

NNEKA VIVIAN IDUU

**COMBINATORIAL EFFECTS OF BOVICIN HC5 AND EPSILON-POLYLYSINE
AGAINST *Alicyclobacillus acidoterrestris* IN ORANGE JUICE**

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Hilário Cuquetto Mantovani

Co-adviser: Maria Cristina Dantas Vanetti

**VIÇOSA - MINAS GERAIS
2022**

Ficha catalográfica elaborada pela Biblioteca Central da
Universidade Federal de Viçosa - Campus

T

I21c
2022

Iduu, Nneka Vivian, 1991-
Combinatorial effects of bovicin HC5 and epsilon-polylysine
against *Alicyclobacillus acidoterrestris* in orange juice / Nneka Vivian
Iduu. - Vicosa, MG, 2022.
1 dissertação eletrônica (68 f): il. (algumas color.).
Texto em inglês.
Orientador: Hilário Cuquetto Mantovani
Dissertação (mestrado) - Universidade Federal de Viçosa,
Departamento de Microbiologia, 2022.
Referências bibliográficas: .
DOI: <https://doi.org/10.47328/ufvbbt.2022.715>
Modo de acesso: World Wide Web.

1. *Alicyclobacillus acidoterrestris*; 2. Peptídeos antimicrobianos;
3. Suco de frutas - Conservação; I. Mantovani, Hilário Cuquetto II.
Universidade Federal de Viçosa.. Departamento de Microbiologia.
Programa de Pós-Graduação em Microbiologia Agrícola III. Título

CDD 22. ed. 579.362

Bibliotecário(a) responsável: ALICE REGINA PINTO PIRES CRB-6/2523

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APPROVED: October 27, 2022.

Assent:



Nneka Vivian Iduu
Author



Documento assinado digitalmente
HILARIO CUQUETTO MANTOVANI
Data: 02/12/2022 17:50:17-0300
Verifique em <https://verificador.itl.br>

Hilario Cuquetto Mantovani
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To God and to my mum.

ACKNOWLEDGEMENTS

To God, for His love.

To my mum, for her continuous prayers.

To the Federal University of Viçosa, for the opportunity to complete this postgraduate program.

To Professor Hilário Cuquetto Mantovani (adviser) and Professor Maria Cristina Dantas Vanetti (co-adviser) for their guidance, commitment, and immense support.

To members of the BIOTAN Laboratory, Nivea, Ana Julia, Rodrigo, Katialaine, Juliana, and Yasmin, for all their positive contributions.

To my friends, for all their moral support and advice.

To the Tertiary Education Trust Fund (TETFund), Nigeria, and Forum for Agricultural Research in Africa (FARA), for the financial support and organization of the Agricultural Research and Innovation Fellowship for Africa (ARIFA) program.

To Nnamdi Azikiwe University (NAU), Awka, Nigeria, for nominating me to be a beneficiary of this fellowship program.

To my fellow ARIFA scholars, for all the shared motivation

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for granting the scholarship.

To the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), for granting the scholarship.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), to granting the scholarship.

"For nothing will be impossible with God".
(Luke 1:37)

ABSTRACT

IDUU, Nneka Vivian, M.Sc., Universidade Federal de Viçosa, October 2022. **Combinatorial effects of bovicin HC5 and epsilon-polylysine against *Alicyclobacillus acidoterrestris* in orange juice.** Adviser: Hilário Cuquetto Mantovani. Co-adviser: Maria Cristina Dantas Vanetti.

Alicyclobacillus acidoterrestris is a thermo-acidophilic spore-forming bacterium that can survive pasteurization procedures and cause spoilage of fruit juices and acidic beverages in the food industry. Antimicrobial peptides (AMPs) are considered an alternative strategy to control the growth of this spoilage microorganism in acidic beverages. AMPs can be used alone or in combination with other antimicrobials to enhance the shelf-life of foods. In this study, the antibacterial effect of bovicin HC5 and ϵ -polylysine were evaluated individually and in combination against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 grown in AAM broth and orange juice. A significant decrease ($P < 0.05$) in the microbial growth and specific growth rate, and an increase in lag phase ($P < 0.05$) of vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 were observed with the addition of increasing concentrations of both antimicrobial peptides in AAM broth. Both AMPs exhibited strong inhibition of the target bacterium with a minimum inhibitory concentration (MIC) of 2.50 and 5.00 $\mu\text{g/mL}$ of bovicin HC5, and 7.81 and 7.81 $\mu\text{g/mL}$ of ϵ -polylysine against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498, respectively. Further studies showed that bovicin HC5 possessed bactericidal properties having a minimum bactericidal concentration (MBC) of 5.00 $\mu\text{g/mL}$ against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498. The antibacterial activity of both AMPs in combination was evaluated using the checkerboard assay. A fractional inhibitory concentration (FIC) index of 1 was obtained against the vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 indicating an additive effect between the two AMPs. The AMPs alone and in combination reduced ($P < 0.05$) the viability of *A. acidoterrestris* DSMZ 2498 germinated endospores that were inoculated (10^6 CFU/mL) in concentrated orange juice (11 °Bx). However, the antimicrobial effect was more pronounced with bovicin HC5 as viable cells and germinated endospores reduced

below the detection limit after 36 h of treatment. The exposure time required to kill 90 % of the initial microbial population at 95 °C ($D_{95^{\circ}\text{C}}$) was used to assess the effect of the AMPs in the thermal reduction of *A. acidoterrestris* DSMZ 2498 endospores. Concentrations of 80, 160 µg/mL of bovicin HC5, 125, and 250 µg/mL of ε-polylysine and their combination respectively were evaluated. Viable spores were evaluated by spread-plating aliquots taken at different time intervals in AAM medium (pH 4). The results showed that the mean D-value without AMPs was 7.68 min at 95 °C. With the addition of ε-polylysine into the juice, no significant difference was observed ($P > 0.05$) while treatment with bovicin HC5 reduced the D-value ($P < 0.05$) compared to control. This effect was observed with bovicin HC5 alone and in combination with ε-polylysine with a D-value reduction of 71.48 % and 68.36 %, respectively. Additionally, the effect of the AMPs on the morphology and cell structure of *A. acidoterrestris* DSMZ 2498 was evaluated by atomic force microscopy (AFM). The AFM topographical images and cross-sectional images revealed structural changes in the cell morphology of vegetative cells upon a 24 h treatment with 1X and 2X MIC of the AMPs alone and in combination. The findings in this study revealed the potential application of bovicin HC5 and ε-polylysine as natural preservatives in hurdle technologies to control *A. acidoterrestris* and improve the microbiological stability and safety of fruit juices.

