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Antimicrobial peptides: Could cecropin A and nisin be new promising agents for the treatment of anaerobic infections

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Abstract: Antimicrobial peptides (AMPs) may mitigate the danger of increasing antimicrobial resistance. We aimed to determine the activities of catestatin, temporin A, nisin and cecropin A against *Bacteroides fragilis* ATCC 25285, *Prevotella melaninogenica* ATCC 25845, *Cutibacterium acnes* ATCC 6919, *Peptostreptococcus anaerobius* ATCC 27337 and *Peptostreptococcus stomatis* DSM 17678. strains. The susceptibility of all anaerobic bacteria was determined by Kirby-Bauer disc diffusion method, agar dilution and broth microdilution method, recommended by CLSI. By broth microdilution the MIC of temporin A for *P. anaerobius* was 500µg/mL, and MBC >500µg/mL. The MIC of nisin for *P. melaninogenica* was 200µg/mL, with a MBC of 400µg/mL, for *C. acnes*, *P. anaerobius*, and *P. stomatis*, MIC were 40mg/mL. The MIC of cecropin A for *B. fragilis* was 50µg/mL, MBC was 500µg/mL. For *C. acnes*, the MIC was 4µg/mL, MBC was 8µg/mL. The MIC for *P. melaninogenica*, *P. anaerobius* and *P. stomatis* were 8µg/mL, with corresponding MBC values of 16, 32 and 50µg/mL, respectively. Conversely, catestatin proved ineffective against all strains. In conclusion, our study, demonstrated that cecropin A and nisin showed promising results against anaerobic standard strains. We believe thatfurther research conducted to explore those AMPs could hold promise as a treatment option for anaerobic bacterial infections.

Keywords: Antimicrobial peptides, anaerobic bacteria, antimicrobial susceptibility, minimal inhibitory concentrations, minimum bactericidal concentration.

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INTRODUCTION

Anaerobic bacteria can cause endogenous, polymicrobial, and potentially life-threatening infections in various body regions. However, their isolation from clinical samples poses challenges due to their susceptibility to oxygen, the requirement for specialized medium ingredients and slow growth characteristics (Gajdács *et al*. 2017, Humphries and Citron, 2016).

For a long time, the determination of antibiotic susceptibility in anaerobic bacteria using the Kirby-Bauer disc diffusion method lacked standardization. However, in 2022, EUCAST released documents outlining a standardized Kirby-Bauer disc diffusion method for anaerobes. Despite this development, the routine implementation of this test remains infrequent (EUCAST 2022). This is primarily attributed to the time-consuming and labor-intensive nature of the process, making it challenging to incorporate into regular microbiology laboratories. As a result, empirical treatment protocols are frequently relied upon to manage anaerobic infections (NCCLS 2004).

Currently, domestically and globally, the rate of anaerobic bacteria developing antibiotic resistance is rapidly increasing, presenting a significant challenge for clinicians (Veloo *et al.* 2004). On the one hand, the resistance of anaerobic bacteria to anti-anaerobic agents, combined with their ability to form biofilms, has prompted scientists to seek out new antimicrobial agents. Consequently, antimicrobial peptides (AMPs) become particularly noteworthy candidates (Bernard *et al.,* 2011, Cooley *et al.,* 2019, Lei *et al.,* 2019, Silvestro *et al.,* 2000)

AMPs are molecules that are evolutionarily conserved and produced by a wide range of organisms to combat pathogenic microorganisms in their environment (Lei *et al.,* 2019). These proteins typically consist of 10-15 amino acids, possess a positive charge and exhibit amphipathic properties. It is believed that the antimicrobial activities of AMPs disrupt the integrity of microorganisms by binding to their surfaces and forming pores in their membranes, thereby exerting a bactericidal effect (Kang *et al.,* 2017, Weis *et al.,* 1978). Numerous studies have demonstrated the effectiveness of AMPs against antibiotic-resistant bacterial strains, often through synergistic interactions when combined with various antibiotics (Almaaytah *et al*., 2019, Darwish *et al.,* 2022,

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Lu *et al*., 2022, Mhlongo *et al*., 2023) Furthermore, the likelihood of microorganisms developing resistance to AMPs is low due to the complex nature of their mechanisms of action (Hancock *et al.,* 1998, Jenssen *et al.,* 2006; Lei *et al.,* 2019).

Numerous studies have examined the effect of AMPs such as catestatin, derived from the processing of chromogranins in the chromaffin granules of the adrenal medulla; temporin A, isolated from the skin of the amphibian *Rana temporaria*; nisin, a bacteriocin produced by *Lactococcus lactis* bacteria and cecropin A, isolated from the hemolymph of *Hyalophora cecropia*, against various microorganisms (D'Andrea *et al.*, 2023, Di Grazia *et al.,* 2014*,* Jati *et al.,* 2023, Radek *et al.,* 2008, Shin *et al.,* 2016). However, there is limited investigations regarding their effectiveness against anaerobic bacteria (Dong *et al.,* 2024, Enigk *et al.* 2020). Hence, this study aimed to investigate the antimicrobial actcivity of antimicrobial peptides (AMPs), including catestatin, temporin A, nisin, and cecropin A against specific anaerobic bacteria, namely *Bacteroides fragilis, Prevotella melaninogenica, Cutibacterium acnes, Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*.

MATERIALS AND METHODS

Bacterial strains

Anaerobe standard bacterial strains of *Bacteroides fragilis* ATCC 25285, *Prevotella melaninogenica* ATCC 25845, *Cutibacterium acnes* ATCC 6919, *Peptostreptococcus anaerobius* ATCC 27337 were provided from Hardy Diagnostics Headquarters, USA and *Peptostreptococcus stomatis* DSM 17678 standard strain was provided from DSMZ-German Collection of Microorganisms and cell Cultures GmbH, GERMANY.

