Benedito Rodrigues da Silva Neto
(Organizador)

Pesquisa Científica e Tecnológica em Microbiologia 3

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APRESENTAÇÃO

Temos o prazer de dar continuidade ao tema de microbiologia inter-relacionado à pesquisa científica e tecnológica iniciado pela editora no ano de 2019. Apresentamos aqui um novo volume deste contexto, denominado “Pesquisa científica e tecnológica em microbiologia, volume 3” contendo trabalhos e pesquisas desenvolvidas em diversos institutos do território nacional contendo análises de processos biológicos embasados em células microbianas ou estudos científicos na fundamentação de atividades microbianas com capacidade de interferir nos processos de saúde/doença.

A microbiologia é um vasto campo que inclui o estudo dos seres vivos microscópicos nos seus mais vaiados aspectos como morfologia, estrutura, fisiologia, reprodução, genética, taxonomia, interação com outros organismos e com o ambiente além de aplicações biotecnológicas. Como uma ciência básica a microbiologia utiliza células microbianas para analisar os processos fundamentais da vida, e como ciência aplicada ela é praticamente a linha de frente de avanços importantes na medicina, agricultura e na indústria.

A microbiologia como ciência iniciou a cerca de 200 anos, entretanto os avanços na área molecular como a descoberta do DNA elevou a um novo nível os estudos desses seres microscópicos, além de abrir novas frentes de pesquisa e estudo. Sabemos na atualidade que os microrganismos são encontrados em praticamente todos os lugares, e a falta de conhecimento que havia antes da invenção do microscópio hoje não é mais um problema no estudo, principalmente das enfermidades relacionadas aos agentes como bactérias, vírus, fungos e protozoários.

Temas ligados à pesquisa e tecnologia microbiana são, deste modo, discutidos aqui com a proposta de fundamentar o conhecimento de acadêmicos, mestres e todos aqueles que de alguma forma se interessam pela saúde em seus aspectos microbiológicos. Portanto a obra propõe uma teoria bem fundamentada nos resultados práticos obtidos em alguns campos da microbiologia, abrindo perspectivas futuras para os demais pesquisadores de outras subáreas da microbiologia.

Assim, desejo a todos uma ótima leitura!

Benedito Rodrigues da Silva Neto

SUMÁRIO

CAPÍTULO 6

EFFECTS OF SUB-INHIBITORY CONCENTRATION OF ANTIMICROBIALS IN *Bacteroides fragilis* STRAINS ISOLATED FROM INTRA-ABDOMINAL INFECTIONS

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ABSTRACT: *Bacteroides fragilis* is the obligate anaerobe most frequently associated with infectious diseases in humans. Sub-minimal inhibitory concentrations (Sub-MICs) of different antimicrobials are potentially capable of interfering positively or negatively with microbial pathogenicity. Thus, the effects of sub-MIC of Piperacillin+tazobactam (PTZ), Metronidazole (MET) and Clindamycin (CLI) on strains of *B. fragilis* strains were evaluated in this study. One reference and two clinical strains were previously cultured in sub-MIC of these three antimicrobials to investigate alterations in cell morphology, grown curve, capsule production, resistance to hydrogen peroxide, biofilm formation, cell surface hydrophobicity, hemagglutination ability, and hemolytic activity. Sub-MICs of both MET and CLI led to significant delays in the log growth phase of the all strains. Sub-MICs of both PTZ and MET caused changes in cell morphology. Overall, sub-MIC of the three antimicrobials

decreased resistance to the hydrogen peroxide in all strains. All strains showed haemolysis in all blood types tested, except for one clinical strain cultured with sub-MICs of the three antimicrobials in sheep's blood. Therefore, sub-MICs of antimicrobials suggest being able to alter bacterial physiological patters, which reflect on their pathogenicity and may affect the diagnosis and treatment of infectious diseases caused by *B. fragilis.*
KEYWORDS: *Bacteroides fragilis*, pathogenicity, antimicrobial subinhibitory concentrations.

EFEITOS DA CONCENTRAÇÃO SUB-INIBITÓRIA DE ANTIMICROBIANOS EM AMOSTRAS DE *Bacteroides Fragilis* ISOLADAS DE INFECÇÕES INTRA-ABDOMINAIS

RESUMO: A espécie *Bacteroides fragilis* é um anaeróbio obrigatório frequentemente associado a doenças infecciosas em humanos. Concentrações sub-inibitórias mínimas (Sub-CIMs) de diferentes antimicrobianos podem interferir positiva ou negativamente em sua patogenicidade. Assim, os efeitos da sub-CIM de Piperacilina + tazobactam (PTZ), Metronidazol (MET) e Clindamicina (CLI) nas linhagens *de B. fragilis* foram avaliados neste estudo. Uma amostra de referência e clínicas foram previamente cultivadas em sub-CIM desses três antimicrobianos, para investigar alterações na morfologia celular e na curva de crescimento; produção de cápsulas, resistência ao peróxido de hidrogênio; formação de biofilme; hidrofobicidade da superfície celular; capacidade de hemaglutinação; e atividade hemolítica. Sub-CIM de MET e CLI acarretaram atrasos significativos na fase log de crescimento de todas as amostras testadas. As sub-MICs de PTZ e MET causaram alterações na morfologia celular. No geral, as sub-MICs dos três antimicrobianos diminuiram a resistência das amostras ao peróxido de hidrogênio. Todas as bactérias testadas apresentaram hemólise em todos os grupos sanguíneos, exceto uma amostra clínica, cultivada em sub-CIM dos três antimicrobianos, testada com sangue de ovelha. As alterações na patogenicidade apontam para prováveis riscos de uma terapia antimicrobiana inadequada, podendo induzir diferentes interações entre o hospedeiro e a bactéria, além de afetar o diagnóstico e o tratamento de doenças infecciosas causadas por *B. fragilis.*
PALAVRAS-CHAVE: *Bacteroides fragilis*, patogenicidade, concentrações sub-inibitórias de antimicrobianos.