Keywords: Antimicrobial peptides. Orange juice. *Alicyclobacillus acidoterrestris*. Thermoacidophilic. Spoilage. Fruit juice preservation

RESUMO

IDUU, Nneka Vivian, M.Sc., Universidade Federal de Viçosa, outubro de 2022. **Efeitos combinatórios de bovicina HC5 e epsilon-polilisina contra *Alicyclobacillus acidoterrestris* em suco de laranja.** Orientador: Hilário Cuquetto Mantovani. Coorientadora: Maria Cristina Dantas Vanetti.

Alicyclobacillus acidoterrestris é uma bactéria termoacidofílica formadora de esporos que pode sobreviver aos procedimentos de pasteurização e causar deterioração de sucos de frutas e bebidas ácidas na indústria alimentícia. Peptídeos antimicrobianos (AMPs) são considerados uma estratégia alternativa para controlar o crescimento desse microrganismo deteriorante em bebidas ácidas. Os AMPs podem ser usados sozinhos ou em combinação com outros antimicrobianos para aumentar a vida útil dos alimentos. Neste estudo, o efeito antibacteriano de bovicina HC5 e ϵ -polilisina foi avaliado individualmente e em combinação contra células vegetativas e endósporos germinados de *A. acidoterrestris* DSMZ 2498 cultivados em caldo AAM e suco de laranja. Uma diminuição significativa ($P < 0,05$) no crescimento microbiano e na taxa de crescimento específico e um aumento na fase lag ($P < 0,05$) de células vegetativas e endosporos germinados de *A. acidoterrestris* DSMZ 2498 foram observados com a adição de concentrações crescentes de ambos peptídeos antimicrobianos em caldo AAM. Ambos os AMPs exibiram forte inibição da bactéria alvo com uma concentração inibitória mínima (CIM) de 2,50 e 5,00 $\mu\text{g/mL}$ de bovicina HC5, e 7,81 e 7,81 $\mu\text{g/mL}$ de ϵ -polilisina contra células vegetativas e endosporos germinados de *A. acidoterrestris* DSMZ 2498, respectivamente. Estudos posteriores mostraram que a bovicina HC5 possuía propriedades bactericidas com uma concentração bactericida mínima (MBC) de 5,00 $\mu\text{g/mL}$ contra células vegetativas e endosporos germinados de *A. acidoterrestris* DSMZ 2498. A atividade antibacteriana de ambos os AMPs em combinação foi avaliada usando o ensaio checkerboard. Um índice de concentração inibitória fracionada (FIC) de 1 foi obtido contra as células vegetativas e endósporos germinados de *A. acidoterrestris* DSMZ 2498 indicando um efeito aditivo entre os dois AMPs. Os AMPs isoladamente e em combinação reduziram ($P < 0,05$) a viabilidade de endósporos germinados de *A. acidoterrestris* DSMZ 2498 inoculados (10^6 UFC/mL) em suco de laranja concentrado (11 °Bx). No entanto, o efeito antimicrobiano foi mais pronunciado com bovicina HC5 como células viáveis e endosporos germinados

reduzidos abaixo do limite de detecção após 36 h de tratamento. O tempo de exposição necessário para matar 90 % da população microbiana inicial a 95 °C (D95 °C) foi usado para avaliar o efeito dos AMPs na redução térmica de endósporos de *A. acidoterrestris* DSMZ 2498. Foram avaliadas as concentrações de 80, 160 µg/mL de bovicina HC5, 125 e 250 µg/mL de ε-polilisina e suas combinações, respectivamente. Esporos viáveis foram avaliados por alíquotas espalhadas em diferentes intervalos de tempo em meio AAM (pH 4). Os resultados mostraram que o valor D médio sem AMPs foi de 7,68 min a 95 °C. Com a adição de ε-polilisina no suco, nenhuma diferença significativa foi observada ($P > 0,05$), enquanto o tratamento com bovicina HC5 reduziu o valor D ($P < 0,05$) em relação ao controle. Este efeito foi observado com bovicina HC5 sozinha e em combinação com ε-polilisina com uma redução do valor D de 71,48 % e 68,36 %, respectivamente. Adicionalmente, o efeito dos AMPs na morfologia e estrutura celular de *A. acidoterrestris* DSMZ 2498 foi avaliado por microscopia de força atômica (AFM). As imagens topográficas AFM e as imagens transversais revelaram mudanças estruturais na morfologia celular de células vegetativas após um tratamento de 24 h com 1X e 2X MIC dos AMPs sozinhos e em combinação. Os resultados deste estudo revelaram a aplicação potencial de bovicina HC5 e ε-polilisina como conservantes naturais em tecnologias de barreira para controlar *A. acidoterrestris* e melhorar a estabilidade microbiológica e a segurança dos sucos de frutas.

Palavras-chave: Peptídeos antimicrobianos. Suco de laranja. *Alicyclobacillus acidoterrestris*. Termoacidófilico. Deterioração. Preservação de suco de frutas

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LIST OF ACRONYMS AND ABBREVIATIONS

EMR	Expert Market Research
SD	Standard Deviation
SEM	Standard Error of Mean
CLSI	Clinical and Laboratory Standards Institute
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
PC	Pure Culture
CAC	Codex Alimentarius Commission

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
°Bx	Degree Brix
~	Approximately equal to
±	Plus-minus
≤	Less than or equal to
≥	Greater than or equal to
h	Hour
min	Minute

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1. INTRODUCTION

Fruit juice is the unfermented liquid obtained from the edible part of mature and fresh fruit or fruits that have been harvested and properly preserved (CAC 2019). The fruit juice sector shows positive and dynamic growth mostly due to a change in the population's lifestyle and the fact that consumers are becoming more aware of the potential health benefits of using fruit beverages (Licciardello et al. 2018). World fruit production went up 54 % between 2000 and 2019, to 883 million tonnes, which represents an increase of 311 million tonnes (FAOSTAT 2021). The global fruit juice market reached a volume of about 46.8 billion liters in 2020 and is further expected to grow at a Compound Annual Growth Rate (GAGR) of 2.1 % from 2022 to 2027 to reach a volume of 53 billion liters (EMR 2020).

Commercial fruit juices have been regarded as safe from pathogenic and spoilage-causing microorganisms due to their inherent high acidity, which is often unfavorable for microbial survival, growth, and proliferation (Kakagianni et al. 2018). The industrial microbiological control of fruit juices is commonly achieved by subjecting the juices to pasteurization with a temperature range of 80 – 100 °C for less than 30 s (Ağçam et al. 2018). Nonetheless, spores can survive these temperatures, and although juice has a low pH, spores of thermoacidophilic bacteria can germinate and grow into vegetative cells, leading to the spoilage of the food product (Molva and Baysal 2014). Among the spore-forming microorganisms that cause the deterioration of juices are the species of the genus *Alicyclobacillus*, such as *A. acidoterrestris*, *A. pomorum*, *A. herbarius*, and *A. acidiphilus*. Of these species, *A. acidoterrestris* is considered the most important (da Silva et al. 2020).