Bacterial strains were incubated for 72 hours before the experiments and then inoculated on Schaedler agar medium (ThermoFisher Sci.) supplemented with Vitamin K1 (10mg/mL) and 5% sheep blood. The media were incubated at 37ºC for 48-72h in anaerobic jars with anaerobic atmosphere generation bags (BD, GasPak). Following incubation, colonies from pure cultures were transferred to a chocolate agar medium and incubated in an aerobic environment at 37ºC to establish anaerobic and aerobic growth controls. Smears were prepared from colonies grown on anaerobic media and examined under a microscope after Gram staining. For future experiments, pure cultures were transferred to 10% glycerol skimmed milk medium and stored at -80 ºC (NCCLS 2004).

Antimicrobial peptides

Catestatin, nisin and cecropin A AMPs were purchased from Merck/ MilliporeSigma (Germany), and temporin A was purchased from AnaSpec, Inc., (CA-United States). The 100%, 75%, 50% and 25% dilutions of each peptide were prepared for Kirby-Bauer disc diffusion method and portioned into sterile eppendorf. The stock solution of catestatin was prepared at a concentration of 1mg/mL by dissolving 0.5mg of powdered catestatin in 0,5mL of sterile distilled water, following the manufacturer's instructions (Catestatin C6249, 2021). The stock solution of temporin A peptide was prepared using a 0,01% acetic acid solution containing 0,2% bovine serum albumin, as described in the literature review (Ataman, 2016). The stock solution of nisin was prepared according to the manufacturer's instructions by mixing 0,5mL of 1M HCl, 24,5mL of distilled water, and 187,5mg of NaCl in a sterile Falcon tube. The mixture was autoclaved at 121ºC and 1 atm pressure for 15 minutes. Subsequently, 1g of pure nisin was added to the 25mL solution and dissolved (Alves *et al*., 2016, Davies *et al*., 2003; NCCLS, 2004; Rojo- Bezares *et al*., 2007). The stock solution of cecropin A was also prepared following the manufacturer's instructions (Cecropin A C6830, 2021) by dissolving 0,5 mg of cecropin A powder in 0,5mL of sterile distilled water to achieve a concentration of 1mg/mL (NCCLS, 2004).

Different concentrations of catestatin, cecropin A, temporin A, and nisin AMP were prepared for agar dilution and broth microdilution tests. Catestatin and cecropin A dilutions ranged from 500 to 1µg/mL, temporin A dilutions ranged from 500 to 5µg/mL and Nisin AMP dilutions ranged from 40mg/mL to 0.4µg/mL.

All the prepared AMP solutions were filtered through sterile syringe filters with a pore diameter of 0.2 µm and stored at -20ºC for dilution studies (Shin *et al.,* 2016).

Antimicrobial susceptibility testing

(i) Kirby-Bauer disc diffusion method

For the Kirby-Bauer disk diffusion method, dilutions of each peptide were prepared at concentrations of 100%, 75%, 50% and 25%. These dilutions were portioned into sterile eppendorf tubes. Catestatin, temporin A and cecropin A AMP solutions were prepared at concentrations of 500, 375, 250 and 125µg/mL, while nisin solutions ranged from 40 to 10mg/mL. These peptide solutions were impregnated onto sterile blank disks (Thermo-Fisher Scientific, US).

The inocula were prepared by suspending standard bacterial strains in Brucella broth (Thermo Fisher Sci.) to achieve a density equivalent to a 1 McFarland standard (Davies *et al.,* 2003). A volume of 10mL (approximately 10^5 CFU) of each bacterial dilution was spread on Brucella agar (Merck KGaA, Germany) supplemented with vitamin K1 (10mg/mL), 5% defibrinated sheep blood, and hemin (5mg/mL) (Sigma Aldrich, US). Subsequently, antibiotic discs impregnated with different concentrations of AMPs were placed on the surface of the media following the 15-15-15-minute rule of EUCAST (EUCAST 2021, Laura *et al.,* 2016, Nagy *et al.,* 2015).

Imipenem (10µg) and metronidazole (5µg) antibiotic discs were used as the positive control, while a gentamicin (500 µg) disc (Bioanalyse, Turkey) was used as the negative control. The media were incubated at 37ºC for 48-72h in anaerobic jars with anaerobic atmosphere generation bags (Becton Dickinson (BD), US) (NCCLS 2004).

During our study conducted between 2018 and 2020, the Kirby-Bauer disk diffusion method was not an established reference method for determining the susceptibility of anaerobic bacteria according to guidelines such as European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI). Consequently, zone diameter information was not available. In our study, we meticulously recorded the diameter of each inhibition zone, and the absence of an inhibition zone was interpreted as indicative of resistance to the concentration of the AMPs (Laura *et al.,* 2016, Luu *et al.,* 2013, Nagy *et al.,* 2015, NCCLS 2004).

(ii) Agar dilution method

The agar dilution method, considered the gold standard for determining the susceptibility of anaerobic bacteria by CLSI, was utilized for all the tested AMPs (NCCLS 2004). Falcon tubes containing 32 separate dilutions were labeled to indicate the concentrations of the AMPs. Subsequently, 2 mL of the AMP dilutions were added to the corresponding marked Petri plates. Brucella agar supplemented with Vitamin K1 (1µg/mL), hemin (5 µg/mL) and 5% hemolyzed sheep blood was then poured onto the pre-marked Petri plates within the biosafety cabinet (Nüve-MN 120 Class II, TR). To prepare the inoculum, pure standard bacterial isolates from supplemented Schaedler agar medium were cultured in Schaedler broth medium. The density of the suspension was verified using a McFarland densitometer (Biosan Den-1, Biosan, TR) to achieve a 0.5 McFarland standard (1.5×108 CFU/mL) (NCCLS 2004).

