GIOVANNA CAROLINE SANTANA

ATIVIDADE ANTIBACTERIANA E ANTI-BIOFILME *IN VITRO* DE ENTEROCINA ISOLADA E COMBINADA COM VANCOMICINA CONTRA *Enterococcus* VANCOMICINA RESISTENTE (VRE)

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina, como requisito final à obtenção do título de Mestre.

Orientação: Prof[a]. Dr[a]. Márcia Cristina Furlaneto.

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Londrina, 25 de abril de 2019.

Dedico este trabalho...

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota” (Madre Teresa de Calcuta).

SANTANA, Giovanna Caroline. **Atividade antibacteriana e anti-biofilme *in vitro* de enterocina isolada e combinada com vancomicina contra *Enterococcus* Vancomicina Resistente (VRE)**. 2019. 65f. Dissertação (Mestrado em Microbiologia) - Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

Enterococcus sp. são patógenos oportunistas reconhecidos por causar graves infecções hospitalares. A resistência aos antibióticos glicopeptídios, como vancomicina e teicoplanina, é a preocupação atual da resistência dos *Enterococcus* e coincidiu com o aumento da resistência à penicilina e aos aminoglicosídeos, configurando assim um desafio. Combinações entre peptídeos antimicrobianos e fármacos antibacterianos tradicionais, podem amenizar o problema da resistência bacteriana, pois o possível efeito sinérgico se torna estratégico, possibilitando o uso de doses menores dos fármacos no tratamento de doenças infecciosas, reduzindo os custos e diminuindo a toxicidade. Neste trabalho, a ação de enterocinas não concentradas e não purificadas produzidas por *Enterococcus* sp. obtidos de leite e queijo foi avaliada quanto ao seu potencial antibacteriano sobre isolados de *Enterococcus* sp. resistentes à vancomicina, bem como a ação sinérgica *in vitro* entre enterocina, produzida pelo isolado de *Enterococcus* sp. L3, e o antimicrobiano vancomicina (concentrações 16, 32 µg/mL) contra células planctonicas e biofilme de *Enterococcus* sp. resistentes à vancomicina (VRE). Os isolados foram selecionados a partir de ensaio de PCR, onde foram avaliados os perfis de expressão de genes relacionados a produção de enterocina e genes relacionados à resistência à vancomicina. Foi realizado o teste de ação antagônica por *spot on lawn*, seguido da obtenção do sobrenadante livre de células (CFS), o qual foi submetido a testes enzimáticos e de termoestabilidade. O CFS obtido foi exposto aos isolados VRE utilizando a técnica de poço difusão. A visualização de halos de inibição ao redor dos cultivos de *Enterococcus* VRE demonstrou a potencial ação antibacteriana dessas enterocinas sobre os isolados resistentes à vancomicina. A análise da interação entre enterocina e vancomicina foi realizada pela metodologia de *checkerboard.* Os resultados foram mensurados pela contagem celular e análise de densidade optica para compor o Indice de Concentração Inibitória Fracionada. A viabilidade celular também foi mensurada a partir da visualização da coloração de Iodeto de propídeo em microscopia de fluorescência. Foi demonstrado ação sinérgica contra 80% dos isolados VRE, sendo que para um isolado foi verificada interação parcial, os resultados obtidos trazem uma nova abordagem e perspectiva ao tratamento de infecções por VRE.

Palavras chave: Bacteriocina. Glicopeptídeo. Resistência. Sinergismo.

SANTANA, Giovanna Caroline. **Antibacterial and anti-biofilm activity of enterocin isolated and combined with vancomycin against Enterococcus vancomycin-resistant (VRE).**2019. 65f. Dissertation (Master degree in Microbiology) – State University of Londrina, 2019.

ABSTRACT

Enterococcus sp. are opportunistic pathogens recognized for causing serious hospital infections. Resistance to antibiotics glycopeptides, such as vancomycin and teicoplanin, is the current concern of *Enterococcus* resistance and coincided with increased resistance to penicillin and aminoglycosides, thus posing a challenge. Combinations of antimicrobial peptides and traditional antibacterial drugs may alleviate the problem of bacterial resistance because the possible synergistic effect becomes strategic, allowing the use of lower doses of drugs in the treatment of infectious diseases, reducing costs and reducing toxicity. In this work, the action of non-concentrated and non-purified *Enterococcus* sp. obtained from milk and cheese was evaluated for its antibacterial potential on *Enterococcus* sp. resistant to vancomycin, as well as the synergistic action in vitro between enterocin, produced by *Enterococcus* sp. L3, and the antimicrobial vancomycin (concentrations 16, 32 µg / mL) against planktonic cells and *Enterococcus* sp. vancomycin resistant (VRE). The isolates were selected from the PCR assay, where the expression profiles of genes related to the production of enterocin and genes related to resistance to vancomycin were evaluated. The antagonist action was performed by spot on lawn, followed by the cell free supernatant (CFS), which was submitted to enzymatic and thermostability tests. The obtained CFS was exposed to the VRE isolates using the well diffusion technique. The visualization of inhibition halos around the *Enterococcus* VRE cultures demonstrated the potential antibacterial action of these enterocines on the vancomycin resistant isolates. The analysis of the interaction between enterocin and vancomycin was performed by the checkerboard methodology. The results were measured by cell counting and optical density analysis to compose the Index of Fractional Inhibitory Concentration. Cell viability was also measured from the visualization of propidium iodide staining under fluorescence microscopy. A synergistic action was demonstrated against 80% of the VRE isolates, and for one isolate, partial interaction was verified, the results obtained bring a new approach and perspective to the treatment of VRE infections.

Keywords: Bacteriocin. Glycopeptide. Resistance. Synergism.

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1. INTRODUÇÃO

Enterococcus são patógenos oportunistas reconhecidos por causar graves infecções hospitalares, destacando-se endocardite, infecções do trato urinário e feridas cirúrgicas. A crescente prevalência de resistência antimicrobiana deste gênero tem um resultado crítico no sistema de saúde devido ao aumento da mortalidade e por falta de alternativa terapêutica de antimicrobianos.

A resistência aos antibióticos glicopeptídios, como vancomicina e teicoplanina, é a preocupação atual da resistência dos enterococos e coincidiu com o aumento da resistência à penicilina e aos aminoglicosídeos, configurando assim um desafio. As concentrações bactericidas mínimas para vancomicina *in situ* não podem ser alcançadas na prática clínica devido à toxidade do antibiótico, sendo necessário a atividade combinada de duas drogas.

Ainda, este gênero tem capacidade para a formação de biofilme, que confere maior facilidade de colonização de superfícies inertes e biológicas, protegendo o microrganismo dos agentes antimicrobianos e do sistema imunitário, intervindo também na ação de adesão e invasão das células do hospedeiro e interferindo na terapêutica.

Uma opção para minimizar essa situação é a combinação de bacteriocinas com antimicrobianos. Além da função sinérgica entre ambos componentes químicos, a combinação pode diminuir a concentração de antibióticos necessários para eliminar determinado patógeno alvo, além da diminuição dos efeitos colaterais adversos associados ao antibiótico.

Diante o exposto, este trabalho propõe avaliar a ação de enterocinas sobre isolados *Enterococcus* sp. resistentes à vancomicina (VRE), em células planctônicas e biofilme, além da ação sinérgica com o antimicrobiano vancomicina.

2. REVISÃO BIBLIOGRÁFICA

2.1. Características gerais do gênero *Enterococcus* sp.

Os *enterococos* são bactérias Gram-positivas ubíquas que foram isoladas do solo, águas superficiais e água do mar; em associação com plantas; em produtos alimentares fermentados; como parte da microbiota intestinal de ambos os vertebrados e invertebrados, e como agentes causadores de doenças humanas (ARIAS et al., 2012). O gênero *Enterococcus* tem até o momento 58 espécies descritas de acordo com informações compiladas da Lista de Nomes Procarióticos com Permanente em Nomenclatura (http://www.bacterio.net/enterococcus.html#r) (PARTE, 2014).

Sendo os *enterococos* considerados organismos comensais do trato gastrintestinal humano, também podendo ser patogênicos, estão comumente associados à infecção do trato urinário (ITU), bacteremia, endocardite, queimaduras e infecções da ferida operatória, infecções do abdome e vias biliares e infecção de cateteres e outros dispositivos médicos implantados. Na maioria dos levantamentos, *enterococos* são a terceira causa mais comum de endocardite, após *Staphylococcus aureus* e *Streptococos viridans* (MURDOCH et al., 2009; SLIPCZUK et al., 2013).

São extremamente resistentes e versáteis, podendo sobreviver em condições adversas de temperaturas e pH (entre 10ºC e >45ºC, pH entre 4,0 e 9,6) e sal (NaCl 6,5%) (ARIAS, 2012; FOULQUIÉ-MORENO, 2006; FACKLAM et al., 2002).

A patogenicidade de *Enterococcus* é multifatorial, complexa e ocorre a partir de uma sequência de fatores de virulência. Dentre os determinantes de virulência associados à patogenicidade destacam-se a resistência a antibióticos, como vancomicina (genes *van*A, *van*B, *van*C) e a produção de proteínas extracelulares como: hemolisina (genes *cyl*), gelatinase (gene *gel*E), proteínas de superfície (gene *esp*), adesinas de parede celular (gene *efa*), adesinas de colágeno (gene *ace*), substância de agregação (gene *asa*), *DNAse* e termonuclease (EATON & GASSON, 2001; FOULQUIÉ-MORENO et al., 2006; JOHANSSON &RASMUSSEN, 2013).

MacCallum e Hastings relataram pela primeira vez uma infecção enterocócica em 1899, descrevendo um caso de endocardite e oferecendo uma descrição detalhada das bactérias isoladas, que nomearam de *Micrococcus zymogenes*. Thiercelin, 1899, descreveu bactérias entéricas circulares comensais (um entérocoque) capazes de causar doença diarréica e septicemia.

De acordo com Top (2007), a capacidade dos enterococos em se estabelecerem como agentes de Infecções Relacionadas à Assistência à Saúde (IRAS) deve-se em parte à sua resistência intrínseca a muitos antimicrobianos e à sua capacidade de adquirir novas características de resistência. As espécies mais prevalentes nas IRAS são *E. faecalis* e *E. faecium*. Historicamente, *E. faecalis* foi isolado em cerca de 50,3% de todas as IRAS enterocócicas; no entanto, há uma tendência crescente para infecções causadas por *E. faecium*, principalmente associadas à elevação de cepas de *E. faecium* resistentes à vancomicina. Uma linha do tempo destacando os principais eventos no estabelecimento de enterococos como agentes de IRAS é mostrada na Figura 1.