1 I INTRODUCTION

The genus *Bacteroides* is composed of pleomorphic obligate anaerobes, Gram-negative rods and *B. fragilis* is the most prominent species found in human infections disease (LIU *et al.*, 2003). Several virulence abilities contribute to the survival and success of this species in infectious processes, such as capsule production (NAKANO *et al.*, 2008). The presence of fimbriae is associated with adhesion and hemagglutination ability (CHANDAD & MOUTON, 1995). *B. fragilis* can also, produce hemolysins whose main functions are the destruction of erythrocytes and leukocytes (ROBERTSON *et al.,* 2006;

LOBO *et al.*, 2013;) and to form biofilms, important to the host's immune response and antimicrobial agents (REIS *et al.*, 2014). *B. fragilis* uses efflux pumps to expel harmful agents including antimicrobials (DONELLI *et al.*, 2012). It is also able to withstand the reactive oxygen species produced by the host's defenses and persists in oxygenated tissues (TEIXEIRA *et al.*, 2018).

Species of the genus *Bacteroides* are generally isolated from mixed infectious diseases, such as intra-abdominal infections (IAIs), usually in combination with aerobic or facultative bacteria. The most commonly prescribed antimicrobials for IAIs caused by *Bacteroides* species include beta-lactams (which may be combined with a beta-lactamase inhibitor), carbapenems, metronidazole (MET) and clindamycin (CLI) (DOS SANTOS *et al.*, 2004).

To achieve satisfactory levels of antimicrobials in abscesses with low perfusion or necrotic tissues, larger doses are recommended for extended periods of time. Poor management of this parameter may result in lower levels of antimicrobial efficacy and consequently reaching sub-minimal concentrations (sub-MICs) at the site of infection. This may lead to failure in eliminating the bacterial pathogens. Moreover, bacteria exposed to sub-MIC have several aspects of microbial physiology altered, including changes in cell surface hydrophobicity and biofilm formation (VELOSO *et al.*, 2013; DE ANDRADE *et al.*, 2016; CLSI, 2017).

In this regard, the effect of sub-MICs on the physiology of *B. fragilis* during infection is still not completely understood. Thus, the aim of this study is to evaluate the effects of sub-MICs of piperacillin + tazobactam (PTZ), metronidazole (MET) and CLI on the physiology of *B. fragilis* which seem to be involved in its pathogenicity traits, such as cell morphology, capsule presence, resistance to hydrogen peroxide (H_2O_2), biofilm formation, cell surface hydrophobicity, haemagglutination, and hemolytic activity. We also evaluatedthe presence of the genes for hemolysins A, B, C, E and III by conventional polymerase chain reaction (PCR).

2 I MATERIAL AND METHODS

2.1 Bacterial strains

One reference strain (*Bacteroides fragilis* ATCC 25285) and two clinically isolated from patients with IAIs were evaluated (named P34 and P50). The clinical strains were isolated, identified and had their susceptibility profile stablished in a previous study (FERREIRA *et al.*, 2016) approved by the Research Ethics Committee of Minas Gerais University, under the number ETIC 0097.0.203.000-10. *B. fragilis* identification was performed by the automated system Vitek II (Biomerrieux®) and by PCR amplification with 16S rRNA primers, according to Liu *et al.* (2003).

2.2 Determination of minimum inhibitory concentration (MIC) of PTZ, MET and CLI for the *B. fragilis* strains studied

The followed antimicrobials were used: *piperacillin/tazobactam* (Aurobindo Pharma, Andhra Pradesh, India), *MET* and *CLI* (Sigma-Aldrich São Paulo, Brazil). The criteria used for antimicrobial selection occurred according to the proposition of the Infectious Disease Society of America – IDSA (SOLOMKIN *et al.*, 2010). The MIC pattern was determined by the broth dilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017).

The MIC was considered the antimicrobial concentration at which no visible growth of the strains was detected, and the obtained results were interpreted according to the CLSI (2017), while the *su-binhibitory concentrations* corresponded to1/2 of the MIC of the antimicrobials (PTZ, MET and CLI). This test was repeated three times to confirm the result. For quality control of the tests, the reference strain *B. fragilis* ATCC 25285 and *Eggerrthella lenta* ATCC 43055 were included in all experiments.

2.3 Growth curves of *B. fragilis* strains without and with sub-inhibitory concentrations of PTZ, MET or CLI

To determine the time at which the cultures would reach between the intermediate log and the beginning of the stationary phase (required for all experiments), growth curves of *B. fragilis* were constructed in the absence or presence of 1/2 MIC of the antimicrobials.

Pre-inoculum of each bacteria grown in BRU-S under anaerobic conditions at 37°C for 24 h were diluted in 90 mL of the same medium with and without sub-MICs of each antimicrobial (PTZ, MET and CLI), to an optical density (OD) of 0.1 at 550 nm using a spectrophotometer (Thermo Scientific Multiskan® Spectrum, Vantaa, Finland). The flasks were then incubated in anaerobic chamber at 37°C and 100 µL aliquots of each bacterial culture were removed in triplicate to read the OD at 550 nm every 3 h up to 72 h (DOS SANTOS *et al.*, 2007; VELOSO *et al.*, 2013; DE ANDRADE *et al.*, 2016). For quality control of the tests the reference strain *B. fragilis* ATCC 25285 and *Eggerthella lenta* ATCC 43055 were included in all experiments.