Each of the 32 Petri dishes prepared for agar dilution was divided into five sections and marked accordingly. Then, 1 mL of bacterial suspensions (approximately 10^5 CFU per culture) was pipetted and cultured onto the plates containing different AMP concentrations, ranging from the lowest to the highest peptide concentration. After allowing the inoculum on the plates to dry, the plates were inverted. The aerobic control plates were incubated at 35ºC in a CO_2 incubator for 48h, while all AMP-containing plates and anaerobic control plates were incubated under anaerobic conditions for 48-72h with their lids facing downward.

E-test strips (bioMerieux, Marcy-l'Etoile, France) of imipenem and metronidazole were used as positive control, gentamicin E-test strip was used as negative control. Because *Cutibacterium acnes* is naturally resistant to metronidazole, only the imipenem E-test (0,016-256µg) was used as positive control for this bacterium (Brook *et al.,* 2013, Humphries *et al.,* 2016, NCCLS 2004).

(iii) Broth microdilution method

The broth microdilution method, recommended by CLSI standards for testing only the *Bacteroides fragilis* group, was utilized in determining the susceptibility of all anaerobic bacteria included in our study (NCCLS 2004). For inoculum preparation, pure colonies of anaerobic bacteria isolated from 48h Schaedler agar medium supplemented with 5% sheep blood and vitamin K1 were collected. A homogeneous suspension of each colony was prepared in Scaedler broth enriched with vitamin K1 (10 mg/mL), and the turbidity was assessed using a McFarland Densitometer to achieve 0.5 McFarland standard (1.5×10^8 CFU/mL) (NCCLS 2004). Once the inoculums were prepared, they were promptly inoculated onto the MIC plate within 15 mins. The first two wells of the sterile polypropylene microplate were designated as positive and negative control wells.

In each microplate, except for the control wells, 100µL of bacterial suspensions with approximately 5×10^4 CFU/mL were inoculated into Brucella Broth medium. Furthermore, 100µL of the tested AMP stock solutions were added. The MIC plates were then placed in anaerobic jars and incubated at 37ºC for 48-72h, using the GasPak system (Becton Dickinson (BD), US) as an anaerobic environment provider (Roe-Carpenter 2010).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

Following incubation of the MIC plates in anaerobic jars at 37ºC for 48-72h, each MIC plate was positioned on a black, non-reflective surface and arranged in order from Petri plates with the lowest to highest concentrations of AMP. Following the CLSI M-11 guidelines, the MIC value was determined as the lowest concentration of an antibiotic at which bacterial growth is completely inhibited (NCCLS 2004, Roe-Carpenter 2010).

In liquid microdilution experiments, the MIC, following the CLSI M-11 guideline, was defined as the lowest concentration of the antimicrobial agent showing no visible growth compared to the positive control well (NCCLS 2004). From each well without visible growth, 10 µl of samples were extracted and inoculated onto an enriched Brucella blood agar plate. The plates were then incubated in anaerobic jars at 37ºC for 48-72h with the assistance of anaerobic media providers. The lowest peptide concentration that killed 99.9% of bacterial growth was accepted as the MBC value (Roe-Carpenter 2010).

RESULTS

The effectiveness of antimicrobial peptides on anaerobic standard strains

The antimicrobial activities of the AMPs on anaerobic standard bacterial strains are shown in table 1. The MIC values of cecropin A, determined using the agar dilution method, were 50µg/mL for *B. fragilis* ATCC 25285, 16µg/mL for *P. melaninogenica* ATCC 25845, 8µg/mL for *C. acnes* ATCC 6919, 8µg/mL for *P. anaerobius* ATCC 27337 and 4µg/mL for *P. stomatis* DSM 17678 (fig. 1A).

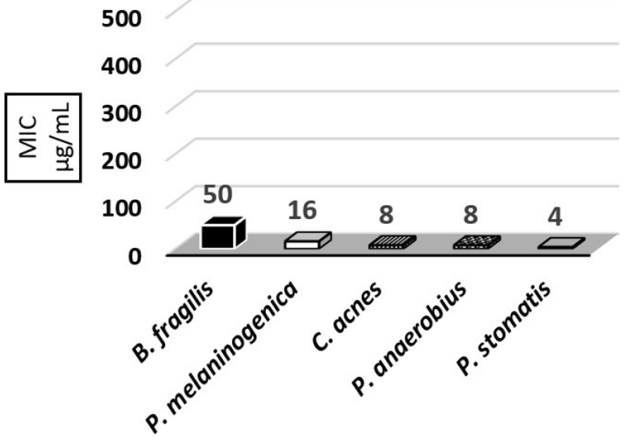

Fig. 1A: Cecropin A. Antimicrobial activity to anaerobic bacteria tested by agar dilution method.

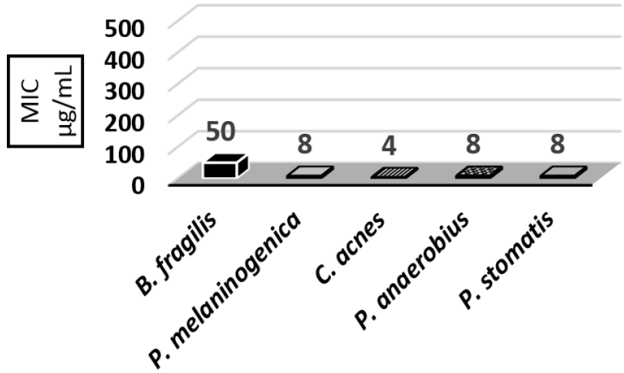

Fig. 1B: Cecropin A. Antimicrobial activity to anaerobic bacteria tested by broth microdilution method.