Figura 1. Cronograma de eventos relevantes na história dos *enterococos* como patógenos humanos (retângulos azuis), aparência de resistência a antibióticos (retângulos verdes) e estreia clínica antibiótica (retângulos vermelhos). A linha do tempo começa em 1899 com a primeira descrição formal de *enterococos* como bactérias entéricas redondas. A linha do tempo, em seguida, salta para 1964 para a primeira descrição da transferência de resistência ao cloranfenicol, apenas 15 anos após a sua introdução clínica. Histórias semelhantes ocorreram para aminoglicosídeos e glicopeptídeos. Desde o final da década de 1980, a prevalência de *E. faecium* resistente à vancomicina tem aumentado, assim como a porcentagem geral de IRAS enterocócicas. A resistência aos novos antibióticos introduzidos, linezolida e daptomicina, surgiu muito rapidamente após a sua introdução clínica, mas a maioria dos *enterococos* continua suscetível.

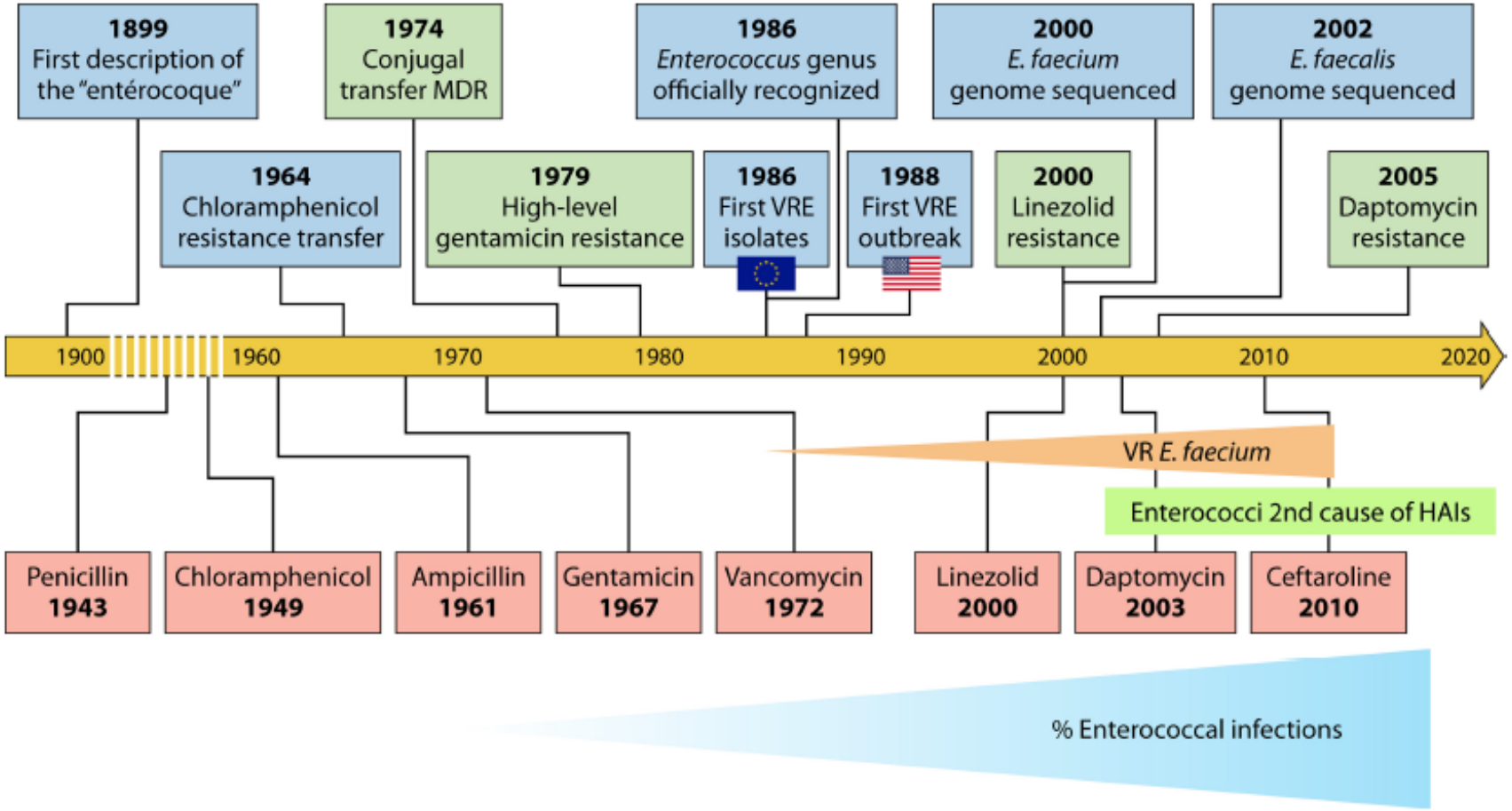

Imagem retirada de: The *Enterococcus:* a Model of Adaptability to Its Environment. rev. Clinical Microbiology Reviews, 2019.

Em contrapartida, são bactérias consideradas do grupo bactérias ácido lático (BAL) com aplicabilidade na indústria alimentar, devido a sua capacidade de inibir microrganismos patogênicos, pela produção de compostos antimicrobianos, e pela característica probiótica (DU TOIT, 2000; SONOMOTO, 2012; WANG, 2009).

2.2. *Enterococcus* sp. resistente à vancomicina

A propagação de bactérias resistentes aos antibióticos se tornou um grande problema de saúde pública em todo o mundo (MARTINEZ, 2002) sendo os fatores de risco para aquisição nosocomial de *Enterococcus* sp. resistente à vancomicima (VRE): internação prolongada, gravidade da doença de base, cirurgia prévia, hipoalbuminemia e o uso de antibióticos de largo espectro (TACCONELLI & CATALDO, 2008). A resistência antimicrobiana, juntamente com os fatores de virulência em VRE, contribuem para o desenvolvimento e disseminação dessas bactérias pelo ambiente hospitalar (PALAZZO et al., 2011).

A vancomicina é um antibiótico glicopeptídeo tricíclico e age inibindo a biossíntese do principal polímero estrutural da parede celular bacteriana, o peptidoglicano. Adicionalmente, a vancomicina afeta a permeabilidade da membrana citoplasmática e pode prejudicar a síntese de RNA. Devido a este mecanismo incomum, muitos pensavam que seria impossível a geração de resistência à vancomicina (MANDEL, 2009).

O antibiótico glicopeptídeo vancomicina permaneceu praticamente universalmente ativo contra *E. faecalis* e *E. faecium* por quase três décadas após sua introdução clínica. No início dos anos 80, começaram a surgir cepas, primeiro na Europa e depois nos Estados Unidos, que expressavam uma resistência de alto nível à vancomicina e à teicoplanina (SHLAES, 1989). A resistência foi atribuída à aquisição de operons que alteraram a natureza dos precursores de peptidoglicano, substituindo um terminal D-alanina pela terminal D-lactato (ARTHUR, 1993). No processo de estabelecimento da reticulação peptídica essencial para a estabilidade da parede celular, a D-alanina terminal é removida da cadeia para fornecer a energia para a reação de transpeptidação.

A vancomicina liga-se à alanina D terminal do precursor da

parede celular, impedindo o acesso PBP (a vancomicina, devido ao seu grande tamanho, também interfere um pouco com a reação de transglicosilação adjacente). A vancomicina liga-se a hastes pentapeptídicas que terminam em D-lactato com uma afinidade aproximadamente 1.000 vezes menor do que àquelas que terminam em D-alanina e, portanto, não é um inibidor efetivo da síntese da parede celular nessas cepas. O primeiro operon de resistência ao glicopeptídeo que foi descrito foi o operon vanA (ARTHUR, 1993), e este continua sendo o operon mais comumente encontrado no cenário clínico. O operon consiste em sete genes cuja finalidade combinada é substituir o pentapeptídeo suscetível ao glicopeptídeo que termina em D-Ala-D-Ala com um precursor que termina em D-Ala-D-Lac (ARTHUR, 1997).

A resistência aos antibióticos glicopeptídios, como vancomicina e teicoplanina, é a preocupação atual da resistência dos enterococos e coincidiu com o aumento da resistência à penicilina e aos aminoglicosídeos, configurando assim um desafio para os médicos que tratam pacientes com infecções causadas por estes microrganismos. Ainda como agravante, as concentrações bactericidas mínimas (CBM) requeridas para os enterococos resistentes são pelo menos 32 vezes maiores que as concentrações inibitórias mínimas (CIM) necessárias para essas bactérias quando sensíveis, concentrações que não podem ser alcançadas na prática clínica devido à toxidade do antibiótico. No entanto, a atividade combinada com outra droga tem sido uma opção (MANDEL, 2009).

Portanto, o aparecimento de resistência antimicrobiana, particularmente às bactérias resistentes a múltiplos antibióticos, junto à falta de antimicrobianos mais novos e com diferentes mecanismos de ação dos antibióticos atuais, tendem a ser um problema sério no futuro (BASSETTI, 2011; BUTTER, 2011). Sendo assim, novas estratégias para o controle dessas bactérias são urgentemente necessárias e a descoberta de novos agentes antimicrobianos, como bacteriocinas, por exemplo, podem ajudar a reduzir o desenvolvimento dessas infecções (DONADIO, 2010).

2.3. Biofilme em *Enterococcus* sp.

Os *enterococos* também são conhecidos por sua capacidade de formar biofilmes, os quais, são um fator chave nas infecções associadas aos

dispositivos em cateteres, materiais dentários e outros implantes médicos, bem como nas superfícies mucosas na cavidade oral e no trato urogenital, por isso, representam um problema clínico significativo, estando associado a aproximadamente 80% de todas as infecções médicas no mundo, além de contribuírem para a resistência bacteriana aos antibióticos e à fagocitose, tornando sua erradicação extremamente difícil (DUNNY, 2014; DESAI, 2014).