A. acidoterrestris is a Gram-positive, non-pathogenic, spore-forming, thermoacidophilic, and strictly aerobic bacterium (Molva and Baysal 2015). It grows at temperatures ranging from 26 to 60 °C, with an optimal growth temperature between 42 and 53 °C, and a pH ranging from 2.0 to 6.0, with an optimum pH between 3.5 and 5.0 (da Silva et al. 2020). These physiological characteristics of *A. acidoterrestris* allow its survival during the commercial pasteurization process and also make this bacterium a key quality control target for pasteurized fruit juices and relevant beverages (Cai et al. 2015). *A. acidoterrestris* produces substances that promote odors in juices, such

as guaiacol, 2,6-dibromophenol, 2,6-dichlorophenol, and the halophenols (Osopale et al. 2016). The off-flavors of these compounds compromise the quality of the products (Pornpukdeewattana et al. 2020), which leads to unaccepted products and substantial economic losses for the beverage industry.

Since pasteurization ensures the inactivation of vegetative cells but does not affect endospores, alternative approaches to control the germination of endospores in juice have been sought. Among these, antimicrobial peptides have been proposed as an effective and safe alternative to control food-borne pathogens and spoilage bacteria in fruit juices (Anjos et al. 2016; Barbosa et al. 2017)

Epsilon-polylysine (ϵ -polylysine) is a natural antimicrobial cationic peptide that is generally regarded as safe (GRAS) as a food preservative (Li et al. 2014). This compound exhibits a wide antimicrobial spectrum against Gram-negative and Gram-positive bacteria, yeasts, and molds (Tan et al. 2019). ϵ -Polylysine is characterized as being edible, non-toxic to humans, water-soluble, and stable at high temperatures (Tan et al. 2019; Wang et al. 2021) and can inhibit bacterial growth, mainly through electrostatic adsorption to the bacterial cell surface and its cationic properties which can damage the cell membrane and lead to cell death (Chang et al. 2010). Hu et al. (2020) reported that ϵ -polylysine had antibacterial effects on cells and spores of *A. acidoterrestris* ATCC 49025 and *A. acidoterrestris* OSYE.

Bovicin HC5, a bacteriocin from *Streptococcus equinus* HC5, was also able to inhibit several Gram-positive bacteria, including spoilage and pathogenic bacteria commonly found in foods (Barbosa et al. 2017; Garsa et al. 2019). Bacteriocins are ribosomally synthesized peptides that display antimicrobial activity against bacteria closely related to the producer strain, but to which the producer strain itself is resistant (Logan et al. 2020). Bovicin HC5 is very stable at high temperatures (121 °C / 20 min) and in acidic environments and binds to bacteria that are susceptible at pH values < 6.0 (Houlihan and Russell 2006; de Carvalho et al. 2008) and these characteristics are suitable to control thermo-acidophilic spoilage bacteria in acidic drinks that have been heat-treated. Bovicin HC5 has been reported to be bactericidal and sporicidal against *A. acidoterrestris* under laboratory conditions, but the concentration required to achieve inhibition is higher in the fruit juice matrix (de Carvalho et al. 2008).

Therefore, this work aimed to evaluate the combinatory antibacterial effect of bovicin HC5 and ϵ -polylysine in controlling the growth of vegetative cells and spore germination of *A. acidoterrestris* in orange juice.

2. LITERATURE REVIEW

2.1. *Alicyclobacillus* sp.

Alicyclobacillus species are Gram-positive, nonpathogenic, rod-shaped, thermophilic, and acidophilic spore-forming bacteria (Pornpukdeewattana et al. 2019). *Alicyclobacillus* spp. produce endospores that can be terminal, subterminal, or central, with or without swollen sporangium which may be oval, ellipsoidal, or round and present high thermal and chemical resistances at acidic conditions (De Souza Sant'Ana et al. 2014). Most species are strictly aerobic, however *A. pholiae* is sometimes facultatively anaerobic (Sokołowska et al. 2020). *Alicyclobacillus* can grow in a temperature range of 20–70 °C, with an optimum between 40 – 60 °C, and in a wide pH range (2.0 – 6.0), with an optimum between 3.5 and 4.5 (Sourri et al. 2022).

The unique characteristic that distinguishes *Alicyclobacillus* species from other spore-forming bacteria is the presence of ω -alicyclic fatty acids in their membranes which according to researchers, may be responsible for their heat and acid tolerance (Sourri et al. 2022). It has been also stated that the presence of ω -cyclohexane fatty acids decreased permeability and increased the acyl chain density which causes the lipids in the membrane core to be more tightly packed and leads to the stabilization of the membrane structure and protection of the microorganism against high temperatures and high acid conditions by the formation of a protective coating with strong hydrophobic bonds (Ciuffreda et al. 2015).

Another characteristic that may contribute to the resistance to extreme environments is the presence of hopanoids in the cells of most *Alicyclobacillus* strains. The hopane glycolipids are structurally similar to cholesterol, and with their condensing action, provide a more stable membrane due to a decreased mobility of the acyl chain of the lipids in the cytoplasmic membrane. Furthermore, this action is more advantageous at low pH values since passive diffusion of protons and solutes through the membrane would be diminished (Sourri et al. 2022).

The heat resistance of *Alicyclobacillus* endospores has been associated with several other factors including temperature, pH, and water activity (a_w) (Sourri et al. 2022). Specifically, the temperature of thermal treatment exerts the greatest influence

on the heat resistance of endospores since the D-value decreases with increasing temperature. In addition, pH and Total Soluble Solids (TSS) also affect the heat resistance with a linear decrease in D-value with decreasing pH, and a linear increase in D-value when the content of TSS increases. Water activity also has an impact, since it has been shown that bacterial spores become more resistant as the values of a_w decrease (Sourri et al. 2022). Moreover, endospore resistance to heat can also be influenced by the presence of heat-stable proteins, stronger Ca^{2+} and Mn^{2+} binding capacity, and the mineralization of dipicolinic acid (DPA) with divalent cations (Sokołowska et al. 2020). It needs to be noted that different strains even of the same species of *Alicyclobacillus* may have different D-values (Tianli 2014). Furthermore, the cell number, the cell age, the sporulation temperature, and the state of the endospore protoplast cortex can influence the heat resistance of the endospores (Steyn et al. 2011).