Using the broth microdilution method, the MIC and MBC values of cecropin A were determined as follows: for *B. fragilis* ATCC 25285, the MIC value was 50 µg/mL, and the MBC value was 500µg/mL; for *C. acnes* ATCC 6919, the MIC value was 4µg/mL and the MBC value was 8 µg/mL; for *P. melaninogenica* ATCC 25845, the MIC value was 8 µg/mL and the MBC value was 16µg/mL; for *P. anaerobius* ATCC 27337, the MIC value was 8 µg/mL, and the MBC value was 32µg/mL; for *P. stomatis* DSM 17678, the MIC value was 8 µg/mL and the MBC value was 50µg/mL (fig. 1B).

By using the disc diffusion method, it was observed that the strain *P. melaninogenica* ATCC 25845 exhibited a susceptibility zones of 12 mm around discs impregnated with 100% and 75% diluted nisin and cecropin A respectively, a susceptibility zones of 10 mm around discs impregnated with %75 and 50% diluted cecropin A and 50% diluted nisin (fig. 1C).

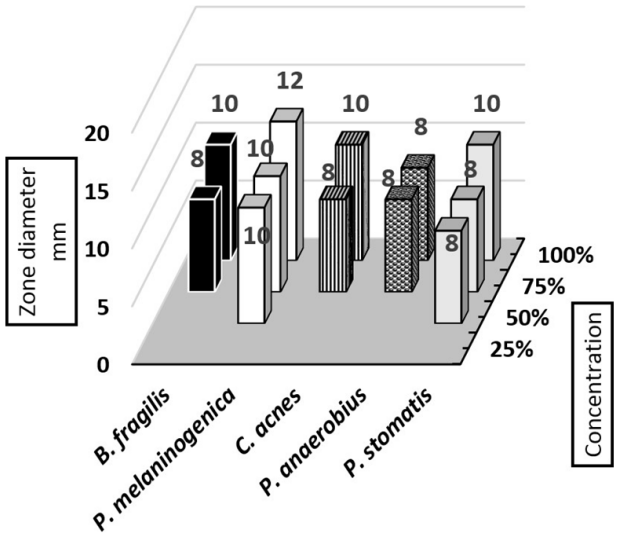

Fig. 1C: Cecropin A. Antimicrobial activity to anaerobic bacteria tested by disc diffusion method.

The MIC value of temporin A for *P. anaerobius* ATCC 27337 was determined to be 500µg/mL by both agar and broth dilution methods, with an MBC value greater than 500 µg/mL. However, it was found to be ineffective against other anaerobic bacterial strains (fig. 2A, fig. 2B).

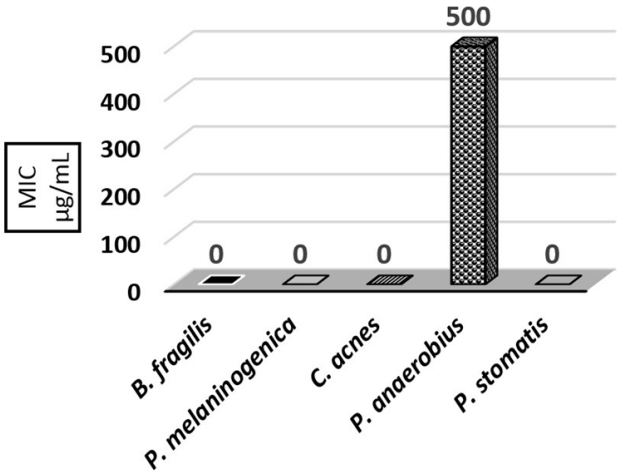

Fig. 2A: Temporin A. Antimicrobial activity to anaerobic bacteria tested by agar dilution method.

By using the disc diffusion method, it was observed that *P. anaerobius* ATCC 27337 exhibited a susceptibility zone of 6 mm around discs impregnated with 100% diluted temporin A (fig. 2C). The MIC value of nisin, determined by the agar dilution method, was found to be 400µg/mL for *P. melaninogenica* ATCC 25845 and 40mg/mL for *C. acnes* ATCC 6919, *P. anaerobius* ATCC 27337 and *P. stomatis* DSM 17678 respectively (fig. 3A).

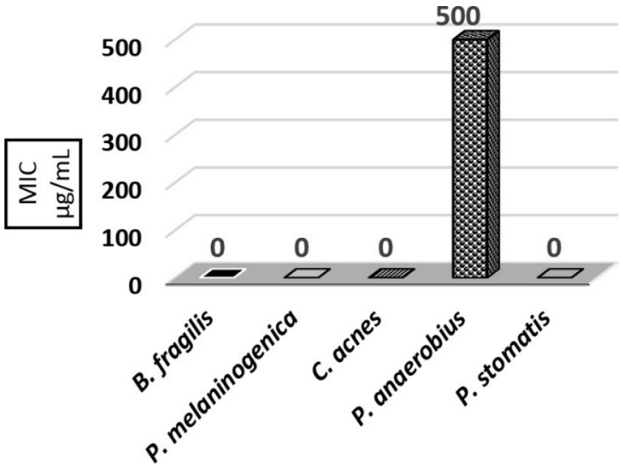

Fig. 2B: Temporin A. Antimicrobial activity to anaerobic bacteria tested by broth microdilution method.

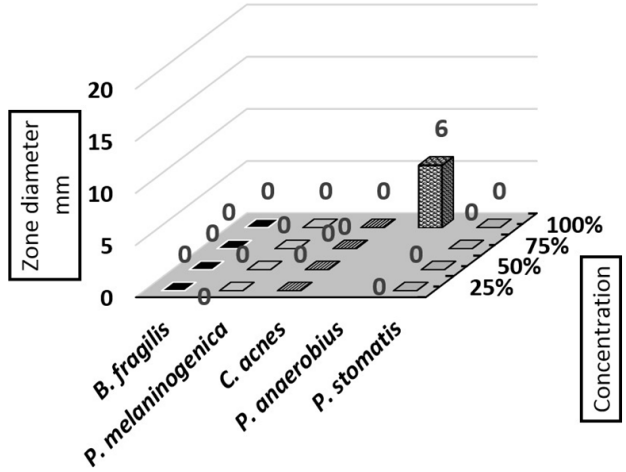

Fig. 2C: Temporin A. Antimicrobial activity to anaerobic bacteria tested by disc diffusion method.