A habilidade destas bactérias em multiplicarem e desenvolverem em biofilme pode ser influenciada por vários fatores como temperatura, pH, concentração de carboidrato e disponibilidade do ferro e do CO_2 (MOHAMED & HUANG, 2007).

Em geral, a formação de biofilmes está associada à detecção de *quorum-sensing*. O sistema *quorum-sensing* é o mecanismo regulador dependente da densidade populacional, e pelo qual as bactérias se comunicam via moléculas de sinalização, chamadas de auto-indutores (BASSLER, 2006). Como descrito por Rutherford (2012), geralmente, em *quorum-sensing*, as bactérias produzem auto-indutores, e essas moléculas se acumulam no ambiente com o aumento da densidade celular. O papel desses auto-indutores depende da localização de seus receptores, que estão presentes na superfície celular ou no citoplasma. Um auto-indutor ativa especificamente seu receptor, que então, ativa a transcrição de genes que detectam *quorum*. Esse fenômeno fornece um mecanismo para que as bactérias sincronizem seu comportamento social, se comuniquem entre si e regulem a expressão gênica em resposta à sua densidade populacional.

Portanto, uma vez que os biofilmes se formam nas superfícies de dispositivos médicos, como cateteres vasculares residenciais, articulações prostéticas e marcapassos cardíacos, podem ser extremamente difíceis de erradicar e podem ser necessários procedimentos invasivos, como a remoção do dispositivo infectado. As infecções associadas ao biofilme são frequentemente crônicas ou redicivantes; portanto, a prevenção e o tratamento bem sucedidos dessas infecções são questões importantes devido ao impacto causado pela formação desse biofilme e a resistência aos antimicrobianos, sendo extremamente desafiadora a busca por novas estratégias para prevenir e erradicar biofilmes patogênicos (DONLAN, 2001).

2.4. Enterocinas

As bacteriocinas são sintetizadas ribossomicamente e liberam peptídeos antimicrobianos extracelularmente produzidos por bactérias que variam em estrutura, propriedades bioquímicas, modo de ação e espectro de atividade (CLEVELAND et al., 2001; SNYDER, 2013). As bacteriocinas produzidas pelas bactérias ácido lácticas (BAL) são mais cuidadosamente caracterizadas por sua qualidade e importância industrial. Estes peptídeos são principalmente ativos contra bactérias Gram-positivas e podem inibir vários microrganismos, como os de deterioração e patogênicos de origem alimentar.

Várias bacteriocinas foram caracterizadas e seu potencial como conservantes naturais de alimentos tem sido demonstrado (GRANDE et al., 2005; CARVALHO, 2007), o fato de as bacteriocinas geralmente não causarem alterações nos produtos alimentícios as tornam grandes candidatas para a conservação de produtos na área de alimentos (ELSSER-GRAVESEN, 2014).

Embora muitas bacteriocinas possuam características a serem utilizadas na conservação de produtos alimentícios, até o momento, apenas nisina (KOMITOPOILOU et al., 1999), enterocina AS-48 (SNYDER, 2013; GRANDE et al., 2005), bovicina HC5 (CARVALHO, 2008), enterocina 416K1 (ANACARSO et al., 2011), bificina C6165 (PEI, 2014) e pediocina (NARSAIAH et al., 2015) foram testadas contra microrganismos deteriorantes e patogênicos nesses tipos de produtos.

Bacteriocinas produzidas por BAL podem ser agrupadas em quatro grandes grupos, com base em sua estrutura, como por exemplo, similaridades entre a sequência primária, propriedades físico-químicas, sequência-líder e número de peptídeos que constituem sua atividade; podem ser também agrupadas, principalmente, com base em seu modo de ação (AYMERICH, 1996).

As bacteriocinas produzidas por *Enterococcus*, chamadas Enterocinas, são divididas em 4 classes. A Classe I refere-se aos lantibióticos, que constitui um grupo de peptídeos pequenos, que sofrem extensa modificação pós-traducional. A Classe II inclui os peptídeos pequenos (de 4 a 6 kDa), termoestáveis, e que não sofrem extensa modificação pós-traducional, exceto a clivagem do peptídeo durante o transporte para fora da célula. A

Classe II é dividida por Klaenhammer (1993) em três subgrupos: IIa, IIb e IIc. As bacteriocinas de Classe III são peptídeos termolábeis maiores que 30 kDa e as da Classe IV compreendem complexos de bacteriocinas que contêm lipídeos essenciais ou porções de carboidratos ligados a proteínas (FRANZ, 2007).

A síntese das bacteriocinas envolve quatro genes: (1) o gene responsável pela produção do pré-peptídeo; (2) o gene de imunidade específica que confere a produção de uma proteína de imunidade à célula produtora; (3) o gene que codifica proteínas do transportador ABC, responsável por exteriorizar a bacteriocina, e (4) o gene que codifica uma proteína acessória essencial para a exteriorização da bacteriocina, cuja função não está totalmente esclarecida. Esses genes estão organizados em um ou dois operons (NES, 2006). Em espécies *E. faecium*, a produção de bacteriocina é regulada por indutores específicos, os peptídeos feromônios. A regulação da expressão de bacteriocinas ocorre quando o peptídeo feromônio, sintetizado nos ribossomos como pré-peptídeos, sofrem clivagem e são transportados extracelularmente.

Quando os feromônios atingem um limiar de concentração específico, se ligam aos receptores da proteína quinase histidina (HPK) presentes na membrana celular, fosforilando o resíduo de histidina e o regulador de resposta que se liga à região promotora do gene de bacteriocina, ativando a expressão (NES, 2006).

Além da aplicabilidade como bioconservante de alimentos, as bacteriocinas também são de grande interesse clínico e farmacêutico, pois atuam como alternativa aos antibióticos convencionais no tratamento de doenças associadas a patógenos microbianos (YANG, 2014).

Diversas enterocinas já foram identificadas a partir de isolados de *E. faecalis* e *E. faecium* derivados de infecção, sendo bacteriocina 31 (TOMITA et al., 1996) e bacteriocina 41 (TOMITA, KAMEI e IKE, 2008) de *E. faecalis*, e bacteriocina 43 (TODOKORO et al., 2006), bacteriocina 32 (NES et al., 1996) e bacteriocina 51 (YAMASHITA et al., 2011) de *E. faecium*. Contudo, o estudo de enterocinas é mais comum a partir de isolados bacterianos de origem alimentar, a exemplo de enterocina L50A / L50B (CINTAS, 1998), Enterocina Q (CINTAS, 2000; CRIADO, 2006), Enterocina A (NILSEN, 1998),

Enterocina P (GUTIÉRREZ, 2005), Enterocina B (CASAUS, 1997).

Embora a maioria das enterocinas tenham sido caracterizadas de *E. faecium* e *E. faecalis*, algumas também já foram isoladas de *E. mundtii*, *E. avium*, *E. hirae* e *E. durans* (INGOLF, 2014).

As combinações sinergísticas de bacteriocinas com antibióticos também podem reduzir a carga financeira associada à síntese e à administração dos antibióticos mais caros. As interações sinérgicas bem sucedidas entre bacteriocinas e outros antimicrobianos podem ampliar o espectro de atividade, o que pode ser útil no tratamento de infecções clínicas de etiologia desconhecida. Uma variedade de métodos diferentes, que avaliam a sinergia antimicrobiana em condições de laboratório foram descritos na literatura, exemplos de tais testes incluem o ensaio de tabuleiro de damas (*checkerboard*), que permite a determinação do índice de concentração inibitória fracionada (FIC) (ORHAN, 2005; TSUJI e RYBAK, 2007; FOWERAKER, 2009; SOPIRALA, 2010; SOLTANI, 2012).

3. JUSTIFICATIVA

Nas últimas décadas, *Enterococcus* sp emergiu como um importante patógeno associado à saúde devido à sua capacidade de colonizar e causar doenças em pacientes de alto risco. Muitos desses problemas decorrem da capacidade dos enterococos apresentarem características de resistência a vários antibióticos, dentre eles à vancomicina, limitando as opções terapêuticas, tornando o controle da infecção mais difícil. Considerando a problemática dos isolados de *Enterococcus* sp resistentes à vancomicina em ambiente hospitalar, e tendo em vista o risco de disseminação, juntamente com a dificuldade de tratamento desses casos, se faz necessário estudos que busquem novas alternativas terapêuticas mais eficazes e acessíveis. Uma das alternativas diz respeito a testes com enterocinas, que vem apresentando grande potencial antimicrobiano contra diversos patógenos, de forma isolada ou associada a outro composto.

4. OBJETIVOS

Objetivo geral

Avaliar a potencial ação bactericida de enterocinas sobre células e biofilme de isolados de *Enterococcus* resistente à vancomicina, bem como a ação sinérgica com o antimicrobiano vancomicina.

Objetivo especifico

- determinar o potencial inibitório de enterocinas sobre células planctonicas de VRE;

- determinar a unidade arbitraria de enterocinas sobre células planctonicas de VRE;

- determinar o potencial inibitório de enterocinas sobre biofilme formado por VRE;

- determinar a concentração inibitória mínima da vancomicina frente aos isolados clínicos de VRE;

- averiguar a ação combinada de enterocina com vancomicina sobre células planctonicas e biofilme de VRE.

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ARTIGO 1

Short-Communication

Antibacterial activity of enterocin against vancomycin-resistant *Enterococcus*

ABSTRACT

The emergence and dissemination of antibiotic-resistant pathogenic bacteria such as vancomycin-resistant *Enterococcus faecalis* (VRE) has been an increasingly serious problem in public health worldwide. New strategies for controlling these bacteria are urgently needed. The use of bacteriocins as replacement for currently used antibiotics is promising, and the isolated bacterium in the genus *Enterococcus* might be a potential candidate strain for use as an alternative source of peptide antibiotics in the future. The present study evaluated the action of enterocins produced by isolates MF2, MF5 and L3 on planktonic cells of *Enterococcus* resistant to vancomycin. The genotypic profile of the enterocin-expressing genes were evaluated by PCR, and revealed the presence of *ent*A, *ent*B and *ent*X genes. The antagonist action was performed by spot on lawn, followed by the cell free supernatant (CFS), which was submitted to enzymatic and thermostability tests. The CFS obtained by isolates MF2, MF5 and L3 presented significant antagonistic action against *Enterococcus* VRE cultures. The treatment with proteolytic enzymes confirmed the proteinic character of the enterocin, as well as its resistance to heat treatment. The results demonstrated the potential antibacterial action of these enterocins on vancomycin resistant isolates, bringing a new approach and perspective to the treatment of these infections.