2.4 Phenotypic tests to evaluate the pathogenicity of *B. fragilis* strains cultured previously in sub-MICs of PTZ, MET or CLI

Cell morphology - Bacteria were cultured on BRU-S, in the presence or absence of each antimicrobial at sub-MICs and incubated in an anaerobic chamber at 37°C for different times (determined on the growth curve: 30, 48, 72 and 96 h). Then, a smear was made from the colonies grown, and Gram staining was performed for morphotintorial evaluation under optical microscopy with immersion objective (1000x magnification) (FREITAS et al., 2015). The reference strain *B. fragilis* ATCC 25285 was used as quality control.

Capsule Detection – The Hiss staining method was used with modifications (NAKANO *et al*., 2004). *B. fragilis* strains were cultured anaerobically in yeast extract and peptone with 1% glucose in the presence or absence of antimicrobials in sub-MICs and incubated in anaerobiosis at 37°C for different times (determined on the growth curve: 30, 48, 72 and 96 h). One drop of this bacterial suspension was deposited on a microscopic slide. Then this was covered with crystal violet for 5 minutes. Each slide was washed with 20% copper sulfate and allowed to air dry. The capsule was visualized by optical microscopy with immersion objective (1000X magnification) by means of the verification of a free area around the bacteria.

Resistance to H2O2 - This test was performed using disk diffusion assays (DE ANDRADE *et al*., 2016). The strains were cultured on BRU-S, in the presence or absence of antimicrobials in sub-MICs and incubated in anaerobiosis at 37°C for different times (determined on the growth curve: 30, 48, 72 and 96 hours). Thereafter, a bacterial suspension at 1.5 x 10^8 CFU/mL was inoculated into BRU-S plates. Test carried out with the strains grown in the medium with sub-MICs of each antimicrobial and inoculated in Brucella agar without the drugs, was called 1X. On the other hand, when the strains were grown and platted in the agar medium with the respective antimicrobials in sub-MICs, it was called 2X. Sterile filter paper discs of 6 mm were placed on the plate added with 5 µL of the H2O2 agent (Merck, Darmstadt, Germany) at concentrations of 1%, 5%, 10% and 20%. After overnight incubation in anaerobiosis at 37°C, the diameters of the growth of the zones of inhibition (ZI) were measured. The tests were performed in duplicate and repeated on three different occasions.

Biofilm Formation - The microplate adhesion method was used (DONELLI *et al*., 2012). After growth in anaerobiosis on BRU-S at 37°C for 48 h, a bacterial suspension with turbidity of 0.5 on the McFarland scale in 0.85% sterile saline was prepared. A total of 20 µL of this suspension was added to 180 µL of BRU-S, with the presence or absence of pre-determined sub-MICs and packed in a flat bottom 96-well microplate, in quadruplicate. The microplates were incubated in anaerobiosis at 37°C for different times (determined on the growth curve: 30, 48, 72 and 96 h). After the recommended incubation and washing steps with phosphate buffered saline (PBS), the remaining bacteria were stained with 300 µL of crystal violet (NewProv, Pinhais, PR, Brazil) for 10 min at room temperature. The absolute ethanol (Merck, Darmstadt, Germany) was then added to solubilize the crystal violet that stained the biofilm. The solution contained in each well was transferred to a new, dry and clean plate. The absorbance was quantified in the spectrophotometer (Thermo Scientific Multiskan® Spectrum, Vantaa, Finland) at a wavelength of 550 nm (DOS SANTOS *et al*., 2007; FERREIRA *et al*., 2016). The tests were performed in quadruplicate and repeated on three different occasions. As a negative control, only BRU-S was used. The interpretation of the test was obtained by dividing the OD of the strains (ODa) by the OD of the negative control (ODc). The strains were also classified according to the

OD_c into the following groups: OD≤OD_c=non-adherent; OD_c<OD≤2xOD_c=weakly adherent; 2xOD_c<OD≤4xOD_c=moderately adherent; 4xOD_c<OD=strongly adherent (DONELLI *et al*., 2012).

Cell surface hydrophobicity - It was evaluated by the Microbial Adhesion to Solvents (MATS) method, according to Kos *et al*. (2003). The bacteria were cultured in BRU-S in the presence or absence of the antimicrobial agents in sub-MICs and incubated in anaerobiosis at 37°C for different times (previously determined on the growth curve: 30, 48, 72 and 96 h). Subsequently, they were washed twice with 1 mL of PBS and resuspended in 0.1 M KNO_3 solution (pH 6.2) to obtain the OD at 600 nm. Subsequently, 60 µL of xylene (Labsynth, Diadema, SP, Brazil) was added to 360 µL of the bacterial suspension, which was adjusted to an OD of 0.100 (A0). After 10 min preincubation at room temperature, the two-phase system was vortexed for 2 min and then held for 50 min. After this period, the aqueous phase was removed and the OD at 600 nm was evaluated (A1). MATS were calculated as the percentage of xylene associated bacteria according to the formula: MATS = [1 - (A1 / A0)] x 100. The strains were classified as highly hydrophobic (MATS> 70%), moderately hydrophobic (MATS 50-70%) or slightly hydrophobic (MATS <50%). The tests were performed in triplicate and repeated on two different occasions.