Alicyclobacillus species have been isolated from various environments, such as hot springs and soils, as well as beverages, fruit concentrates, and fruit juices (Połaska et al. 2021). The contamination of fruit juices by *Alicyclobacillus* species is most likely caused by soil, during harvest, as well as by fallen and unwashed or poorly washed fruits. Handling of fruits by employees in manufacturing facilities and the water used in the processing environment could also represent potential sources of contamination (Sourri et al. 2022). The contamination by *Alicyclobacillus* that can lead to spoilage is difficult to detect because no gas production or package swelling is observed in condemned juices, hence making *Alicyclobacillus* a serious problem for the fruit juice industry (Pornpukdeewattana et al. 2019).

The evident sign of spoilage after consumption is an off flavor described as medicinal, phenolic, and antiseptic (Uchida et al. 2017) associated mainly with the production of guaiacol (2-methoxyphenol), but also with the halophenols 2,6-dibromophenol and 2,6-dichlorophenol (Osopale et al. 2016). Guaiacol, which is the major metabolite associated with off-flavors in fruit juices, can be produced during ferulic acid metabolism, from a non-oxidative decarboxylation of vanillic acid, catalyzed by vanillate decarboxylase (Sourri et al. 2022).

So far, 26 species of *Alicyclobacillus* have been identified and nine of these – *A. acidiphilus*, *A. acidoterrestris*, *A. daucy*, *A. herbarius*, *A. cycloheptanicus*,

A. pomorum, *A. contaminans*, and some strains of *A. hesperidum*, and *A. fastidious* were reported to have the ability to produce guaiacol (Połaska et al. 2021).

2.2. *A. acidoterrestris* and its association with spoilage in the fruit industry

Among all the *Alicyclobacillus* species, *A. acidoterrestris* was found to be the most prevalent spoilage microorganism responsible for relevant product and economic losses in the beverage and juice industry, and hence the heat resistance of its spores have been intensively studied (Połaska et al. 2021; Huertas et al. 2021; Ribeiro et al. 2022).

A. acidoterrestris is a spore-forming bacterium that forms distinctive, easily recognizable, translucent, cream-colored colonies, round with flat interiors and raised centers that become darker and more opaque and umbonate with age. The rod-shaped cells are 2.9–4.3 μm long and 0.6–0.8 μm wide, while the oval-shaped spores are 1.5–1.8 μm long, 0.9–1.0 μm wide (Sokołowska et al. 2020). It can survive thermal treatment during pasteurization, grow at low pH, germinate, and spoil the juice, hence, has been proposed as a target microorganism to control the effectiveness of the pasteurization process in acid fruit juices (Sourri et al. 2022). The maximum accepted concentration of *A. acidoterrestris* spores as defined by the fruit juice industry is 10^2 CFU/mL of raw material (Casas et al. 2012).

A. acidoterrestris spores are very heat resistant and this depends on the conditions of thermal treatment and bacterial strain. The D-value (the time necessary—to at a specific temperature—to reduce the overall microbial population by 90%) of *A. acidoterrestris* in fruit juices at 90 °C range from 5.95 to 23.10 min (Smit et al. 2011; Ribeiro et al. 2022). For different strains of *A. acidoterrestris*, the $D_{95^\circ\text{C}}$ values in various fruit juices are usually between 1 and 10 min, and z-values (temperature increase required to result in one-log cycle decrease of D-value) between 6 and 22 °C in different fruit products and 6 and 10 °C in buffers (Tianli et al. 2014), while Huertas et al. (2021) reported a $D_{95^\circ\text{C}}$ value of 5.24 min and a z-value of 11.2 °C in orange juice.

TSS (expressed in °Bx) influences the microbial resistance to pressure and heat as an increase in the TSS increases the resistance of the spores (Uchida et al. 2017).

In malt extract broth, at 600 MPa, 55 °C; D10 °Bx = 4.2 min, D20 °Bx = 7.6 min, D30 °Bx = 13.7 min (Uchida et al. 2017).

According to Cerny et al. (2000), the addition of 100 mg/L of ascorbic acid into apple juice encouraged the growth of *A. acidoterrestris*, whereas 150 mg/L or higher concentration of ascorbic acid inhibited their growth. On the other hand, Bahçeci and Acar (2007), at a fixed pH of 3.5, reported that an increase in the ascorbic acid concentration resulted in a slight decrease in D-values especially at the low temperatures tested, although it was not found to be significant within the concentration studied.

The mineralization of divalent cations (especially Ca²⁺) with DPA to form the Ca-DPA complex also affect the heat resistance of spores. Remineralizing spores with divalent cations, such as manganese or calcium, can increase the heat resistance of the demineralized spores (Sokołowska et al. 2020).

The first association of *A. acidoterrestris* with spoilage in fruit juices occurred in 1984 (Cerny et al. 1984). Since then, this bacterium has been considered a challenge for the industrial processing of fruit juices worldwide (Sourri et al. 2022). *A. acidoterrestris* has been isolated from a variety of juices and concentrates including apple, orange, lemon, mango, grapefruit, pear, tomato, white grape, pineapple, passion fruit, blueberry, pomegranate, cherry, strawberry, chokeberry, raspberry, watermelon, blackcurrant, kiwi and banana, and from various locations which include, Japan, USA, Australia, South Africa, Brazil, Europe, Nigeria and Argentina (Osopale et al. 2016; Sokolowska et al. 2020).

Several studies have found that a critical cell concentration of *A. acidoterrestris* that must be present before spoilage compounds are produced in detectable concentrations. Bahçeci et al. (2005) found that a slightly lower concentration, 10⁴ CFU/mL was necessary for detectable guaiacol production to occur while according to Steyn et al. (2011), it was proven that when the concentration of *A. acidoterrestris* cells ranges between 10⁵ and 10⁶ CFU/mL, it produces enough guaiacol to spoil the juice.

A. acidoterrestris can only produce guaiacol when vanillic acid or vanillin are available as substrates, and the speed of converting vanillic acid into guaiacol is much

faster than vanillin (Witthuhn et al. 2012). These authors reported that *A. acidoterrestris* cannot decompose ferulic acid into vanillic acid (Figure 1) and further reported that 100 mg/L vanillin resulted in a significant increase in the guaiacol concentration from days 0 to 5. The 100 mg/L vanillin was almost used up by day 5 and the highest concentration of guaiacol produced was 61.7 mg/L whereas, for 1000 mg/L vanillin samples, a higher amount of guaiacol of 170.8 mg/L was produced after 7 days. The authors also reported that guaiacol increased faster in the first 24 h of incubation with 100 mg/L vanillic acid than in samples with 1000 mg/L vanillic acid. In this latter case, a delay in guaiacol production was observed during the first 24 h. In addition, a very high concentration of vanillic acid can inhibit the growth of bacteria (Witthuhn et al. 2012).