Keywords: vancomycin, resistance, *Enterococcus,* enterocins.

INTRODUCTION

Vancomycin-resistant enterococci (VRE) is one of the major pathogens that causes hospital infections and is notable for urinary tract infections, surgical site infections and bacteremia (BOURDDON et al., 2011; ORSI & CIORBA, 2013). Factors of virulence and resistance to antibiotics provide bacteria with the opportunity to adapt to hospital environments and acquire resistance genes to antibiotics, hindering therapeutic efficacy in their treatment (MARTINEZ et al., 2002; ZHU et al., 2010; RATHNAYAKE, 2012; KWON et al., 2012; HUMPHREY, 2014).

Enterococcus has an intrinsic resistance to several antimicrobials and also progressive acquired resistance to antimicrobials commonly used to treat enterococcal infections, and vancomycin is generally required for the treatment of invasive enterococci infections (PINHOLT et al., 2016).

However, the increasing resistance of Gram-positive bacteria to broad spectrum antibiotics and the lack of new antimicrobial molecules, advocates the need for new anti-VRE agents and / or other therapeutic options (DONADIO et al., 2010; TÄNGDÉN, 2014).

Antimicrobial peptides (AMPs) have been accepted as potential alternatives to traditional antibiotics in order to control the increase of bacterial infections. Several bacterial groups are known as potential sources of AMPs, such as lipopeptides and bacteriocins. Unlike lipopeptides, bacteriocins are protein-based AMPs, ribosomally synthesized primarily by lactic acid bacteria (LAB), and have been used in the food industry for more than 50 years and are generally recognized as safe (GRAS) (CLEVELAND et al., 2001). The genus *Enterococcus* produces a large number of bacteriocins (enterocins). Several species of this genus are enterocin-producing, such as *Enterococcus faecalis* and *E. faecium* (GOTO & YAN, 2011), *E. muntii*, *E. avium*, *E. durans* and *E. hirae* (SAAVEDRA et al., 2004; BATDORJ et al., 2006).

Studies reported a wide range of enterocin activities, inhibiting the growth of many undesirable pathogens (CINTAS et al., 2000) have highlighted the potential of *E. faecium* L50 in synthesizing three different enterocins called enterokin L50A and L50B, enterocin P and enterocin Q that act synergistically and inhibit the growth of many Gram-positive bacteria. Notably, enterocins were

also produced by enterococci from the human gastrointestinal system, sources of infection and feces (KANG et al., 2005; SAWA et al., 2012). E-760 enterocin to inhibit the growth of *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter jejuni* and *Staphylococcus aureus* (LINE et al., 2008). These data show that bacteriocins are sustainable antimicrobials that could be used alone or in combination with antibiotics.

Most of the bacteriocins act on the membrane of the target cell, that is, interrupting the integrity of the membrane, leading to the leakage of intracellular solutes and cell death, not discriminating between antibiotic resistant and sensitive bacteria (COTTER et al., 2013; KJOS et al., 2011), as with antimicrobials. Furthermore, there are reports of cell wall inhibitory action, protein synthesis and DNA degradation at nanomolar peak concentrations (RILEY, 1998).

Several in vitro studies have reported that the combination of bacteriocin and antibiotics or alone may be a promising option in the management of antibiotic use and treatment of life-threatening infections (BASSETTI & RIGHI, 2015; NAGHMOUCHI et al., 2013), and the relationship between the time and the duration of the study. In addition to potent inhibitory activity, increased interest in bacteriocins is due to the fact that they are safe for humans, their stability and resistance to bacteriocins have been rarely observed (GIULIANI et al., 2007; RUSSELL & MANTOVANI, 2002).

Within this premise, the objective of this study was to expose VRE isolates to the action of enterococci produced by *Enterococcus* from food samples.

MATERIAL AND METHODS

Bacterial strains

Initially a bacteriogenic action screening was performed between *Enterococcus* sp strains from foods (MF2, MF5, L3) previously identified for their antibacterial action. All strains were stored at -20ºC in Brain Heart Infusion medium (BHI) (Acumedia) supplemented with 20% (v/v) of glycerol. Total DNA was extracted according to the *boiling* method as described by Marques and Suzart (2004). The most common enterocins structural genes were amplified by polymerase chain reaction (PCR). PCR amplification was performed using specific primers (Table 1). The reactions contained 1% (v/v) total DNA , 10% (v/v) Taq buffer (10x), 2.5 mM MgCl, 0.17 mM dNTP, 20 pmol of each primer oligonucleotide, 1 U Taq DNA polymerase and qsp of ultrapure water. The cycles were programmed for 5 minutes of denaturation at 95°C, followed by 30 cycles of 95°C for 30 seconds, hybridization temperatures (Table 1) for 30 seconds and 72°C for 30 seconds, in a thermal cycler (Esco Swift MaxPro). The amplicons were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and the visualization was by observation in UV light transilluminator (Loccus).

Genotypic characterization of vancomycin resistant enterococci (VRE)

Five clinical isolates of *Enterococcus* (Ef28, Ef29, Ef32, Ef33, Ef34) belonging to the collection of strains from the Laboratory of Physiology and Molecular Biology of Fungi, State University of Londrina, previously identified as resistant to vancomycin were selected for this study. The confirmation of susceptibility was assessed by National Committee for Clinical Laboratory Standards (CLSI, 2017) criteria using Mueller-Hinton agar (HiMedia) containing vancomycin 256 µg/mL. *Staphylococcus aureus* ATCC 25923 was included as a positive control strain (WIMLEY, 2011). The genotypic basis of vancomycin resistance was determined by using polymerase chain reaction to amplify sequences coding for resistance, using oligonucleotide primers for *van*A (Table 1).

Table 1 – Specific primers for PCR amplification of genes that code for enterocin and resistance to vancomycin.

Target Gene	Sequence (5´→3´)	Annealing temperature (°C)	Amplicon size (pb)	References
EntA	GGTACCACTCATAGTGGAAA CCCTGGAATTGCTCCACCTAA	55	138	OZDEMIR et al., 2011
EntB	CAAAATGTAAAAGAATTAAGTACG AGAGTATACATTTGCTAACCC	56	201	DE VUYST et al., 2003
EntP	GCTACGCGTTCATATGGTAAT TCCTGCAATATTCTCTTTAGC	55	87	OZDEMIR et al., 2011
***entL*50A/B**	ATGGGAGCAATCGCAAAATTA TAGCCATTTTTCAATTTGATC	55	274	OZDEMIR et al., 2011
***Enterocin* 1071 AB**	GGGGAGAGTCGGTTTTTAG ATCATATGCGGGTTGTAGCC	50	243	MARTINEZ et al., 2002
***Enterocin* 31**	CCTACGTATTACGGAAATGGT GCCATGTTGTACCCAACCATT	50	122	DU TOIT et al., 2000
***Enterocin* AS48**	ATATTGTTAAATTACCAA GAGGAGTATCATGGTTAAAGA	50	185	DU TOIT et al., 2000
***Enterocin* X**	CCTCTTAATCATTAACCATAC GTTTCTGTAAAAGAGATGAAAC	50	500	EDALATIAN et al., 2012
VanA	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	56	231	BELL et al., 1998

Screening for enterocin production against VRE

Isolates with presence at least one enterocin structural genes (Ent+) were screened for their capability to produce bacteriocins against VRE. Therefore, *Enterococcus* Ent+ were streaked in plates containing BHI agar that were incubated at 37°C for 24 hours. The plates were inverted to receive 1ml of chloroform in the covers, and remained closed for 20 minutes. Residual chloroform was evaporated by opening of plates. Through the pour plate method, each indicator strain VRE (10^8 cells.mL^{-1}) was inoculated into soft BHI agar (0.8%) and poured into *Enterococcus* plates forming an overlay, and then plates were incubated at 37°C for 24 hours. Isolates were considered able to produce bacteriocins if inhibitions zones were found around the colonies (LEWUS et al., 1991).

Cell-free supernatant (CFS) preparation from *Enterococcus* and antimicrobial potential against vancomycin resistant enterococci (VRE)

Enterococcus MF2, MF5 and L3 strains was inoculated (1% v/v) into MRS broth (Acumedia) and incubated aerobically for 18 hours at 37°C. Then, the culture was centrifuged at 5.000 rpm for 15 minutes at 4°C (Jouan MR1822), and the supernatant was collected. To avoid the inhibitory effects of organic acids and hydrogen peroxide (H_2O_2), the pH of the CFS was adjusted to 6.5 with 1 M NaOH. The CFS was filtered through a 0.22-µm pore size filter (Millipore, Merck) (OGAKI et al., 2016).

Antagonistic action of enterocin on VRE isolates was performed according to well-diffusion methodologies (DE VUYST et al., 2007) and in microtiter plates (TOBA et al., 1991). BHI soft (0.8%) was inoculated with $1x10^8$ CFU/mL of the target bacterium, and after solidification, wells of approximately 5 mm in diameter were aliquoted 30µL of the sterile CFS was added. The plates were incubated at 37°C or 24 hours, and the results were based on observation of inhibition halos. For the antagonism test on the microplate, 100µL of bacterial inoculum ($1x10^8$ CFU/mL) were transferred to the polystyrene microplate along with 100µL of each CFS, incubation occurred at 37°C for 24 hours and the results were measured by optical density reading at 600nm. The control was the inoculum without CFS.

Thermal stability and protein character confirmation

The thermal stability of the CFS was performed after treatment at 100°C for 20 minutes (AMMOR, 2006). The sensitivity of CFS to proteolytic enzymes was determined by the treatment of supernatant with α-chymotrypsin, protease, trypsin and proteinase K at the final concentration of 1 mg/mL (GARRIGA, 1993). Subsequently, the antagonistic action test was performed according to the previously described methodology.

Statistical analysis

Data was analysed using the program Prisma®. The results are expressed as Optical Density (OD). Statistical significance between groups was assessed by ANOVA followed by Dunnett's test with the level of significance $p < 0.05$.