Hemagglutination – The bacterium was cultured in BRU-S in the presence or absence of antimicrobials in sub-MICs and incubated in anaerobiosis at 37°C for different times (previously determined on the growth curve: 30, 48, 72 and 96 h). Pellets were then collected, centrifuged (12,000 rpm/10,000 g, 5 min) and washed three times in PBS. The bacterial suspension was adjusted to a concentration of 1.5 x 10^8 CFU/mL. In parallel, erythrocytes of adult volunteers (A+, B+, AB+ and O+), horse and sheep were washed three times (5,000 rpm/600 g, 4°C, 5 min) and finally resuspended in PBS. Haemagglutination was qualitatively tested by mixing 50 *µ*L of bacterial suspension with 5 *µ*L of erythrocyte suspension in a 96-well (U-shaped) microtiter plate. Two serial dilutions of the bacterial suspension, with a final volume of 50 *µ*L/well, were performed. Subsequently, 50 *µ*L of erythrocytes were added to each dilution and the plate was gently shaked and incubated first at 37oC for 1h, then at 4oC overnight. Hemagglutination titers were expressed by the reciprocal of the highest activity dilution showing bacterial agglutination (NAKANO & AVILA-CAMPOS, 2004; HRV et al., 2016).

Hemolytic activity – It was investigated in trypcase soy agar (TSA, Becton Dickinson - BD, Sparks, USA) enriched with 0.1% hemin and menadione and 5% blood from horse, sheep or human volunteers (A +, B +, AB + and O +). The strains were cultured on BRU-S, in the presence or absence of antimicrobials in sub-MICs and incubated in anaerobiosis at 37°C for different times (determined on the growth curve: 30, 48, 72 and 96 h). Plates were inoculated in duplicate using Steers replicator, with final inoculum of 105 CFU/spot, and then incubated in anaerobiosis at 37°C for 1 week. The hemolytic activity was identified by checking a clear zone around the bacterial growth. *Streptococcus pyogenes* ATCC 19615

was used as a positive control (NAKANO & AVILA-CAMPOS, 2004; HRV *et al*., 2016).

2.5 Statistical Analysis

For the descriptive statistics SPSS (version 19.0), Excel and Stata version 12.0 (STATA Corp., TX, USA) were used. Stata was also used to perform linear regression analysis in an attempt to compare the dependent variables (inhibition halo size [mm], biofilm formation and hydrophobicity) with the independent variables. Three bacterial strains submitted or not to sub-MICs of PTZ, MET or CLI and the concentration of H_2O_2 (1%, 5%, 10% and 20%). Values of $p < 0.05$ were considered statistically significant.

3 I RESULTS

3.1 Minimum Concentrations of PTZ, MET and CLI capable of inhibiting the growth (MIC) of *B. fragilis* strains evaluated

The MIC of the three antimicrobials tested for the B. fragilis reference (ATCC 25285) and the two clinical strains (named P34 and P50) were determined according to CLSI (2017) guidelines. The sub-MIC was defined as ½ of the MIC, except for the P50 strain cultured in the presence of CLI, whose subinhibitory concentration was 1/4 of the MIC due to the technical impossibility to test this bacterial strain against the ½ of the CLI MIC (Table 1).

Strains	PTZ (μg/mL)	MET (μg/mL)	CLI (μg/mL)
***B. fragilis* ATCC 25285**	1.0	1.0	0.5
***B. fragilis* P34**	1.0	2.0	0.25
***B. fragilis* P50**	1.0	4.0	1.024

Table 1: Minimal Inhibitory Concentration (MIC) of Piperacillin/Tazobactam, Metronidazole and Clindamycin for *B. fragilis* strains described in this study.

Legend: PTZ: Piperacillin/Tazobactam; MET: Metronidazole; CLI: Clindamycin.

3.2 Growth curves of *B. fragilis* strains cultured with sub-MICs of PTZ, MET or CLI

When the growth curves were compared for the transition from mid-log to stationary phase between the strains, it was observed that it took 30 h after inoculation for the reference strain (ATCC), P34 and P50 grown without antibiotics to attain this stage. The same strains cultured with PTZ (ATCC+PTZ, P34+PTZ) and the P50 strain cultured with CLI (P50+CLI) also attained that state after 30 h. The P34 strain cultured with CLI (P34+CLI) took about 72 h. The ATCC+MET strain took 48 h. The ATCC+CLI, P34+CLI and P34+MET strains

took 72 h, while the P50+MET strain took 96 h.

A delay of 15 h for *B. fragilis* ATCC 25285 grown in medium with sub-MIC of MET, and 33 h cultured with sub-MIC of CLI (Figure1A) was observed as compared to the same bacterium grown without antimicrobial. The clinical strain P-34 showed a delay of 36 h when grown in medium with sub-MIC of MET and 30 h cultured in sub-MIC of CLI as compared to the same strain cultured without antimicrobial (Figure 1B). The clinical strain P50 had a delay of 60 h when cultured in medium with METY, and 9 h in medium with CLI as compared to the same strain cultured without antimicrobials (Figure 1C).