Vegetative cells instead of spores must be present for spoilage compounds to be produced. Thus, dormant spores must be activated and germinate to form vegetative cells. Spore activation can be brought about through exposure to a heat shock treatment. Various heat shock treatments have been suggested for the activation of *Alicyclobacillus* spores. Walls and Chuyate (2000) investigated several heat shock regimes and also found that heating at 80 °C for 10 min yielded the highest endospore recovery and was more effective than treatment at 60 °C for 10 min or 100 °C for 5 min. Chang and Kang (2004) reported that spore germination will be much accelerated by a heat shock treatment, leading to a higher concentration of vegetative cells and a higher rate of guaiacol production. Also, Terano et al. (2005) found that spores were unable to germinate unless they had been exposed to a heat shock treatment.

Since most species of *Alicyclobacillus* are aerobic, their growth and guaiacol production could, therefore, be influenced by the amount of oxygen that is available in the growth medium. Although a reduced oxygen supply slows the bacterium growth rate, it does not negatively influence the production of taint compounds (Siegmund and Pöllinger-Zierler 2007). These authors also found that, in a limited oxygen supply, *A. acidoterrestris* was still able to produce guaiacol and 2, 6-DBP in detectable amounts and concentrations even exceeded those produced at a free oxygen supply.

The rate of guaiacol production seems to increase with an increase in the incubation temperature. Bahçeci et al. (2005) found that in apple juice inoculated with

10^3 - 10^5 CFU/mL *A. acidoterrestris*, maximum guaiacol concentrations were formed after 75 h in cultures incubated at 46 °C, while little or no guaiacol was detected in cultures incubated at 25 °C. In a juice-inoculation study conducted by Jensen (2000), higher concentrations of guaiacol were produced by *A. acidoterrestris* at 46 °C than at 37 °C.

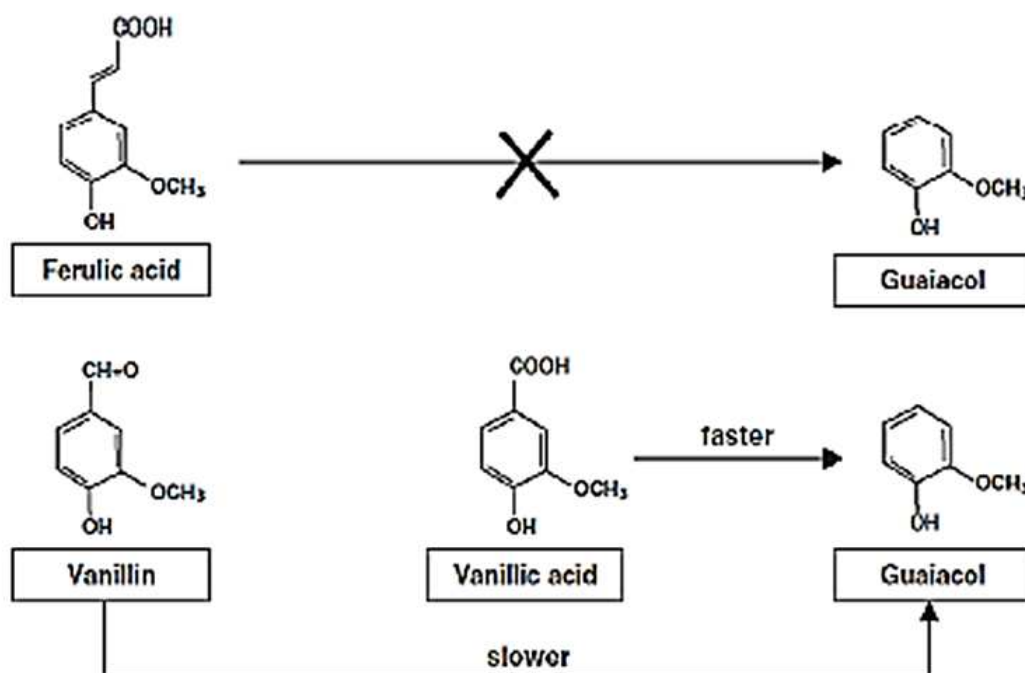


Figure 1: The pathway of guaiacol production by *A. acidoterrestris*. *A. acidoterrestris* is unable to directly metabolic ferulic acid to produce guaiacol but produces guaiacol in substrates containing vanillin or vanillic acid with a faster yield in the presence of vanillic acid (Witthuhn et al. 2012).

The ability of *A. acidoterrestris* spores to survive thermal pasteurization processes and cause spoilage can therefore lead to serious economic impact. As an important control target in the juice industry, research on controlling *A. acidoterrestris* with various methods has been reported (Jia et al. 2021).

2.3. Potential interventions for controlling *A. acidoterrestris*

Conventional thermal treatment accompanied by hot-fill-hold pasteurization processes is ineffective in controlling spoilage initiated by *A. acidoterrestris* spores, instead, the high heat resistance of *Alicyclobacillus* spores allows them to survive

commercial pasteurization, germinate and cause spoilage (Pornpukdeewattana et al. 2019).

A wide spectrum of chemical and physical treatments for the control of *A. acidoterrestris* has been reported. Physical treatments such as the use of high hydrostatic pressure (HHP), ultra-high-pressure homogenization (UHPH), ultraviolet (UV)-C light inactivation, irradiation, microwaves and ultrasonic waves, ohmic heating technique (OHT) have been reported for the control of *Alicyclobacillus* (Pornpukdeewattana et al. 2019; Sourri et al. 2022). Although these treatments have been proven effective, some weaknesses include low penetration into food matrices, lack of sporicidal activity, generation of off-odors, low acceptance by consumers, or negative impact on organoleptic properties (Pornpukdeewattana 2019). Chemical methods such as the use of gaseous ozone (O₃), chlorine dioxide (ClO₂), organic acids, potassium sorbate, sodium benzoate, and poly dimethyl ammonium chloride (PDAC) have also been studied (Osopale et al. 2017; Pornpukdeewattana et al. 2019).

In recent years, there has been an increasing demand from customers for natural additives in food products (Nieva et al. 2022). Natural antimicrobials of microbial, animal, and plant origin such as essential oil, plant extracts, and antimicrobial peptides like nisin have been used or studied for food preservation (Barboza et al. 2022), and the control of the *Alicyclobacillus* (Sourri et al. 2022). Natural antimicrobials are easy to incorporate into production lines and their use does not impinge large costs for implementation (Nieva et al. 2022). In addition, the combination of antimicrobials with other preservatives or control methods by a hurdle technology approach has proved to be a promising strategy to improve food safety and preservation, as it enhances antimicrobial activity, lowers effective doses, save energy, reduce overall costs and expand the shelf life of food products (Liu et al. 2022).