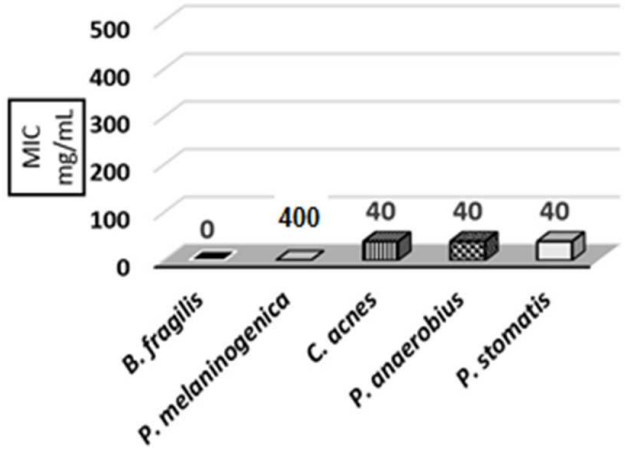

Fig. 3A: Nisin. Antimicrobial activity to anaerobic bacteria tested by agar dilution method.

The MIC value of nisin, determined by the broth microdilution method, for *P. melaninogenica* ATCC 25845 was 200µg/mL, with an MBC value of 400µg/mL. While the MIC value for *C. acnes* ATCC 6919, *P. anaerobius* ATCC 27337, *P. stomatis* DSM 17678 were 40 mg/mL. Furthermore, the MBC value for *P. stomatis* was determined to be greater than 40 mg/mL (fig. 3B).

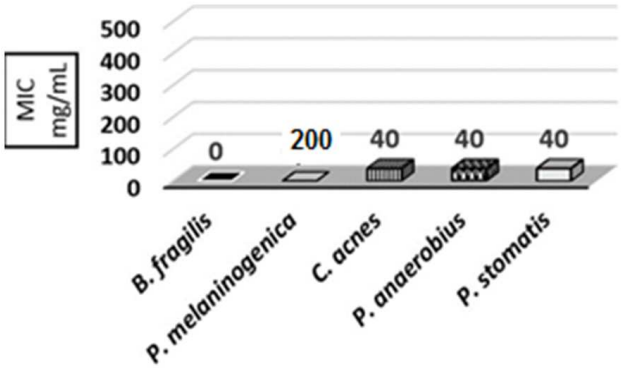

Fig. 3B: Nisin. Antimicrobial activity to anaerobic bacteria tested by broth microdilution method.

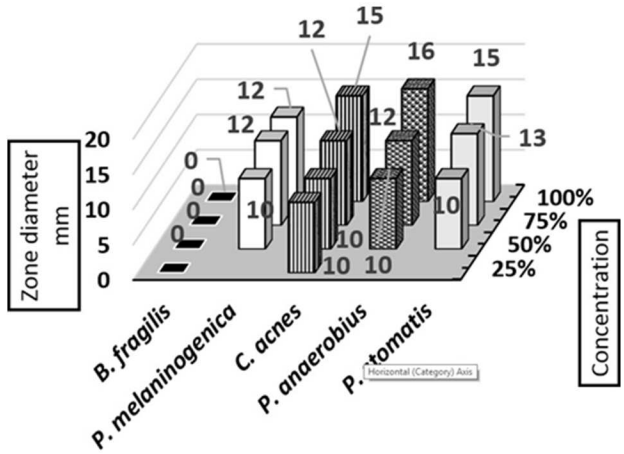

Fig. 3C: Nisin. Antimicrobial activity to anaerobic bacteria tested by disc diffusion method.

In the case of *C. acnes* ATCC 6919, the susceptibility zones for 100% and 75% nisin dilution-impregnated discs were 15 mm and 12 mm, respectively, while the 50% and the 25% diluted disc had a 10 mm susceptibility zone. For cecropin A the susceptibility zones were 10 mm and 8 mm respectively on the 100% and 75% peptide dilution-impregnated discs. For *P. anaerobius* ATCC 27337, the 100% nisin dilution-impregnated disc had a susceptibility zone of 16 mm, the 75% disc had a susceptibility zone of 12 mm, the 50% disc had a susceptibility zone of 10 mm (fig. 3C).

Similarly, in the case of *P. stomatis* DSM 17678, the susceptibility zones were observed to be 15 mm for the 100% nisin dilution-impregnated disc, 13 mm for the 75% disc, 10 mm for the 50% disc. *B. fragilis* ATCC 25285 displayed a 10 mm susceptibility zone around the 100% and 8 mm susceptibility zone around the 75% disc.

Catestatin showed no effectiveness against any of our anaerobic bacterial strains when tested using the agar dilution and broth dilution method (fig. 4A, fig. 4B).

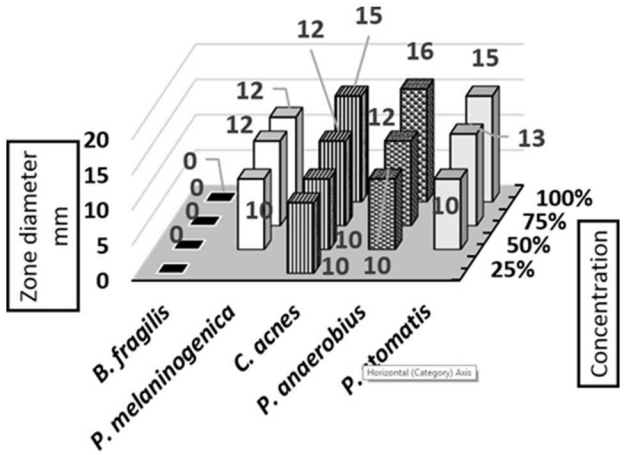

Fig. 4A: Catestatin. Antimicrobial activity to anaerobic bacteria tested by agar dilution method.