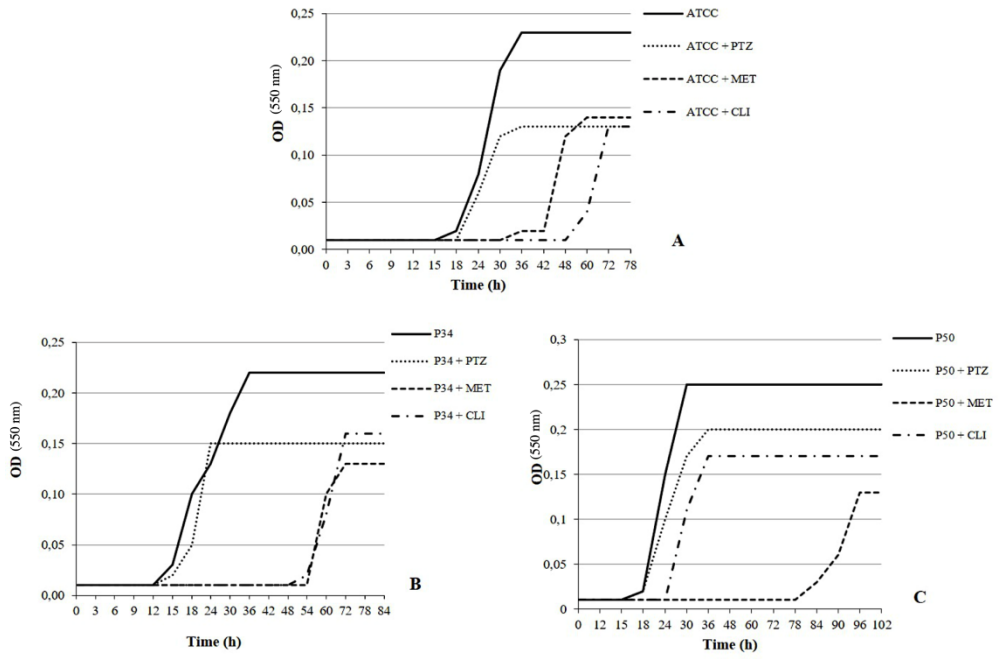

Figure 1 - Growth curve of the type strain *Bacteroides fragilis* ATCC 25285 (A) and fresh clinical *B. fragilis* P34 (B) and *B. fragilis* P50 (C) strains with or without addition of sub-MIC concentration of piperacillin/tazobactam, metronidazole or clindamycin antibiotics. Bacterial strains were grown in Brucella media containing the following antibiotics; piperacillin/tazobactam, metronidazole or clindamycin, at 1/2 concentration of the MIC previously determined. No antibiotic was added into the control cultures. Bacterial growth were determined by measuring OD at 550 nm.

Legend: OD: optical density; h: hours; PTZ: piperacillin/tazobactam, MET: metronidazole; CLI: clindamycin.

3.3 Evaluation of the pathogenicity of *B. fragilis* strains cultured in sub-inhibitory concentrations of PTZ, MET and CLI

3.3.1 Cell morphology

The typical morphology of the *B. fragilis* ATCC25285, a Gram-negative rod, is presented on Figure 2A. The clinical strains (ATCC25285, P34 and P50), cultured in the presence of sub-MICs PTZ (Figure 2B, C and D respectively) and MET (Figure 3A, B and C, respectively) exhibited cells with filamentous appearance.

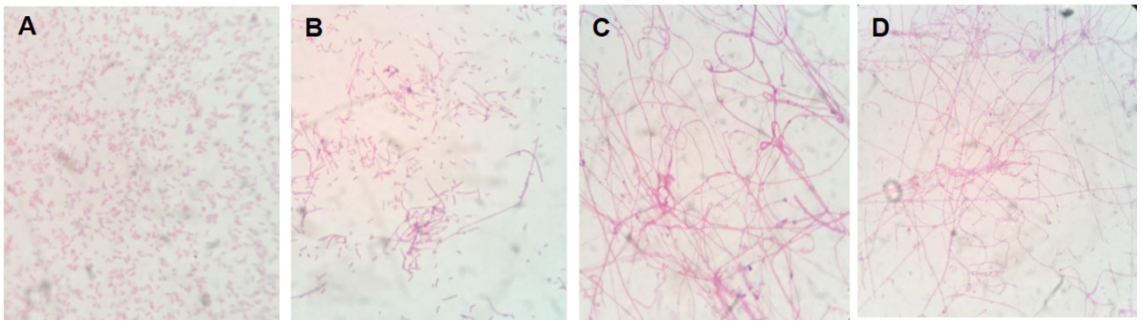

Figure 2. Gram staining of the *Bacteroides fragilis* ATCC 25285 reference strain and clinical isolates of *Bacteroides fragilis* strains (P34 and P50) cultivated in Brucella media containing piperacillin/tazobactam at 1/2 concentration of the MIC previously determined (0.5 μg/mL)

Legend: A- *B. fragilis* ATCC 25285 strain cultured without antimicrobial; B- *B. fragilis* ATCC 25285 strain cultivated in sub-MIC of PTZ; C- B. *fragilis* P 34 cultivated in sub-MIC of PTZ; and D- B. *fragilis* P 50 cultivated in sub-MIC of PTZ.

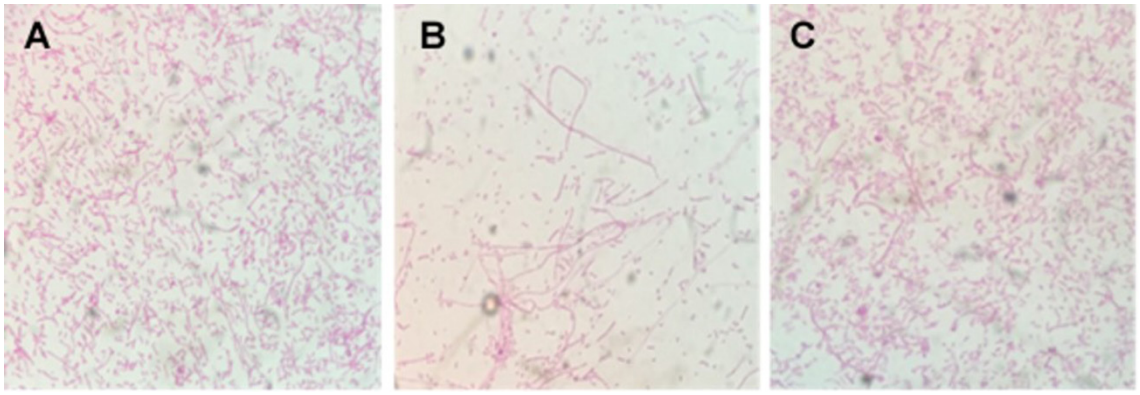

Figure 3. Gram staining of the *Bacteroides fragilis* ATCC25285 and clinical *Bacteroides fragilis* s