2.4. Antimicrobial peptides

There is increasing interest in the use of antimicrobial peptides to preserve food products due to consumers' demand for fresh-tasting and better-preserved chemical-free food products and research on antimicrobial peptides is continuously developing (Barbosa et al. 2017; Huan et al. 2020). The major benefit of using antimicrobial

peptides is that it preserves the food without changing its quality and it is not harmful (Kiny et al. 2022). Antimicrobial peptides (AMPs) are a class of small peptides that commonly exist in nature, and they are a significant part of the innate immune system of different organisms (Huan et al. 2020). These low molecular weight proteins have a broad spectrum of antimicrobial and immune-modulatory activities against Gram-positive and Gram-negative bacteria, viruses, and fungi (Boparai et al. 2019). AMPs are a diverse group of proteins that are classified based on their source, activity, structural characteristics, and amino acid-rich composition (Huan et al. 2020). AMPs are present in animals, plants, insects, bacteria, and fungi, among other organisms (Rai et al. 2016).

AMPs are grouped into four structural categories which include linear α -helical peptides, β -sheet peptides, linear extension structure, and both α -helix and β -sheet peptides (Lei et al. 2019). Also, progressively cyclic peptides and AMPs with more complex topologies have been reported (Koebach and Craik 2019). AMPs have also been grouped by the net charge, among which the cationic α -helical and anionic peptides have been described (Boparai et al. 2019). Furthermore, AMPs have been classified into ribosomally and non-ribosomally synthesized peptides (Moravej et al. 2018).

Based on their mechanism of action, AMPs can be grouped into membrane-targeting and non-membrane-targeting peptides (Huan et al. 2020). AMPs that target the cell membrane of susceptible organisms are mainly cationic molecules that cause membrane disruptions, whereas non-membrane targeting peptides are capable of translocation across the membrane without damaging it (Boparai et al. 2019).

2.4.1. ϵ -Polylysine

ϵ -Polylysine is a cationic antimicrobial peptide that consists of 25–35 residues of L-lysine linked by amide bonds formed by ϵ -amino and α -carboxyl groups (Dodd et al. 2018). The ϵ -polylysine linkage is directly generated by ϵ -polylysine synthetase (PIs), a membrane protein with six transmembrane domains (TM1 to TM6) as well as both an adenylation domain and a thiolation domain, characteristic of the nonribosomal peptide synthetases (Wang et al. 2021). ϵ -polylysine was discovered as an

extracellular material produced by filamentous actinomycetes such as *Streptomyces albulus* ssp. *lysinopolymerus* (Tan et al. 2019).

ϵ -polylysine has generally been recognized as a safe food preservative (GRAS No. 000135) by the Food and Drug Administration (FDA) (Lan et al. 2019) and is reported to display high water solubility and thermal stability (Chen et al. 2018). It is biodegradable, edible, non-toxic, and innocuous. Due to its safety properties and efficacy, ϵ -polylysine has been widely used as a preservative in foods (Tan et al. 2019; Wang et al. 2021).

In fruit-flavored drinks, ϵ -polylysine has been used up to 250 ppm (Food and Drug Administration 2004 and 2010). It can inhibit the growth of a wide range of microorganisms, including Gram-negative and Gram-positive bacteria, yeasts, and molds (Tan et al. 2019). ϵ -polylysine has a positive charge in solution, thus because of electrostatic adsorption and osmotic pressure between cell membranes, cationic ϵ -polylysine enters the cell membrane and disrupts the balance of neutral charge in the cytoplasm causing damage to the cell (Chang et al. 2010).

In previous work, Cai et al. (2015) reported that ϵ -polylysine was effective in controlling growth and guaiacol formation by vegetative cells of *A. acidoterrestris* that were cultivated in AAM broth. In apple juice, 100 mg/L ϵ -polylysine, reduced *A. acidoterrestris* counts to 4.78 log colony forming unit (CFU/mL), and no guaiacol was detected after 14 days of storage at 45 °C. Hu et al. (2020) also reported that ϵ -polylysine had antibacterial effects on vegetative cells and endospores of *A. acidoterrestris* ATCC 49025 and *A. acidoterrestris* OSYE in apple juice during incubation at 37 °C and also achieved a MIC of 74 μ g/mL against both *Alicyclobacillus* strains in Sucrose-supplemented Yeast Starch Glucose (SSYSG) (pH 3.7) at 37 °C.

Lan et al. (2019) reported that ϵ -polylysine acts on the cell membrane, thereby increasing membrane permeability and inhibiting enzyme involved in respiratory and cell metabolism which prevents cell growth, and eventually causes cell death (Figure 2). Tan et al. (2019) investigated the mechanism of action of ϵ -polylysine and reported that ϵ -polylysine induced structural changes of peptidoglycan, which resulted in a more fragile cell wall and a cell membrane with greater permeability. Additionally, different concentrations of ϵ -polylysine caused various effects on metabolic pathways, with high

concentrations directly restraining the central carbon metabolism and low concentration inhibiting primarily the glycolytic pathway (Tan et al. 2019).

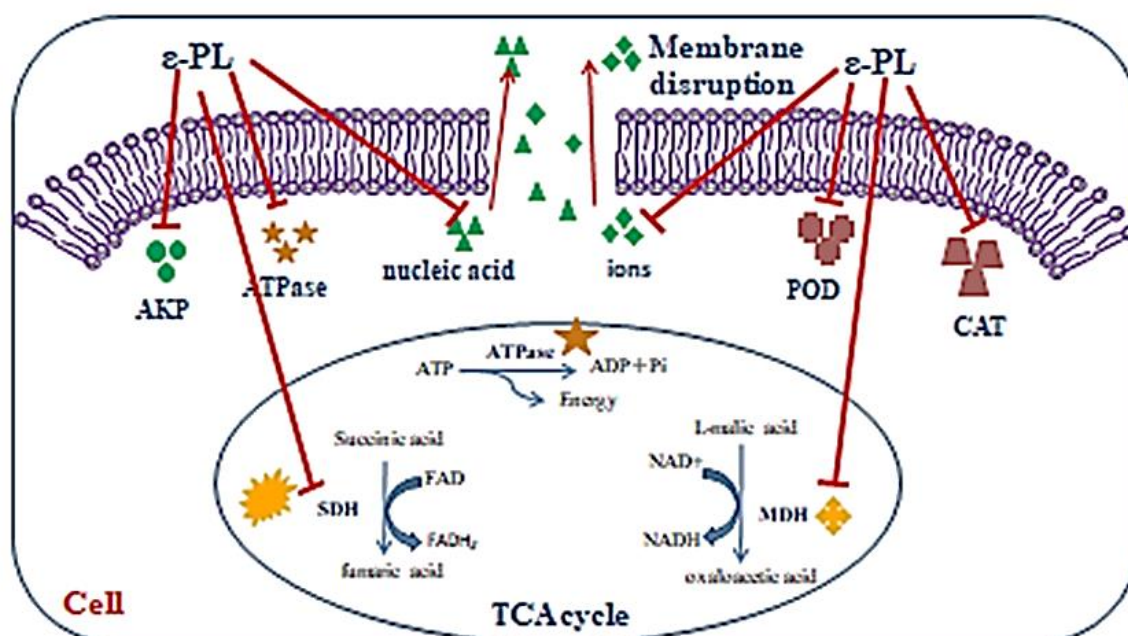


Figure 2: Schematic representation of the possible antimicrobial mechanism of ε-polylysine. ε-polylysine acts by disrupting cell membrane, increasing its permeability, and therefore inhibiting enzyme activity in relation to respiratory and cell metabolism which leads to inhibition of cell growth, and may eventually lead to cell death (Lan et al. 2019).