RESULTS

Three isolates of *Enterococcus* from food were selected in this study, because they presented antagonistic action against VRE (Figure 1A) and enterocin-encoding genes (Ent +) (table 2). Ent + isolates were identified in previous studies as belonging to the *E. durans* species (MF3 and MF5); the L3 isolate was confirmed only at the genus level. These isolates had at least two genes expressing enterocins and showed an antagonistic effect against the five VRE isolates.

The VRE isolates Ef28, Ef29, Ef32, Ef33 and Ef34 were confirmed belonging to the *E. faecium* species and had the *van*A gene (Table 2).

The enterocin present in CFS obtained from isolates L3, MF2 and MF5 showed antimicrobial activity against VRE (Ef29, Ef32, Ef33 and Ef34) (figure 1B), presenting significant difference ($p < 0.05$), except for the isolate Ef28 that was inhibited only when using enterocin L3 (Figure 2).

When CFS was treated with proteolytic enzymes, they lost the antagonistic effect, but this effect was maintained when treated at high temperature, confirming the protein character and thermostability, consistent with the characteristics of group II bacteriocins.

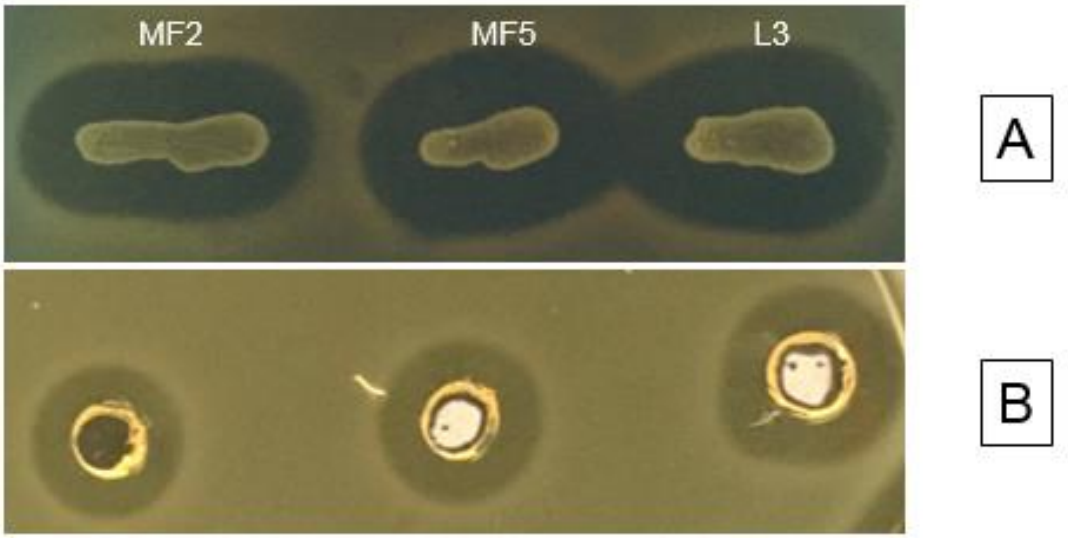

Figure 1 - Representation of the antagonistic tests by the spot on lawn (A) and well diffusion (B) isolates of *Enterococcus durans* (MF2 and MF5) and *Enterococcus* sp (L3) against clinical isolates *Enterococcus faecium* VRE; highlight the inhibition halo around the wells.

Table 2 - Source and identification of species of *Enterococcus* sp. and genotyping for vancomycin resistance and enterocin production.

Strain	Source	Identification	Genotyping
		Enterocin production	
MF2	cheese	*Enterococcus durans*	*ent*A, *ent*B
MF5	cheese	*Enterococcus durans*	*ent*A, *ent*B, *ent*X
L3	milk	*Enterococcus* sp.	*ent*A, *ent*X
		VRE	
Ef28	Clinical	*Enterococcus faecium*	*van*A
Ef29	Clinical	*Enterococcus faecium*	*van*A
Ef32	Clinical	*Enterococcus faecium*	*van*A
Ef33	Clinical	*Enterococcus faecium*	*van*A
Ef34	Clinical	*Enterococcus faecium*	*van*A

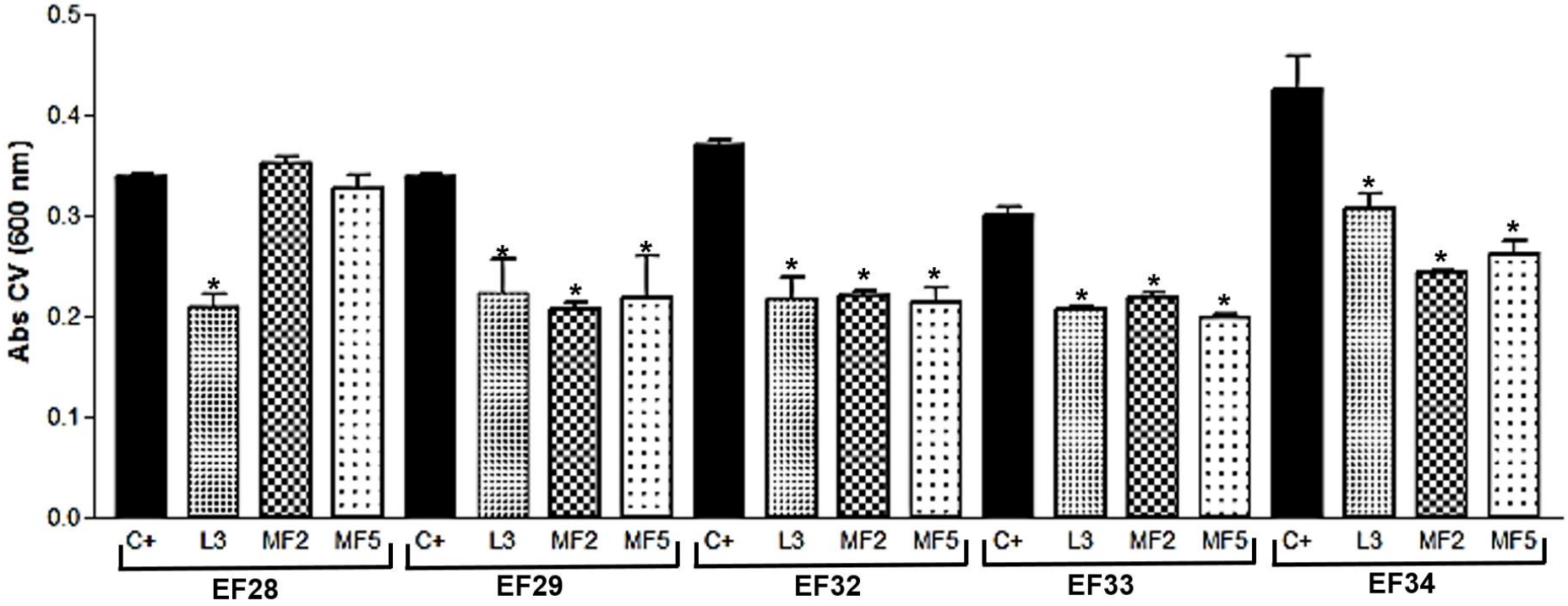

Figure 2. Representation of optic density observation antagonist tests of *Enterococcus durans* (MF2 and MF5) and *Enterococcus* sp (L3) isolates against clinical isolates of *Enterococcus faecium* VRE (Ef28, Ef29, Ef32, Ef33 and Ef34). The significant differences were determined by Anova followed by the Dunnett's test to compare the effect of CFS on the growth of each isolate against its respective resistant control (* $p < 0.05$).

DISCUSSION

Vancomycin-resistant enterococci (VRE) are one of the most important aetiologies of nosocomial infections worldwide, mainly due to their typical profile of multiple drug resistance and the tendency to cause serious infections in critically ill patients (KANG et al., 2013). The increasing prevalence of antimicrobial resistance in the genus *Enterococcus* sp. has a critical effect on the health system due to the increase in mortality due to the lack of a therapeutic alternative of effective antimicrobials in severe infections, such as endocarditis (LINDEN et al., 2007). These facts have encouraged scientists in the development of alternative therapeutic techniques, for example, the use of bacteriocins in the control of antibiotic-resistant bacteria (HOLMES et al., 2016).

In this study we evaluated the potential antagonistic effect of enterocin produced by *Enterococcus* strains called MF2, MF5 and L3 against VRE (Figure 1). The isolates present several genes encoding enterocins, and the results demonstrated the sensitivity to proteolytic enzymes and thermostability of the bacteriocin present in CFS, demonstrating to be enterocin. Also, by neutralizing the CFS with NaOH, we discard the possibility of the action of acids derived from bacterial metabolism. These data corroborate with the tests used by several authors (BENOUOUNA, 2018; JAOUANI et al., 2014; FOULQUIÉ-MORENO et al., 2006).

The *van*A gene expresses high resistance to vancomycin and teicoplanin, and is usually acquired through transposon (IWERIEBOR et al., 2010; ARSHADI, 2018). This fact and the presence of this gene on the isolates Ef28, Ef29, Ef32, Ef33 and Ef34 highlights the need to seek alternative forms of treatment for this microorganism. In addition, there are reports of isolates of *Enterococcus* vancomycin-dependent, where there is an increase in the number of cells in the presence of the antibiotic (TAMBYAH et al., 2004).

Enterococci have already been well documented for bacteriocin production, which gives them an environmental advantage against susceptible bacteria (NES et al., 2006). Several isolates producing enterocins were isolated from fermented, raw and processed foods (REHAIEM et al., 2014), and the mammalian intestinal microbiota (KURUSHIMA et al., 2013).

The presence of multiple genes encoding bacteriocin suggests a potent antimicrobial mixture, however, does not necessarily mean that all are

expressed together (LIU et al., 2011; PEREZ et al., 2012) indicated that some of the multiple bacteriocins in *E. faecium* were not expressed simultaneously and some regulated the expression of others.

Although it was not the purpose of this study to verify the expression of the enterocin-encoding genes, all the isolates presented the entA gene. According to several authors, the *ent*A and *ent*B genes appear to be commonly associated, since they use the same carrier responsible for the outsourcing of enterocin (DE VUYST et al., 2003; FRANZ et al., 1999; OZDEMIR et al., 2011) , as observed for the MF2 and MF5 isolates, except for the L3 strain that presented the *ent*A and *ent*X genes.