Legend: A- *B. fragilis* ATCC 25285 strain in sub-MIC of MET (0.5 μg/mL); B- *B. fragilis* P34 cultivated in sub-MIC of MET (1.0 μg/mL); C- *B. fragilis* P50 cultivated in sub-MIC of MET. trains (P34 and P50) cultivated in Brucella media containing metronidazole at concentrations ranging from 5 to 2.0 μg/mL

3.3.2 Capsule detection

With the methodology used, it was not possible to visualize the presence of a capsule in all *B. fragilis* strains cultured in medium with or without sub-MICs of the three antimicrobials.

3.3.3 Resistance to H_2O_2, Biofilm formation and Cell surface hydrophobicity

Table 2 shows that linear regression was used to compare the dependent variable of 'diameter of the zone of inhibition' with the independent variables of H_2O_2 concentrations (1%, 5%, 10% and 20%) and *B. fragilis* strains (reference and clinical). The constant (_ cons) that represents the medium size of ZI (mm) obtained by the reference strain without exposure to antimicrobials in the presence of the lowest concentration of hydrogen peroxide (1%). P50 strain previously cultured in a sub-MIC of PTZ showed the highest

halo (p <0.01), which is considered the most sensitive of all tested strains.

Table 2 also exhibits the result of linear regression analysis considering biofilm formation as a dependent variable (value obtained by dividing the OD_a by the OD_c) and comparing that to the independent variable (bacterial strains) and the constant (_cons) which was represented by *B. fragilis* ATCC 25285 without antimicrobial exposure, and the mean OD division found was 1.47. The dependent variable hydrophobicity defined by MATS was compared with the bacterial strains. The constant represents the average percentage of hydrophobicity (46%) of the *B. fragilis* ATCC 25285 strain without previous exposure to antimicrobials (Table 2).

Independent variables	**Dependent variables**					
	ZI size	*p value*	**Biofilm**	*p value*	***MATS***	*p value*
_cons[a]	17.13	-	1.47	-	46	-
H_2O_2 (%)					-	
5	38.98	<0.01	-	-	-	
10	45.92	<0.01	-	-	-	
20	52.91	<0.01	-	-	-	
***B. fragillis* Strains**						
ATCC + PTZ	17.68	0.46	2.07	0.58	44.5	0.66
ATCC +MET	16.55	0.44	1.2	0.80	25	<0.01
ATCC + CLI	14.81	<0.01	1.2	0.80	22.5	<0.01
P34	16.64	0.51	5.54	<0.01	50.5	0.44
P34 + PTZ	15.6	0.07	2.3	0.95	55	0.13
P34 + MET	15.71	0.09	4.4	0.01	33.5	0.05
P34 + CLI	16.65	0.52	2.84	0.22	36.5	0.11
P50	18.00	0.90	5.07	<0.01	48	0.72
P50 + PTZ	19.65	<0.01	5.2	<0.01	56.5	0.08
P50 + MET	14.72	<0.01	2.57	0.32	39.5	0.27
P50 + CLI	16.98	0.84	4.4	0.01	49.5	0.54

Table 2. Statistical analysis using linear regression of dependent variables (diameter of zone of inhibition in millimeters) and the independent variables (hydrogen peroxide at 1, 5, 10 and 20%, the ability of biofilm formation – represented by division of the OD of the strains by the OD of the negative control, Microbial Adhesion to Solvents – MATS and all tested Bacteroides fragillis strains – ATCC, P34 and P50).

Legend: ZI: zone of inhibition; MATS: Microbial Adhesion to Solvents; [a]_cons: constant (represents the average [mean] size of ZI (mm) obtained by the reference strain without exposure to antimicrobials, in the presence of the lowest concentration of hydrogen peroxide (1%); H_2O_2: hydrogen peroxide; PTZ: Piperacillin/Tazobactam; MET: Metronidazole; CLI: Clindamycin; ATCC: *B. fragilis* ATCC 25285; P34 and P50: clinical strains.

3.3.4 Hemagglutination and haemolitic activity

None of the bacterial strains (reference or clinical) showed hemagglutination in the different blood types tested: human (A +, B +, AB + and O +), sheep and horse. All strains hemolyzed all tested blood types after 6 days of incubation in anaerobiosis at 37°C.

4 I DISCUSSION

There are several situations during the infectious process in which the microorganism is subjected to sub-MICs of antimicrobials for varied periods of time. This may not be able to eliminate the microorganism but could interfere with the expression of some pathogenicity factors and may result in the selection of resistant bacteria. These changes in the pathogenicity of *B. fragilis*, in the presence of sub-MICs of antimicrobials demonstrate their pathogenic potential, being able to provoke different interactions between the host and the bacterium (DE SOUZA FILHO *et al.*, 2012; VELOSO *et al.*, 2013).

According to studies on antimicrobial resistance with *B. fragilis*, rates of resistance to CLI in several countries are reported between 24% and 56% (KANGABA *et al.*, 2015; KOUHSARI *et al.*, 2019). On the other hand, there are few reports of resistance to PTZ and MET (SZÉKELY *et al.*, 2015; YIM *et al.*, 2015). In the present study, the clinical strain P34 was susceptible to the three tested antimicrobials, and the clinical strain P50 showed only resistant to clindamycin (MIC=1.024 µg/mL).