2.4.2. Bacteriocins

Bacteriocins are ribosomally synthesized and extracellularly release antimicrobial peptides produced by bacteria that vary in structure, biochemical properties, mode of action, and spectrum of activity (Barbosa et al. 2017). The bacteriocins produced by lactic acid bacteria (LAB) have molecules that are cationic, hydrophobic and often have a length of 20 to 60 amino acids (Kumariya et al. 2019) and are more thoroughly characterized on account of their “food grade quality” and industrial importance. According to Liu et al. (2022), bacteriocins are categorized on the basis of their chemical compositions, types of producing organisms, physical characteristics, modes of action, stability, and molecular size. Class I are heat-stable, lanthionine-containing bacteriocins, typically range in size from 19 - 50 amino acids and are made up of amino acids that have undergone extensive post-translational

modification, Class II are non-lanthionine-containing bacteriocins that group small, heat-stable, unmodified peptides that can be further divided into four subclasses; (Class IIa), two-peptide unmodified bacteriocins (Class IIb), circular bacteriocins (Class IIc), and unmodified, linear, non-pediocin-like bacteriocins (Class IId) (Wang et al. 2018; Liu et al. 2022). Heat-labile proteins with large molecular weights (>30 kDa) are included in class III bacteriocins while Class IV bacteriocins are large complex proteins incorporating either lipid or carbohydrate moieties (Vogel et al. 2021).

The use of bacteriocins as food additives is widespread, however, little is known about their use in fruit products, especially in minimally processed fruits (Barbosa et al. 2017). Nisin and pediocin PA-1/AcH are the only bacteriocins that have been approved to be used as food additives, but their use in fruit products is still limited to certain countries. Hence, the continuous study of bacteriocins with biochemical characteristics that make them candidates to preserve fruit products are of great interest (Barbosa et al. 2017).

2.4.2.1. Bovicin HC5

Bovicin HC5, a bacteriocin synthesized by *Streptococcus equinus* HC5, a facultative anaerobic bacterium isolated from the bovine rumen, is a lantibiotic that has a molecular mass of 2449 Da and shows post-translational modified amino acids, with a pattern of lanthionine rings similar to nisin and streptin (Mantovani et al. 2002). Its extraction can be performed using acidic solutions of NaCl (100 mmol, pH 2.0) (Moreira et al. 2020). The crude extract is resistant to α -chymotrypsin, proteinase K, and heat, which are advantageous properties for industrial applications (Mantovani et al. 2002).

Bovicin HC5 has been reported to be able to inhibit several Gram-positive bacteria, including several strains of food-borne pathogenic and spoilage microorganisms and has shown effectiveness in preserving fruit juices and pulps, hence, is suggested as an additive for food preservation (Barbosa et al. 2017; Garsa et al. 2019, Ribeiro et al. 2022). When the antimicrobial activity of bovicin HC5 was tested in mango pulp against *A. acidoterrestris* DSMZ 2498, the bacteriocin showed a bactericidal effect against vegetative cells and endospores of this microbe (de Carvalho et al. 2008). At concentrations of 40-160 AU/mL, it was able to completely inhibit growth in AAM broth (pH 4.0) at 40 °C for as long as 15 d. The MIC of bovicin

HC5 was determined to be 5.0 AU/mL and 2.50 AU/mL for vegetative cells and spores, respectively. When mango pulp with a pH ranging from 4.0-7.0 was inoculated with *A. acidoterrestris* cells or spores at a level of 10^5 - 10^6 CFU/mL and treated with 80-100 AU/mL bovicin HC5, a bactericidal and sporicidal effect was observed. When bovicin HC5 was added to mango pulp (pH 4.0) at a concentration of 80 AU/mL, the D80-95 values of *A. acidoterrestris* spores decreased to 77-95%, and the z-value to 48.7% (de Carvalho et al. 2008).

The ability of bovicin HC5 and nisin to inhibit the growth of vegetative cells and reduce the thermal resistance of endospores of *A. acidoterrestris* inoculated (10^7 CFU/mL) in different fruit juices was also evaluated (Ribeiro et al. 2022). When vegetative cells of *A. acidoterrestris* ($\sim 10^6$ CFU/mL) were inoculated into fruit juices with pH values ranging from 2.78 to 3.55 with the addition of increasing concentrations of bovicin HC5, 60-80 AU/mL reduced the viable cells below the detection limit in most fruit juices. Also, even in the lower concentration of bovicin HC5 evaluated (10 AU/mL), a statistically significant reduction in *A. acidoterrestris* viability was detected in all fruit juices when compared to the control ($P < 0.05$). The addition of 80 AU/mL bovicin HC5 or nisin to the juices reduced the $D_{90^\circ\text{C}}$ value by up to 1.5 log and no statistical difference was observed. Ribeiro et al. (2022) also reported substantial structural changes in the cellular framework of vegetative cells upon treatment with bovicin HC5 or nisin using atomic force microscopy.

The antibacterial mechanism of action of bovicin HC5 is similar to that of nisin which involves specific binding to Lipid II on the target cells but the mode of action also depends on the membrane thickness of the target cell (Moreira et al. 2020). In thin membranes, bovicin HC5 is able to assemble with Lipid II leading to the formation of pores on cell membrane and also impair the cell wall biosynthesis by the segregation of Lipid II into domains (Figure 3), consequently, target cells lose intracellular potassium and intracellular ATP is depleted, via the activity of F1F0-ATPase (Moreira et al. 2020).

