Bacillus thuringiensis* SF361 [23], and *Bacillus subtilis* EMD4 [24] have been reported for their Anti-bacterial activity against several human pathogens.

In the present study, the growth of methicillin-resistant *Staphylococcus aureus* was strongly inhibited by Bac-SM01. Similarly, Mersacidin, produced by *Bacillus* sp. strain HIL Y85, was also active against methicillin-resistant *Staphylococcus aureus* [25]. Recently, subtilosin A produced by *B. subtilis* KKU213 showed diverse Anti-bacterial activity against Gram-positive bacteria, including *S. aureus* [26].

Incubation time plays an important role in bacteriocin activity. After 24 hours maximum Anti-bacterial activity of Bac-SM01 was achieved in BHIB medium. The Anti-bacterial activity of Bac-SM01 could not be detected at 72 hours which showed that Bac-SM01 produced either lost its activity during incubation or become unstable at 72 hours. The present finding concerning the effect of incubation time on the Anti-bacterial activity of Bac-SM01 was in agreement with those of Xie, et al. [16] and Sirtori, et al. [27] who stated that the maximum bacteriocin activity from *B. subtilis* LFB112 and *Bacillus* sp. strain P45 was attained at 16 and 30 hours, respectively.

Bac-SM01 had maximum activity at neutral pH 7.0. Bacteriocin retained its activity when the pH was changed from 3 to 11. Similar studies have been reported; bacteriocin BAC YAS 1 from *Bacillus* sp. YAS 1 strain showed Anti-bacterial activity over a wide range of pH (1-13) [28]. Amylolysin from *Bacillus amyloliquefaciens* showed Anti-bacterial activity at pH ranging from 2-9 and sonorensin produced by a marine isolate, *B. sonorensis* MT93, had Anti-bacterial activity at a variety of pH levels (2-12) [18,29]. Furthermore, bacteriocin activity from *B. subtilis* was stable within a pH range of 3-9 as reported by Liu, et al. [24] and Joseph, et al. [30].

The optimum temperature for the Anti-bacterial activity of Bac-SM01 was 37°C. There was no activity observed

at 67°C against the tested pathogens. These findings were in agreement with the results obtained with bacteriocin produced by *B. subtilis* KIBGE IB-17 [31]. To complement the above findings, it would be appropriate to further research the purification, molecular characterization, and large-scale production of Bac-SM01 using *B. subtilis* SM01.

CONCLUSION

An important finding in this study is that the bacteriocin Bac-SM01 produced by the marine isolate *B. subtilis* SM01 has the ability to inhibit the *ESBL* producing Gram-negative strains *A. baumannii*, *P. aeruginosa*, and *E. coli*, and the Gram-positive MRSA strain. It can be concluded that the bacteriocin Bac-SM01 could be used as an alternative Anti-bacterial agent in pharmaceutical products to control drug resistant bacteria. We look forward further to improving the efficacy and characterization Bac-SM01 bacteriocin.

DECLARATIONS

Acknowledgment

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Conflict of Interest

We declare that we have no conflict of interest.

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