Independent of the presence of several genes that expression of enterocin in our isolates, all presented anti-VRE activity. As observed in the tests performed with CFS (containing enterocin), the *E. faecium* VRE 29, 32, 33 and 34 isolates had a significant reduction in the number of cells after 24 hours of enterocin incubation. It is worth noting in this study the high number of CFU used in the experiment ($1x10^8$) and that the enterocin used was not concentrated. The present study allowed to shed light on the potential of enterocins in VRE control.

The anti-VRE results of enterocins obtained in our study corroborate with several authors (KATHRYN, 2018; INOUE et al., 2006). Khan et al (2010) describe the activity against various pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*, *Mycobacterium* spp., *Escherichia coli* and *Myxococcus* spp.

The in vivo antimicrobial action of bacteriocins has been reported for other groups of microorganisms, such as microcin J25 against *Salmonella* (LOPEZ et al., 2007), bacteriocin OR-7 against *Campylobacter jejuni* and bacteriocin E 50-52 against *Salmonella* enteritidis (SVETOCH et al., 2008). In addition, the bacteriocin P126 class IIa exhibits activity against *Listeria* (*Pseudomonas aeruginosa*), which has been shown to be highly effective against MRSA infection (MOTA-MEIRA et al., 2005) and mersacidin (KRUZEWSKA et al.,2004). In the present study, the bacteriocin was used as a protective agent against bacterial infections. In addition, bacteriocin has been shown to be effective against *Clostridium difficile* (REA et al., 2007).

Vancomycin-resistant *Enterococcus* has emerged and has become a major clinical problem mobilizing the search for new potent antimicrobial agents (DONADIO et al., 2010; MARTINEZ et al., 2002). Given the limitations of the pharmaceutical industry in launching new drugs in the market, and in search of alternative methods for the control of infections caused by antibiotic-resistant bacteria already commercialized, bacteriocins appeared as a proposal to control infections (REHAIEM et al., 2014).

CONCLUSION

The approach to the issue of antimicrobial resistance in hospital strains is of paramount importance, in view of the search for control and new ways of fighting infections. In this study we show the anti-bacterial action of enterocin MF2, MF5 and L3 on vancomycin-resistant isolates of *E. faecium*, showing high efficiency even though they are neither concentrated nor purified.

ACKNOWLEDGMENTS

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ARTIGO 2

In Vitro Activities of enterocin and enterocin in combination with vancomycin against Vancomycin Resistant *Enterococcus* Biofilms.

ABSTRACT

The emergence and dissemination of vancomycin resistant pathogens is a global concern, particularly in *Enterococcus*. In addition, higher levels of resistance are conferred to bacteria in biofilms. Based on this, antimicrobial peptides have been an alternative as a potential solution, either as natural inhibitors that can be used alone or in combination with antibiotics acting synergistically, reducing the antimicrobial resistance. Here, we evaluate the potential of the antimicrobial peptide enterocin produced by *Enterococcus* sp L3, to increase the efficacy of the antibiotics vancomycin (MIC 16 e 32 µg/mL), with a focus on their application to prevent planktonic cells and biofilm formation of Vancomycin Resistant *Enterococcus* (VRE). The analysis of the interaction between enterocin and vancomycin was performed by the checkerboard methodology. The results were measured by cell counting and optical density analysis to compose the Index of Fractional Inhibitory Concentration. Cell viability was also measured from the visualization of propidium iodide staining under fluorescence microscopy. Synergistic action was demonstrated against 80% of the VRE isolates, and for one isolate, partial synergism was verified. The enterocin / vancomycin combination showed VRE antibiofilm efficiency. The results reveal that the concentrations of vancomycin that are required to effectively inhibit biofilm formation can be dramatically reduced when combined with enterocin, thereby enhancing efficacy, and ultimately, restoring sensitivity.

Keywords: *Enterococcus faecium*; synergism; antibiofilm.

INTRODUCTION

Worryingly, the horizontal transmission of vancomycin resistance in *Enterococcus* sp, both in hospital and community settings, presents a significant clinical challenge worldwide (STRYJEWSKI et al., 2014). In fact, in addition to resistance, the ability to form biofilm contributes to the success of *Enterococcus* sp as pathogen (FLEMING et al., 2010).

Biofilm is a community of microorganisms that adheres to biotic and abiotic surfaces and produces extracellular matrix consisting by polysaccharides proteins, nucleic acids and lipids (LAPLANTE et al., 2009). Biofilm formation is recognized as an important virulence factor in *Enterococcus*

sp, providing the bacterium with remarkable resistance to various chemical, physical and biological antimicrobial agents and is one of the main causes of persistent infection in the hospital environment (COSTERTON, 2001; DAUTLE, 2003; LEWIS, 2001). Bacterial cells in a biofilm exhibit resistance to the host's immune system and antibiotic treatments (KANIA et al., 2008). Therefore, once biofilms form, they can be extremely difficult to eradicate, and invasive procedures such as removal of the infected device may be required (DONLAN, 2001).

Indeed, such strategies for combinations with bacteriocin and antimicrobial to address issues related to prevent and eradicate bacterial biofilms formed by multidrug-resistant bacteria show great promise (REFFUVEILLE et al., 2014; DE LA FUENTE-NÚÑEZ et al., 2015).

In keeping with this line of enquiry, there has been a particular focus on assessing and enhancing the benefits of applying bacteriocin in clinical settings (COTTER et al., 2013; FIELD et al., 2015). Bacteriocin are ribosomally synthesized peptides and have become the focus of much biomedical and pharmaceutical research due to their high potency *in vitro*, numerous modes of action and capacity to destroy target cells rapidly (COTTER et al., 2005; TIWARI et al, 2015).

Enterocin (bacteriocins) have a broad spectrum of antimicrobial action against Gram positive and Gram negative bacteria, including *Listeria monocytogenes*, *Salmonella* enterica, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter jejuni* and *Staphylococcus aureus* (KANG & LEE, 2005).

Indeed, several studies have demonstrated synergistic relationships between conventional antibiotics and a more commun bacteriocin. The majority of these studies have involved Gram-positive bactéria such as Staphylococci, including methicillin-resistant forms (DOSLER et al., 2011; OKUDA et al., 2013), enterococci (TONG et al., 2014), including vancomycin resistant enterococci, and streptococci (LEBEL et al., 2013).

Bacteriocin also can be used as a anti-biofilm therapeutic strategies. In a direct line, combinations of antibiotics and bacteriocins could offer new therapeutic options, supported by different and successful in vitro studies (SOLTANI et al., 2012; HASSAN et al., 2012; MATARACI et al., 2012), search

for new combinations in the control of bacterial biofilm are necessary in the research.

In this premise, our study evaluated the enterocin produced by *Enterococcus* isolated L3 against planktonic cells and vancomycin-resistant *Enterococcus* biofilm (VRE), isolated and combined with vancomycin antimicrobial. In general, combinations of antibiotics and bacteriocins offer a new strategy to combat pathogenic bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Enterococcus sp L3 strains was cultured in Man-Rogosa Sharpe (MRS; Acumedia- Neogen) medium at 37°C with shaking. Five clinical *Enterococcus faecium* strains, named Ef28, Ef29, Ef32, Ef33 and Ef34 were cultured in Brain Heart Infusion (BHI; Acumedia- Neogen) medium at 37°C with shaken at 150 rpm.

Minimum Inhibitory Concentration Assays

Minimum inhibitory concentration determination was carried out in triplicate in 96 well microtitre plates as described by Clinical Laboratory Standards Institute (CLSI, 2017). Briefly, target strains Ef28, Ef29, Ef32, Ef33 and Ef34 were grown overnight in the appropriate conditions and medium. Vancomycin (Sigma) was resuspended in ultrapure H_2O to a stock concentration of 512 mg/mL. The antibiotics were adjusted to 16, 32, 64, 128 and 256 mg/mL starting concentration and two fold serial dilutions of each compound were made in 96 well plates. The target strain was then added and after incubation for 18 hours at 37ºC and the MIC was read at OD at 600nm in a spectrophotometer (Bio Tek, USA), as the lowest antibiotic concentration causing inhibition of visible growth.

Preparation of cell-free supernatant (CFS)

Enterococcus sp L3 strains was cultured in Man-Rogosa-Sharpe (MRS) (Acumedia- Neogen) broth medium at 37ºC for 18 hours. Cultures were adjusted to 0.5 McFarland standard in MRS broth at pH 6.2 following incubation at 37ºC, 180 rpm for 24 hours. The respective CFS was obtained by centrifugation at 5,000rpm for 15 minutes, neutralization with 1 M NaOH to a pH of 6.5, and filtered through a 0.22 µm filter. The CFS was stored at -20°C for further use. The confirmation of the antagonistic activity was done in agar well diffusion assay (AWDA), 30 µL of the CFS were deposited in 5 mm wells on BHI agar containing Ef28, Ef29, Ef32, Ef33 and Ef34 ($1x10^8$ CFU mL^{-1}). Finally, the plates were incubated for 24 hours at 37°C. Inhibition halo ≥ 2 mm was considered positive for bacteriocin production. Each condition was tested in duplicate (OGAKI, 2015).

Quantification of antibacterial activity by arbitrary unit (AU)

To quantify the antibacterial activity of enterocin against Ef28, Ef29, Ef32, Ef33 and Ef34, follow the protocol described by (OGAKI, 2015) was used with modifications. For this experiment, CFS (with enterocin) at 1:2 (v/v) dilutions were deposited on microplates using MRS. Then, 100 µL of each dilution with 100 µL of the indicator bacterium (10^8 CFU mL^{-1}) were deposited on microplate and incubated at 37°C for 24 hours, and bacterial growth was measured by OD at 600nm in a spectrophotometer (Bio Tek, USA). Each condition was tested in triplicate. The unit of arbitrary activity (AU mL^{-1}) was defined as the reciprocal of the last dilution that showed growth compared to the control (bacteria without enterocin) multiplied by 100.