The literature reports an increase in the time of growth of bacteria submitted to the sub-MICs of some antimicrobials and different stresses. Veloso *et al*. (2013) observed a delay of 15 h for the beginning of the log phase of growth of *B. fragilis* grown in sub-MICs of PTZ, a fact that was not corroborated in our study. In addition, another study observed that *Escherichia coli*, when cultured in sub-MICs of PTZ, reached the peak of filamentous cells with 16 h of growth, presenting 82% of cells altered in morphology and complexity (DE ANDRADE *et al.*, 2016).

The growth curve performed in the present study demonstrated delays for the beginning of the log growth phase for the reference and the clinical strains (P34 and P50) when cultured in sub-MICs of both MET and CLI. There was also a decrease in OD when they were cultured in sub-MICs of the three antimicrobials. Some authors believe that this is due to the decrease of enzymes related to the energetic metabolism of the bacterium (VELOSO *et al.*, 2013; DE ANDRADE *et al.*, 2016).

The formation of filamentous cells found in strains cultured in medium with sub-MICs of PTZ may be related to the fact that this antimicrobial interferes with bacterial cell wall synthesis by binding to penicillin-3 binding protein (PBP-3), which is responsible for cell septation during division. Thus, the strains continue the division process when in contact with the sub-MICs, but the septation does not occur (DE ANDRADE *et al.*, 2016). On the other hand, in relation to the sub-MICs of MET, changes in bacterial morphology appear to be related to the inhibition of autolytic enzymes that initiate septation (DINIZ *et al.*, 2000; FREITAS *et al.*, 2015).

However, when strains in the filamentous state were cultured in medium without sub-MICs of antimicrobials, the cells returned to their typical form, indicating that the morphological change was a transitory state. Therefore, it is possible that filamentous

cells represent the most sensitive subpopulations that were inhibited by the antimicrobials. However, the consequences of these morphological changes as pathogenicity factors and for clinical diagnosis are not well established (FREITAS *et al.*, 2015; DE ANDRADE *et al.*, 2016).

The polysaccharide capsule of *B. fragilis* is one of the most important pathogenicity factors of this bacterium, as it is responsible for its success in abscess formation, protection against the host's immune system and cell and mucosal adhesion (NAKANO et al., 2008; REIS *et al.*, 2014). In our study, it was not possible to visualize the presence of a capsule in all the strains with the methodology used; however, it does not mean that these strains do not have capsule. As a result, the Chinese ink staining with diluted fuccin was used (MOLINARO *et al.*, 2009), but it was also not possible to visualize a capsule.

According to the literature, within the first 30 min of exposure to oxygen (O_2) or H_2O_2, *B. fragilis* induces the expression of at least 28 proteins as part of a complex response to oxidative stress, and the magnitude of this response seems to be related to the concentration level of the stressor agent (SUND *et al.*, 2008). These proteins include enzymes that detoxify reactive oxygen species, protect DNA, and provide energy for the synthesis of new proteins. Some of the first genes induced during exposure to O_2 or H_2O_2 are members of the OxyR regulatory gene, providing an immediate protective reaction to stress (SUND *et al.*, 2008).

In the oxidative stress test by exposure to H_2O_2 in our study, when comparing the clinical *B. fragilis* strains without and with sub-MICs of the three antimicrobials, it was possible to verify that the strains, ATCC+PTZ, ATCC+MET, P34+CLI and P50+PTZ, previously cultured in medium with antimicrobials sub-MIC were more sensitive to this substance in relation to the same strains without sub-MICs, mainly in the concentration of H_2O_2 20%. This higher sensitivity was observed in all strains when the culture medium of the test plate also contained antimicrobial sub-MICs of antimicrobials (named by us as 2X test), in agreement with the results of Fonseca et al. (2004).

The increase in the sizes of ZI due to the presence of sub-inhibitory concentrations of antimicrobials in the culture medium is believed to be due to a decrease in the production of enzymes such as peroxidases and catalases. These enzymes are responsible for protecting the bacteria from damage caused by H2O2. The lack or decrease in these enzymes may explain the lower capacity of the bacteria to survive and multiply in the presence of oxidative stress (FONSECA et al., 2004; DE ANDRADE et al., 2016).

It is believed that the increase in the sizes of ZI in the tests in which antimicrobials were used at sub-MICs in the culture media and 2X test may be due to actions of the antimicrobials themselves, although in a sub-MIC concentration, there is a decrease or lack of response to oxidative stress (such as production of catalases and other peroxidases) (FONSECA *et al.*, 2004; DE ANDRADE *et al.*, 2016).

Only the ATCC+CLI and P50+MET strains had statistically significant ($p < 0.01$)

resistance to H_2O_2, that is, they showed a decrease in the size of halos, but the reasons were not elucidated, due to the lack of studies relating sub-MICs with oxidative stress. In addition, since sub-MICS of both CLI and MET did not decrease the size of the halos of the other strains tested, it is not possible to state that these antimicrobials increased the resistance to oxidative stress.

According to the literature, antimicrobials may interfere with bacterial adhesion to both epithelial and intestinal cells, as well as expression of other pathogenicity factors in various bacterial species (FREITAS *et al.*, 2015). Some antimicrobials may significantly decrease the bacterial adhesion process to certain types of surfaces, which can be explained by a change in the hydrophobicity of the cell surface, inhibition of motility, or possible decrease of the adhesins in the bacterial cell surface due to the induction of filamentous cells (FONSECA *et al.*, 2004).

Sub-inhibitory concentrations of antimicrobials could interfere with cell adhesion, hydrophobicity, motility, and the formation of biofilm. It also may affect other necessary factors for the formation of biofilm, such as the quorum sensing system, which is essential for communication between cells and is essential for proper training and maintenance of biofilm (OKAMOTO *et al.*, 2002; FONSECA *et al.*, 2004; DE ANDRADE *et al.*, 2016).