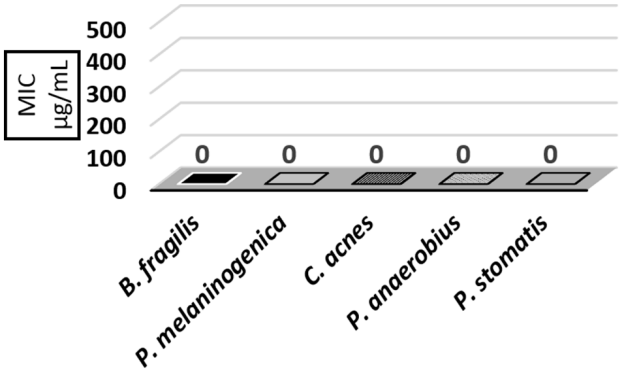

Fig. 4B: Catestatin. Antimicrobial activity to anaerobic bacteria tested by broth microdilution method.

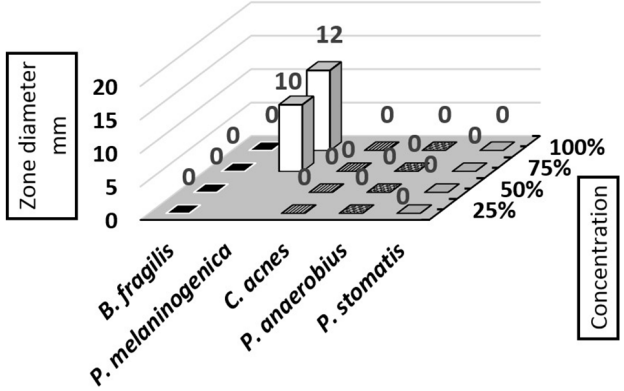

Fig. 4C: Catestatin. Antimicrobial activity to anaerobic bacteria tested by disc diffusion method.

By the disc diffusion method, it was observed that *P. melaninogenica* ATCC 25845 exhibited susceptibility zone of 12 mm and 10 mm around discs impregnated with 100% and 75% diluted catestatin respectively (fig. 4C).

DISCUSSION

Antimicrobial peptides are garnering attention in the fields of microbiology and pharmacology as promising alternatives to antibiotics, being studied as potential cures for infections caused by antibiotic-resistant pathogens and resulting in thousands of deaths yearly (Romero *et al.,* 2019). However, it is believed that further research on AMPs activity is necessary to identify more suitable alternative peptides that can be integrated into the pharmaceutical industry. Studies in this area are highly emphasized.

Our research focused on temporin A, nisin, catestatin and cecropin A, which are AMPs that have been extensively studied for their antimicrobial actcivity against aerobic bacteria in the literature. However, there is limited research on their effect on anaerobic bacteria (Ghapanvari *et al*., 2022, Jati *et al*., 2023, Romero *et al*., 2019).

In our study, Kirby-Bauer disc diffusion, agar dilution and broth micro dilution methods were used to investigate the antimicrobial actcivity of AMPs on anaerobic bacteria of clinical importance. The disc diffusion method, included in the CLSI and EUCAST guidelines for the detection of aerobic bacteria, is typically employed to determine the effectiveness of AMPs on aerobic bacteria. Conversely, the recommended methods for susceptibility tests of anaerobic bacteria include agar dilution, liquid microdilution and the gradient test method (E-test). Since the disc diffusion method is not among the recommended methods in these guidelines, we believe it has not been extensively utilized in studies investigating AMPs on anaerobic bacteria, as observed in the literature (Chakraborty *et al.,* 2021). On the other hand, several publications in recent years have indicated that the disc diffusion method can also be utilized to determine the susceptibility of anaerobic bacteria. Nagy *et al*. (2015) employed the disk diffusion method in 2014 to assess the susceptibility of *B. fragilis* group bacteria. Additionally, the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (2011) published a guide stating that the disc diffusion method can be applied to anaerobic bacteria, providing explanations of the methods involved. Furthermore, Dubreuil (2020) conducted studies to update the values specified in the CA-SFM guideline, while Eminoğlu *et al*. (2021), affirming the safe usage of the disc diffusion method in routine laboratories for determining the antimicrobial susceptibility of *Bacteroides fragilis* group bacteria (NCCLS 2004, EUCAST 2021, Nagy *et al.,* 2015, Dubreuil 2020). By employing the disk diffusion method in our study with AMPs, we aim to contribute to future research and publications in this field (Dong *et al.,* 2023, Enigk *et al.,* 2020, Silvestro *et al*. 2020).

Currently, there is no universally recommended reference method for assessing the antimicrobial actcivity of AMPs against anaerobic bacteria. However, it is noteworthy that the agar dilution and liquid microdilution methods, which are recommended and employed for determining the effectiveness of antibiotics against anaerobic bacteria as