Synergy testing

The study of the interaction between enterocin and vancomycin was carried out using the checkerboard method as proposed by EUCAST (AFST-EUCAST, 2008). In wells of microtiter plates, referring to the columns, 16 to 256µg/mL vancomycin concentrations were added; the CFS (enterocin) in serial dilutions 1: 2 (v/v) were added to the lines. Subsequently, 100µL of the inoculum of VRE isolates at a concentration of $1x10^8$ CFU.mL^{-1} was added. Plates were incubated at 37°C for 24 hours. After this period, an aliquot was withdrawn and cell viability measured in each of the combinations through the CFU count. Potential interactions of enterocin and vancomycin against VRE were evaluated by determination of fractional inhibitory concentration indice (FICIs) as described previously by Zuo et al (2014), as FICI = (MIC of the combination vancomycin and enterocin / MIC of vancomycin) + (MIC of the combination of enterocin and vancomycin / MIC of enterocin). The bacteriostatic interaction mode was judged by FICIs as follows: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 0.75, parcial synergism; 0.75 < FICI < 2.0, indifferent (or no effect) and FICI > 2, antagonism (FADLI et al., 2012).

Biofilm Formation by VRE

Static microtiter plate assays based on a previous study (MARQUES & SUZART, 2004), with modifications. Briefly, 200 µL of log phase cells (10^8 CFU ml^{-1}) in BHI broth were added in wells of a sterile 96-well microtiter plate; 200 µL of BHI was added to a set of wells as a negative control. All wells were seeded in triplicate. Microtiter plates were then incubated at 37°C for 24 hours. Following incubation, the liquid was gently aspirated and replaced with sterile PBS (pH 7.3). Each well was rinsed three times and air dried. Adherent bacteria were then stained with crystal violet (0.5%). The OD at 450nm of stained adherent bacterial films was read with a spectrophotometer (Bio-Tek L 808) The ODs of bacterial films were classified into nonadherent, weakly, moderately, and strongly adherent categories based on multiples of the OD readings as described by Stepanovic et al (2000). The test was carried out in triplicate.

Inhibition of biofilm with enterocin, vancomycin and a combination of both

Biofilm formation followed the protocol described above. Following incubation, the liquid was gently aspirated and replaced with sterile PBS (pH 7.3), and 100 µL of enterocin, vancomycin, and the combination, at the selected concentrations based on tests of MIC and checkerboard, followed by incubation for 24 hours. The evaluation of the antibiofilm activity of the isolated or combined solutions was measured by CFU.

Fluorescence microscopy analysis

Fluorescence microscopy analysis was done to observe cell death of the clinical strains, comparing with CFS-containing enterocin, vancomycin and both (combination), in biofilm on coverslips (13mm). The coverslips were washed twice with PBS and incubated for 15 minutes, at room temperature, in the dark, with 30µg/mL of fluorophores propidium iodide (PI) (Sigma-Aldrich, Germany) in PBS, as described elsewhere (JOHSON & CRISS, 2013). After incubation the staining solution was aspirated, the coverslips were washed in PBS and biofilms were observed using an epifluorescence microscope (Zeiss, Germany). The cells with reddish color were considered not viable.

RESULTS

In this study we evaluated enterocins produced by the *Enterococcus* sp L3 isolate against cells and biofilm formed by 5 isolates of *E. faecium* VRE. The ability of biofilm formation in the polystyrene microtiter plates was confirmed in all the isolates named Ef28, Ef29, Ef32, Ef33 and Ef34, within the characterization of strongly adherent, moderately adherent and non-forming biofilm, were classified as moderately adherent.

For synergism assay we used the lower antibiotic concentrations, together with the enterocin at 200 and 800 AU/mL.

Figure 1 shows the action of vancomycin and vancomycin combination with enterocin on clinical VRE planktonic cells. The AU of the enterocines did not differentiate the CFU, that is, whether or not they were diluted in the CFS, the combination of enterocin with vancomycin showed to be efficient, since there was a decrease in the number of CFU of all the isolates analyzed. However, the most surprising result was that in addition to the reduction of CFU, there was also a considerable reduction of the antibiotic tested to MIC 16 and 32µg/mL, when compared to the cell count using vancomycin alone (MIC 256 µg/mL). Because they were confirmed VRE isolates, cell development was expected in the presence of high concentrations of antibiotic.

When we applied the FICI formula, we obtained for most isolates synergistic action in the combination of enterocin and vancomycin; with exception for the isolate Ef33, which showed partial synergism. Even though enterocin was diluted in CFS, the results were promising, since we proved that the joint action, and especially the decrease of antibiotic used, may be a way to control antibiotic resistance.

In the results related to the action of the drug alone and in combination with enterocin on VRE biofilm (Figure 2), we observed decrease in the biofilm formed by the clinical isolates Ef28 and Ef29 when we used enterocin in combination with vancomycin (MIC 32 µg/mL) when compared only to the action of the separated compounds.

For the other isolates, there was also a reduction in biofilm when the two substances were combined; although the reduction was lower, but it is worth highlighting the decrease in the concentration of vancomycin, emphasizing the joint action of antibiotic and enterocin in the biofilm control by VRE. These

results were very similar to those obtained with planktonic cells, the combination of vancomycin and enterocin reduces the number of VRE cells. A reduction of up to 1log can be observed in both experiments.

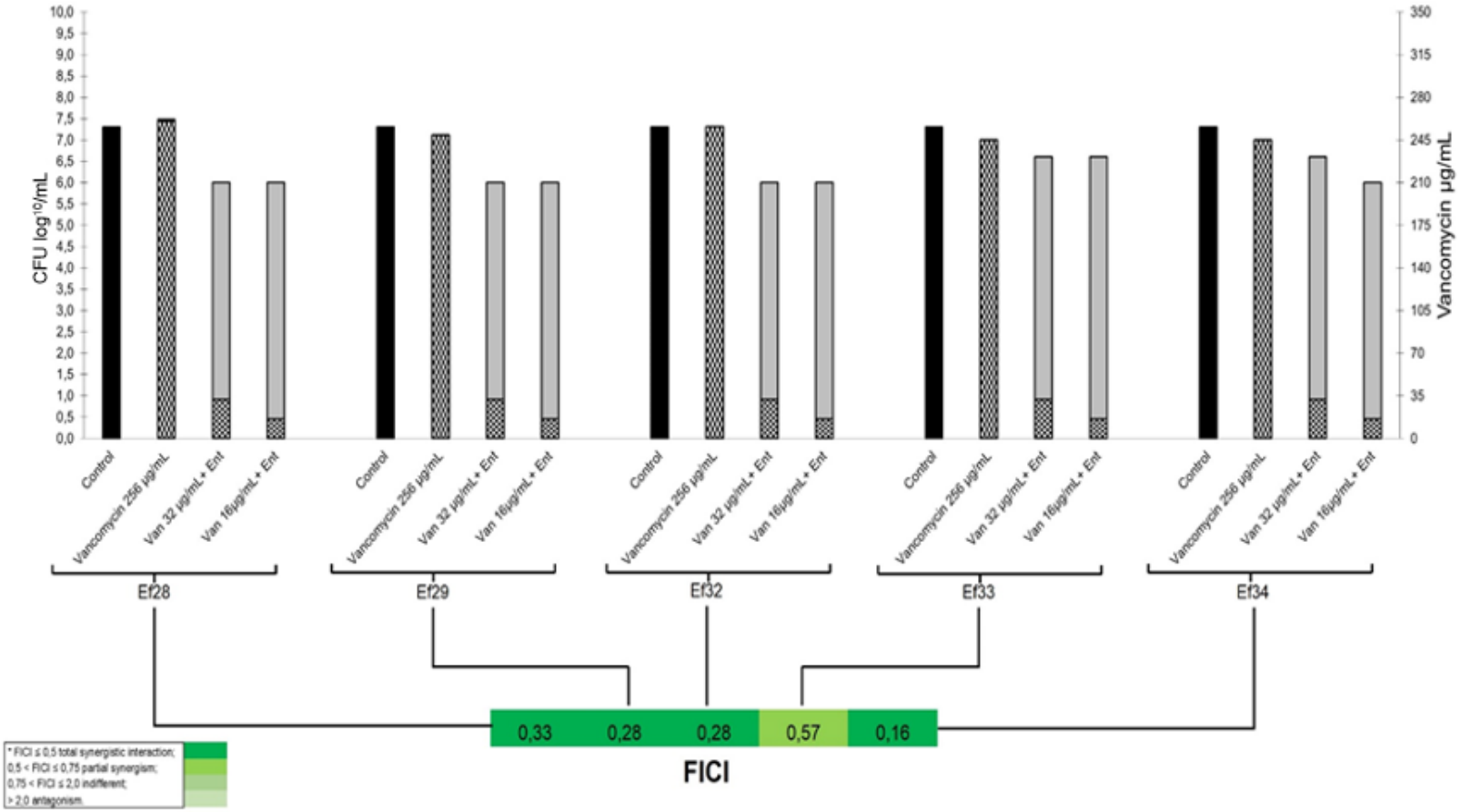

Figure 1 - Action of the combination of vancomycin and vancomycin with enterocin at concentrations determined by arbitrary unit (200 AU for isolates Ef28, Ef32, Ef34; 800 AU for Ef29, Ef33) in VRE clinical planktonic cells. FICI score are highlighted in green color. FICI ≤0.5 total synergistic interation; 0.5 <FICI ≤0.75 partial synergism; 0.75 <FICI ≤2.0 indiffetent; >2.0 antagonism.