Although some studies have shown that sub-MICs of both PTZ and MET decreased the formation of biofilm and that CLI may increase the same, our results were not able to demonstrate these effects, probably due to the low number of strains tested (OKAMOTO *et al.*, 2002; DE SOUZA FILHO *et al.*, 2012; DE ANDRADE *et al.*, 2016).

The three strains previously cultured in the medium with and without sub-MICs of the three antimicrobials showed a hydrophilic character. This may be due to the presence of capsules in *B. fragilis*, which are generally hydrophilic and may contribute to the low hydrophobicity (REIS *et al.*, 2014). Although it was not possible to apply statistical treatment in our study, it was possible to observe that sub-MICs of MET decreased the hydrophobicity of the three strains, and CLI decreased the hydrophobicity of the reference strain and the clinical strain P34 as compared to the strains without sub-MICs of antimicrobials. According to the literature, this decrease in hydrophobicity may occur due to some alterations in bacterial surface structures, especially loss of fimbriae and adhesins (FONSECA *et al.*, 2004). However, there are no studies that relate hydrophobicity to sub-MICs of MET or CLI, and we can not yet clarify this lower hydrophobicity.

Although our study found a 10.5% increase in the hydrophobicity of the P50 strain previously cultured in sub-MICs of PTZ compared to the reference strain without sub-MICs of antimicrobials an increase in hydrophobicity of the two clinical strains in relation to strains without sub-MICs of antimicrobials, Andrade *et al.* (2016) observed a significant decrease in the hydrophobicity of *E. coli* with sub-MICs of PTZ. Fonseca *et al.* (2004) also observed a decrease in the hydrophobicity of *Pseudomonas aeruginosa* with sub-MICs of PTZ, attributing this fact to the changes in the bacterial surface structure, in particular the

fimbriae, which contribute to the hydrophobicity of *P. aeruginosa*. Therefore, no reports dicussing whethwe or not PTZ increases hydrophobicity were found.

Due to the fact that our strains are classified as weakly to moderately hydrophobic, it is possible that hydrophobicity does not exert a significant influence on tissue adhesion and, consequently, the formation of *B. fragilis* biofilms. Okamoto et al. (2002) observed that hydrophobicity in *Fusobacterium nucleatum* was not associated with adhesion in the host's buccal cells and suggested that the mechanism of bacterial adhesion in these species was influenced by the presence of adhesins located in the cell wall or the outer membrane.

While one study observed hemagglutination activity in 84% of clinical strains of *B. fragilis*, others did not significantly observed this ability in strains in different blood types, such as human, horse, sheep, rabbit, dog (NAKANO & AVILA-CAMPOS, 2004; REIS *et al.*, 2014). In our study, no hemagglutination was observed in any strain previously cultured with or without sub-MICs of the three evaluated antimicrobials in all blood types tested. Nakano and Avila-Campos (2004) suggested that the adhesins or outer membrane proteins of the bacterium may be responsible for hemagglutination.

Hemolytic activity is a means of providing bacteria with heme iron from blood cells. However, the precise mechanism and the actions of each hemolysin gene still need to be clarified (SUZUKI *et al.*, 2012). Robertson and coworkers (2006) found that *B. fragilis* hemolysin *hly*A gene produced α-hemolysis and β-hemolysis when combined with *hly*B gene. The authors also suggested that *B. fragilis* genes *hly*A and *hly*B could be combined to form a two-component hemolysin, increasing their individual hemolytic activities. However, Suzuki and colleagues (2012) suggested that hemolysin encoded by the *hly*A gene of *Prevotella intermedia* may act differently from that encoded by the *hly*A gene of *B. fragilis*. We agree that the expression of hemolysin genes and their respective activities should be better investigated in the future in an attempt to clarify the results obtained.

In our study, all evaluated strains previously cultured with or without sub-MICs of all three antimicrobials had hemolysis in all blood types tested after 6 days of incubation. A study by Robertson et al. (2006)2006 found that all strains (reference and clinical), showed hemolysis in human, sheep and horse blood.

However, one strain had hemolysis in horse blood, but not in human and sheep blood. These differences suggested that the hemolytic activity produced by *B. fragilis* can be influenced by the diversity of the strains, the origin of the red blood cells and the growth conditions. In addition, the long period of incubation to visualize hemolysis (6 days) suggests that the apparent lack of hemolytic phenotype in clinical strains of *B. fragilis* may be a consequence of the short incubation period (48 h) which is generally used by laboratories to perform the test (LOBO *et al.*, 2013). This fact was observed by Reis and coworkers (2014), as the hemolysis test in horse blood was negative for the 13 strains of *B. fragilis* used in the study.

5 I CONCLUSIONS

Some alterations found in *B. fragilis* strains due to the presence of sub-MICs of the antimicrobials tested in our study may reflect the pathogenic potential of these microorganisms. There was an interesting reponse of the clinical strain P50 when exposed to sub-MICs of PTZ. After sub-MICs exposition, the strain enhanced its ability of biofilm formation (p=0.002), became more resistant to H_2O_2 exposition (p=0,001) and also increased its hydrophobicity ('p' presented an interesting value: p=0.08, but it was not considered statistically significant for this study, despite clearly demonstrate a tendency to be statistically relevant). In addition, it is important to emphasize that physiological changes may affect the diagnosis and treatment of infections with *B. fragilis*. Therefore, our data highlighted the risks of inadequate treatment, not only related to the selection of antimicrobial resistance but also related to the implications for clinical microbiology and the evolution of infectious diseases.

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ÍNDICE REMISSIVO

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