Table 1: Data on AMPs effective on our anaerobic standard strains

	Cecropin A MIC:µg/Ml,MBC: µg/mL, C:%, ZD:mm			Temporin A, MIC:µg/Ml, MBC: µg/mL, C:%: ZD:mm			Nisin, MIC:µg/Ml,MBC: µg/mL C:%: ZD:mm			Catestatin, MIC:µg/Ml,MBC: µg/mL, C:%: ZD:mm		
	AD MIC	BM MIC /MBC	DD C/ZD	AD MIC	BM MIC /MBC	DD C/ZD	AD MIC	BM MIC /MBC	DD C/ZD	AD MI	BM MIC/MBC	DD C/ZD
B. fragilis ATCC 25285	50	50 /500	100/ 10 75/8	R	R	R	R	R	R	R	R	R
P. melaninogenica ATCC 25845	16	8/16	100/12 75/ 10 50/ 10	R	R	R	400	200/400	100/12 75/12 50/10	R	R	100/12 75/10
C. acnes ATCC 6919	8	4/8	100/ 10 75/ 8	R	R	R	40*	40*/>40*	100/15 75 /12 50/10 25/10	R	R	R
P. anaerobius ATCC 27337	8	8/32	100/ 8 75/ 8	500	500/>500	100/6	40*	40*/ >40*	100/ 16 75/ 12 50/10	R	R	R
P. stomatis DSM 17678	4	8/50	100 / 10 75 / 8 50/ 8	R	R	R	40*	40*/ >40*	100/15 75/ 13 50/10	R	R	R

AD: Agar dilution, BM: Broth microdilution, DD:Disc diffusion, MIC: Minimal Inhibitory Concentration, MBC: Minimal Bactericidal Concentration, ZD:Zone diameter, C: Concentration, R: Resistant,*:mg/mL

specified in the guidelines, have also been utilized in studies to evaluate the effectiveness of AMPs (Citron *et al*. 2011, Jaskiewicz *et al.,* 2016, NCCLS 2004). According to Wiegand *et al*. (Wiegand *et al.,* 2008), the agar dilution and liquid microdilution methods are commonly employed for determining MICs of new antimicrobial agents. One significant advantage of these methods is that they allow for the simultaneous study of multiple bacterial strains on a single agar plate or microplate. These methods were chosen in light of their established reliability and suitability for evaluating antimicrobial activity.

Despite conducting a comprehensive literature review, we could not find any studies that specifically examined the antimicrobial activity of catestatin against anaerobic bacteria. Consequently, we were unable to discuss our MIC results concerning existing literature. Nevertheless, Radek *et al*. (2008) observed that catestatin has demonstrated effectiveness against *S. aureus,* group A streptococci, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger, Aspergillus fumigatus* and *Trichophyton rubrum* in a mouse model. In the same study, catestatin administration led to an increase in the Bacteroidetes phylum and a decrease in the Firmicutes phylum within the mouse fecal microbiota (Radek *et al*., 2008). Mahata *et al*. (2010) established a direct correlation between the skin's antimicrobial defense and catestatin, a neuroendocrine peptide. Furthermore, Mahata *et al* (2000), discovered in another study that catestatin inhibits the growth of Gram-positive bacteria, specifically *M. luteus* and *B. megaterium*. Additionally, in this study, catestatin was tested on Gram-negative bacteria and demonstrated inhibitory effects on the growth of the *E. coli* D22 strain.

Wuersching *et al*. (2020) investigated the impact of catestatin on caries and periodontitis. They examined the effects on both planktonic forms of bacteria and the formation of biofilms, focusing on facultative anaerobic bacteria (*Streptococcus mutans, Streptococcus sanguinis, Actinomyces naeslundii*) and obligate anaerobic bacteria (*Veillonella parvula, Parvimonas micra, Fusobacterium nucleatum*).

Numerous studies have reported the strong regulatory effects of catestatin on the human gut microbiome (Rabbi *et al.* 2021, United States Patent Application Publication 2018) and its ability to influence the ratio of Bacteroidetes to Firmicutes, making it a potential therapeutic option for conditions like Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD) (United States Patent Application Publication 2018, Rabbi *et al.* 2021).

One plausible explanation for the lack of investigation into the *in vitro* activity of catestatin against anaerobic bacteria, may have been less urgency to explore the anti-anaerobic activity of this peptide. Moreover, there is currently no available information regarding whether anaerobes are naturally resistant to catestatin. However, we can speculate that our anaerobic bacteria might possess inherent resistance to this peptide. This assumption is based on our research findings, which revealed that catestatin did not exhibit antimicrobial actcivity against our standard strains at any of the tested concentrations.

Ogawa *et al*. (2020), investigated the mechanism of action of AMPs derived from amphibian skins. Considering the broad mechanism of action exhibited by these peptides, we also utilized temporin A, a peptide isolated from the skin of *Rana temporaria* amphibians, in our study.

Gaiser *et al*. (2020) examined the antimicrobial activity of 12 temporin analog AMPs isolated from amphibian skin against *S. aureus*, MRSA, *Streptococcus suis*, *S. pseudintermedius, P. aeruginosa*, *E. faecium* (VRE), *A. baumannii* (MDR). This pathogenic species is associated with skin infections in both humans and animals and often exhibits multidrug resistance. Additionally, the researchers assessed the effects of these peptides on commensal/ probiotic bacteria, specifically *Lactobacillus*

plantarum, L. rhamnosus, L. salivarus, L. casei, L johnsonii, L. reuteri, L. acidophilus. They discovered that the minimum inhibitory concentration (MIC) values of this peptide against lactobacilli, which are considered commensal probiotics, were approximately 3.5 times lower than those observed against pathogenic bacteria.

While we did not find a specific study on the in vitro activity of temporin A against anaerobic bacteria, valuable insights can be drawn from a study by Urban *et al*. (2007), which examined two AMPs from the temporin family (temporin-1Dra and temporin-1Va). In their research, the antianaerobic activity of these peptides was investigated using the broth microdilution method. The MIC values of temporin-1Dra and temporin-1Va against various anaerobic bacteria are as follows: for *B. fragilis* ATCC 25285, 50µM and >50µM respectively; for *P. melaninogenica* 26117, 12.5µM and 25µM; for *C. acnes* ATCC 11828, both 6 µM; for *C. acnes* 669, 6µM and 12.5µM and for *P. anaerobius*, 12.5µM and 25µM respectively.