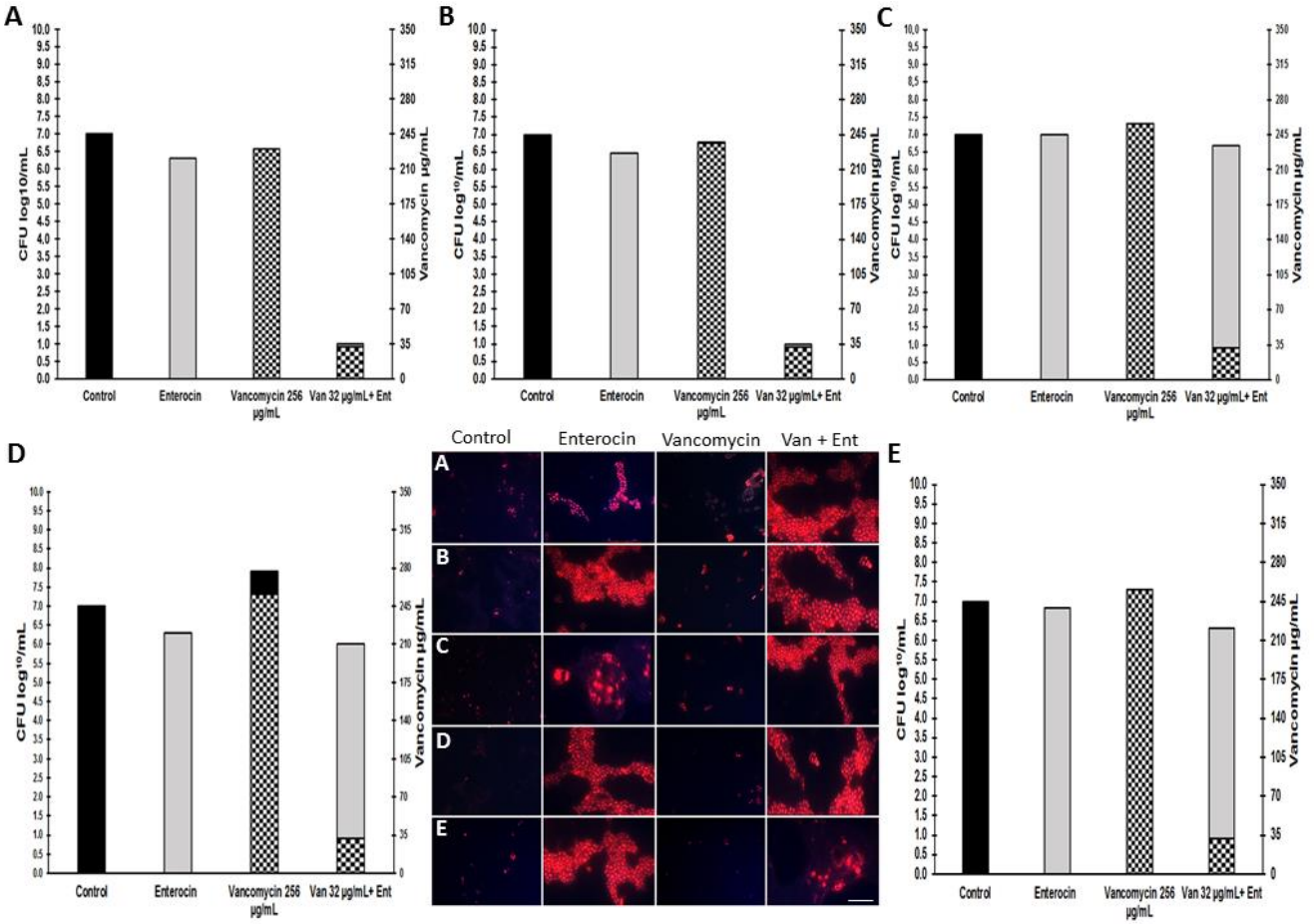

Figure 2 – Antimicrobial activity of vancomycin alone or combined with enterocin mature biofilms of the VRE strains; isolate Ef 28 (A), Ef29 (B), Ef32 (C), Ef33 (D), Ef34 (E). Photos refer to biofilm formation by VRE isolates on glass slides conditioned with enterocin, vancomycin and a combination of both, after 24 hours of incubation. The biofilms were stained with propidium iodide fluorophores (30 µg/mL), and photographed by fluorescence microscopy with a magnification of 1000x. Coloration for dead cells (red). The experiments were performed three times and representative images are shown.

The images obtained by the fluorescence microscopy confirmed the reducing viability of preformed biofilm of *E. faecium* exposed to CFS-containing enterocin are presented at Figure 2. In the absence of enterocin (control) it can be observed a reduced amount of dead cells (reddish color) compared to treated biofilms.

The addition of CFS in developing biofilm resulted in a biofilm structure composed of grouped cells and a larger number of dead; already in the presence of vancomycin, the dead cells are sparse. These results reveal that the combined drug and bacteriocin compounds have a greater potential for antibiofilm action.

Furthermore, antibiotic therapy by these, and other antibiotics, is hindered by the innate antibiotic resistance of bacteria present in biofilms (complex, sessile communities of bactéria embedded in an organic polymer matrix), making novel antibiofilm strategies highly desirable.

DISCUSSION

The rapid emergence and spread of vancomycin-resistant enterococci is a threat to public health globally, as vancomycin is often the last drug of choice for severe enterococcal infections (PAPADIMITRIOU-OLIVGERIS et al., 2014; SALGADO, 2008). In addition to antimicrobial stewardship program to solve the multiresistant microrganisms, as well as many preventive measures, public health research focused on identifying novel antimicrobial compounds. Bacteriocin from lactic acid bacteria (LAB) has gained interest due to its potential as a therapeutic antimicrobial substance, because they have many properties, including low toxicity, that make them suitable for clinical applications (COTTER et al., 2013). This study aimed to discover novel bacteriocins effective against clinical isolates of *E. faecium* and *E. faecalis* VRE, which are the two most common causes of lethal infections.

The ability of a bacterial isolate to form biofilm is considered to be a major cause of nosocomial infections (DAVIES, 2003), and all the Enterococcus isolates tested in this study were able to form biofilm, which makes their eradication even more complicated, emphasizing the need for therapeutic alternatives to control enterococcal infections. These microorganisms are known for their high ability to form biofilms, both on biotic and abiotic surfaces, and are surrounded by a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (COSTERTON, 2001).

The results presented in this paper indicate that enterocin is generally more active than vancomycin against most isolates VRE tested. In fact, following determinations of lower concentrations of MICvancomycin and analysis of combinations by checkerboard in the presence of enterocin and vancomycin, we obtained substantial inhibitory relationships against VRE planktonic cells, showing synergism interactions in 80% of the isolates tested. Some points are worth highlighting: (I) the best interaction results were observed when the lowest vancomycin values were used in combination with enterocin; (II) the use of isolated enterocin had an important impact on VRE reduction; (III) entercoin used in this study is not concentrated, but rather fluid in the supernatant from the cell culture.

Yomna et al (2017) report that the action of vancomycin on *Enterococcus* sp. is isolated-time-dependent, ie the antibiotic does not concomitantly eliminate

all isolates. This result corroborates with those obtained in this study, where we observed that the inhibition of CFU by vancomycin alone was isolated-dependent, and that even using high concentrations of the drug we did not obtain efficiency in the reduction of VRE.

Combinations of bacteriocins with antibiotics may decrease the concentration of antibiotics required to kill a target pathogen, thus decreasing the likelihood of adverse side effects associated with the antibiotic, including nephrotoxic effects, as occurs with vancomycin (MENDES et al., 2009; ABDELRAOUF et al., 2012). Atya et al (2016) also reported decreased concentration of the antibiotic vancomycin when used in combination with enterocin, in the control of MRSA (*Staphylococcus aureus* resistant methicillin). Our study confirms these data, since we obtained satisfactory results using 8 to 16 times less antibiotics.

Hanchi et al (2016) confirmed the synergistic action between duracin 61A with vancomycin against *Clostridium difficile*, *S. aureus* and *Streptococcus* sp, suggesting to be an alternative therapy (requiring less vancomycin) in the treatment of infections caused by these microorganisms. This synergistic combination was also observed against Gram negative bacteria (DICKS & TEN, 2010). Kim et al (2018) associated vancomycin with a conjugate of natural compounds and obtained inhibition of growth of VRE strains as well as antibiofilm activity.

The continuing emergence of multidrug resistant pathogens has been of interest in the search for alternative therapeutic options, including combinatorial antimicrobial therapy of bacteriocins with antimicrobials. In addition, combination therapy may allow the dose of the individual antimicrobials to be reduced and thus counteract the development of drug resistance in bacteria (HARSH MATHUR et al., 2017).

One of the hypotheses of this effectiveness between vancomycin and enterocin is based on the action of these substances, ie both substances affect peptidoglycan. Bacteriocins affect cells by pore formation and inhibition of peptideoglycan biosynthesis (HASSAN et al., 2012; YOUNT & YEAMAN, 2013).

Enterococci are well documented for the production of enterocins that allow them environmental advantage against susceptible bacteria (NES et al., 2006). *Enterococcus* strains producing enterocins have already been isolated

from fermented foods (REHAIEM et al., 2014), the intestinal flora of mammals including humans (KURUSHIMA et al., 2013) and raw and processed foods (GARVER & MURIANA, 1993). The enterocin studied in this work is produced by *Enterococcus* sp isolated L3 from raw milk, and has already been well characterized by our research group, regarding the presence of genes encoding enterocin and biochemical characteristics of the same. Most enterocines are classified in group II, according to Cotter et al (2005).

The data revealed more effective antibiofilm results in the presence of enterocin alone and in combination with vancomycin in all VRE isolates tested; unlike when vancomycin alone was used there was no significant decrease in VRE biofilm, including an increase in the number of CFU counts for isolates Ef32, Ef33 and Ef34. It is known that bacteria associated with biofilm are not affected with therapeutic concentrations of antimicrobial agents, and new control strategies are needed (DOSLER, 2011).

The antibiofilm result was verified by fluorescence microscopy, using a fluorescent probe (Propidium iodide - IP) to evaluate the integrity of the plasma membrane, if the cell integrates IP it can be concluded that there was loss of plasma membrane integrity since in normal situations the cells are impervious to IP. Thus, in Figure 2 the affected cells are exposed, demonstrating the action of the agents on VRE isolates, as described in a similar way by Field (2015).

We emphasize that the enterocin used in this study was neither purified nor concentrated, and even so, it shed light on the antimicrobial activity in planktonic cells and VRE antibiofilm. Therefore, enterocin concentration, purification, and encapsulation studies should be suggested in order to improve this interaction between enterocin /vancomycin.

CONCLUSION

The data collected in this study allowed to prove the antimicrobial activity of enterocin in planktonic cells and biofilm of vancomycin-resistant *Enterococcus.* We also confirmed the synergistic action between enterocin/vancomycin, with increased efficiency when compared to the use of vancomycin alone, as well as reducing the concentration of the antibiotic.

The utilization of from *Enterococcus* L3 alone or in combination with conventional antibiotics may be an effective therapeutic option for the treatment of vancomycin resistant bacteria. However, in future studies, enterocin L3 should be purified and identified. In addition, the antimicrobial effectiveness of enterocin L3 should be evaluated *in vivo*.

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