In our research, we determined the MIC value of temporin A only for *P. anaerobius* ATCC 27337. Both the agar dilution and liquid microdilution methods yielded a temporin A MIC value of 360 µM (500µg/mL) against *P. anaerobius* ATCC 27337. However, we did not observe any activity of temporin A against our other bacterial strains in the study.

In a study carried out by Severina *et al*. (1998), it was demonstrated that nisin possesses bactericidal properties against Gram-positive bacteria, including MRSA, VRE and *S. pneumoniae*. Piper *et al*. (2009) further reported the remarkable effectiveness of nisin against antibiotic-resistant staphylococci, suggesting that continued research on nisin and other lantibiotic compounds holds promise for developing alternative antimicrobial solutions.

In 2020, Enigk *et al*. (2020) conducted a study to examine the antimicrobial activity of five different AMPs against periodontal and non-periodontal pathogenic bacteria, the researchers noted that among the three peptides (nisin, melittin, lactoferrin) they compared, nisin displayed the highest level of antimicrobial activity. Researchers investigated the antimicrobial activity of this peptide using the agar dilution method and reported that the *P. intermedia* DSM20706 strain was completely inhibited at a nisin concentration of 64µg/mL. Furthermore, the standard strains of *P. loescheii* and *P. nigrescens* DSM13386 showed significant reduction at a nisin concentration of 128µg/mL. However, nisin did not affect *P. melaninogenica*.

In our study, employing the agar dilution method, we determined the MIC values of nisin to be 400µg/mL for *P. melaninogenica*, 40µg/mL for *C. acnes*, 40µg/mL for *P. anaerobius* and 40µg/mL for *P. stomatis*.

Our study determined the nisin MIC values using the liquid microdilution method. We found the MIC value to be 200µg/mL for *P. melaninogenica*, 40µg/mL for *C. acnes*, 40µg/mL for *P. anaerobius*, and 40µg/mL for *P. stomatis*. When comparing our results with the study conducted by Enigk *et al*. (2020), we observed that *only P. melaninogenica* matched the bacteria included in our study. While the researchers reported complete inhibition *of P. intermedia* DSM 20706 at a nisin concentration of 64 µg/mL and significant reduction of *P. loescheii* and *P. nigrescens* DSM 13386 at 128µg/mL, nisin did not exhibit effectiveness against *P. melaninogenica* in their study. However, our research determined that nisin was effective against *P. melaninogenica* using both the liquid microdilution and agar dilution methods. This difference may be attributed to variations in the clinical origin of the bacteria included in the respective studies compared to our use of standard strains.

In a study by Edlund *et al*. (1998), the MIC range of the cecropin-melittin hybrid peptide, determined using the agar dilution method, was reported for 24 *B. fragilis* strains to be 2-8mg/L. The MIC50 and MIC90 values were specifically noted as 4 mg/L. The same study found the MIC50 value for 13 strains of *Bacteroides* spp. and *Prevotella* spp. to be 4 mg/L, with a MIC90 value of 8 mg/L. The MIC range for these strains was reported as 2-32mg/L. When analyzing nine strains of *Propionibacterium* spp. using the agar dilution method, the researchers reported a MIC50 and MIC90 value of 4 mg/L for the cecropin-melittin hybrid peptide and the MIC range was specified as 2-4mg/L. For eight strains of *Peptostreptococcus* spp., the study determined the MIC50 value for the peptide as 2mg/L, the MIC90 value as 4mg/L and stated the MIC range for *Peptostreptococci* as 2-4 mg/L.

Oh *et al*. (2000) reported the antimicrobial actcivity of the cecropin-mellitin hybrid peptide analog from clinical samples. They isolated *Bacteroides fragilis*, *Peptostreptococcus* sp., *Propionibacterium* sp., *C. difficile*, *Prevotella* sp. and *F. nucleatum* strains and found that *C. difficile* and *B. fragilis* were the most sensitive species to the hybrid peptide. Furthermore, they noted that 90% of the isolates were inhibited at MIC values ranging from 1 to 4µg/mL, with only one *B. fragilis* isolate having a higher MIC value of greater than 4µg/mL. The researchers also determined the lowest antimicrobial activity of the cecropin-mellitin peptide on *Peptostreptococcus* sp. In this case, they synthesized 16 peptides and found that ten of them had a MIC value of ≥8 mg/mL.

In addition, Oh *et al*. (2000) compared the antimicrobial actcivity of a hybrid peptide using agar and broth microdilution methods. When comparing our findings with their research, it is evident that cecropin A exhibits effectiveness at lower concentrations against *B. fragilis*

and *P. melaninogenica* strains using liquid microdilution and agar dilution methods. This disparity may be attributed to the increased antimicrobial actcivity of the hybrid peptide cecropin A-mellitin on Gram-negative anaerobes. Moreover, our results were consistent with both methods for *C. acnes* and Peptostreptococci.

Moore *et al.* (1996) investigated the antimicrobial activity of cecropin B against various clinical aerobic and anaerobic bacteria using agar dilution and liquid microdilution methods.. The researchers noted that the antimicrobial actcivity of cecropin B was more limited in agar compared to the liquid medium.

Our study found that the MIC value of cecropin A for *B. fragilis* was determined to be 50µg/mL (12.5µM) using both the agar dilution method and the liquid microdilution method. We hypothesize that the observed difference in MIC values could be attributed to the variation in strains used in the two methods.

CONCLUSION

As a result, cecropin A exhibited effectiveness against all anaerobic bacterial strains included in our study. Additionally nisin showed antimicrobial activity against all the studied strains except for *B. fragilis.* However, temporin A was only found to be effective against *P. anaerobius* ATCC 27337 and catestatin was not effective against any of the anaerobic bacteria included in our study.

We believe that AMPs hold promise for the treatment of anaerobic bacterial infections. This can be achieved by synthesizing synthetic variants of AMPs, which may exhibit enhanced effect by utilizing proteomic, bioinformatics and modification strategies.

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