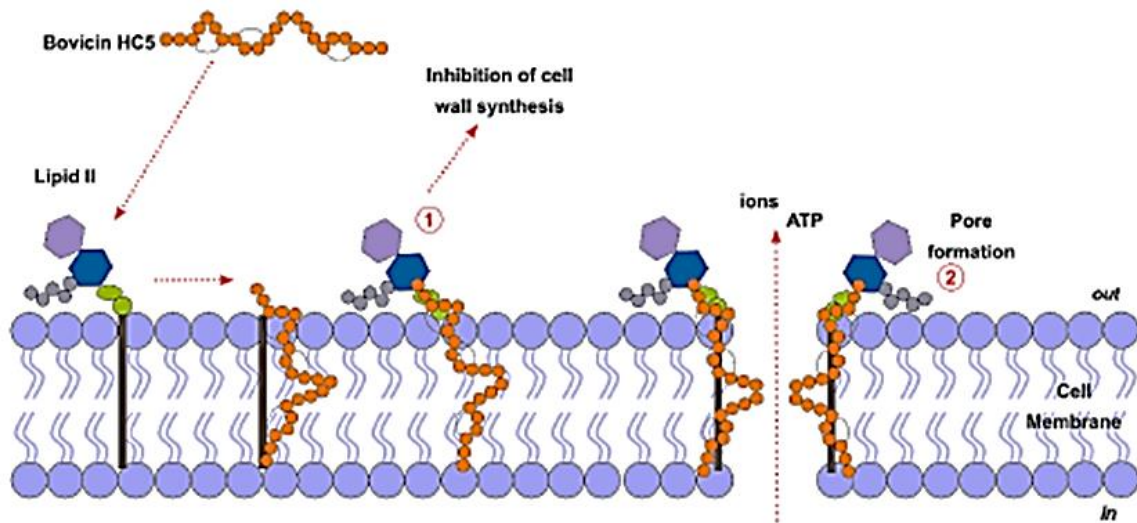


Figure 3: Schematic representation of the mechanism of action of bovicin HC5. Bovicin HC5 is capable of binding to Lipid II domains, which inhibits cell wall synthesis (1). The binding of bovicin HC5 to Lipid II forms pores in the cell membrane causing de leakage of intracellular components such as ions and ATP, which leads to cell death (2). (Moreira et al. 2020).

Since the individual antibacterial activity of bovicin HC5 and ϵ -polylysine against *A. acidoterrestris* has been demonstrated in previous works, we hypothesized that their use in combination could improve the killing effect against *A. acidoterrestris* and the efficacy of thermal processing of fruit juices. Thus, this work evaluated the combined effect of bovicin HC5 and ϵ -polylysine against vegetative cells and germinated endospores of *A. acidoterrestris* in orange juice focusing on improving the prevention of fruit juice spoilage.

3. MATERIALS AND METHODS

3.1. Media preparation, bacteria strains, and culture conditions

Streptococcus equinus HC5 was cultivated anaerobically at 39 °C in a semi-synthetic basal medium (PC) prepared under the flux of oxygen (O₂)-free carbon dioxide flow containing: 0.292 g/L KH₂PO₄, 0.480 g/L (NH₄)₂SO₄, 0.480 g/L NaCl, 0.100 g/L MgSO₄·7H₂O, 0.640 g/L, CaCl₂·2H₂O, 0.500 g/L cysteine hydrochloride, 1 g/L Trypticase (BBL Microbiology Systems, Cockeysville, MD, USA), 0.500 g/L yeast extract, 4g of Na₂CO₃, and 8 g/L glucose. The medium pH was adjusted to 6.5 with sodium hydroxide (NaOH, 3 mol/L) before sterilization (121 °C, 15 min). Bacterial growth (OD_{600nm}) was monitored using a Spectronic 200E spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA).

A. acidocaldarius medium (AAM) was used to culture and enumerate *A. acidoterrestris* DSMZ 2498 under aerobic conditions at 45 °C. The medium was composed of solution A (0.25 g CaCl₂·7H₂O, 0.5 g MgSO₄·7H₂O, 0.2 g (NH₄)₂SO₄, 3.0 g KH₂PO₄); yeast extract (2.0 g); glucose (5.0 g). The volume of the solution was adjusted to 1000 mL (for liquid medium) and 500 mL (for solid medium) with distilled water and the pH was adjusted to 4.0 with 1 mol/L hydrochloric acid (HCL). Solution B contained 0.1 g ZnSO₄·7H₂O; 0.03 g MnCl₂·4H₂O; 0.3 g H₃BO₃; 0.2 g CoCl₂·6H₂O; 0.01 g CuCl₂·2H₂O; 0.02 g NiCl₂·6H₂O, and 0.03 g NaMoO₄·2H₂O, with volume adjusted to 1000 mL distilled water. Solution C was composed of 15 g agar prepared with 500 mL distilled water. All solutions were sterilized separately (121°C/ 15 min). Solution B was sterilized through a 0.22 µm membrane (Millipore 67120; Molsheim, France).

After sterilization, 1 mL of the trace-element solution SL-6 (solution B) was added to solution A (with 1000 mL distilled water) for liquid medium, and also added to solution A (with 500 mL distilled water) combined with solution C for solid medium.

3.2. Preparation of *A. acidoterrestris* DSMZ 2498 spore suspensions

Endospores of *A. acidoterrestris* DSMZ 2498 were obtained in AAM broth as described by Silva et al. (2015) with modifications (Ribeiro et al. 2022). The culture

was incubated at 45 °C for 120 h, under orbital agitation (120 rpm), and subsequently refrigerated at 4 °C for 48 h. Slides were prepared for microscopic observation of spores after malachite green dye staining. The culture was then centrifuged (9000 × g, 10 min, 5 °C) (4 K15, D-37520; Sigma 4K15, SIGMA Laborzentrifugen GmbH, Osterode, Germany). The supernatant was discarded, and the pellet was suspended in 20 mL of phosphate buffer (pH 4.0, 50 mmol/L). The suspension was then incubated in a thermostatically controlled water bath (Thermomix. BM-S, B. Braun Biotech International, Melsungen, Germany) at 80 °C for 10 min to activate the endospores. After heat treatment, the endospore suspension was centrifuged three times (9000 × g, 10 min, 5 °C), and the supernatant was discarded. The pellet was then re-suspended in 10 mL of phosphate buffer (pH 4.0, 50 mmol/L) and stored at 4 °C until use. The endospores were enumerated by counting the colonies resulting from viable spores that germinated in AAM media.

3.3. Preparation of antimicrobial peptides (AMPs)

A commercial powdered ϵ -polylysine from Zhengzhou Binafo Bioengineering Co., Ltd (Zhengzhou, China) was used in this study. The stock solution was prepared in sterile distilled water, then filter sterilized (pore size, 0.22 μ m), and prepared fresh before use. A series of 2-fold dilutions was also prepared using sterilized distilled water (Cai et al. 2015).

Extracts of bovicin HC5 were prepared as described by Mantovani et al. (2002). The pH of the culture was adjusted to 7 followed by a heat treatment at 75 °C for 30 minutes. Stationary-phase *S. equinus* HC5 was harvested by centrifugation (10,000 × g) for 20 min and the supernatant was discarded. The weight of the wet cell mass was recorded. The cell pellet was suspended in 0.1 M acidic NaCl solution (50 mL, pH 2.0) and left overnight at 4 °C with continuous stirring. The suspensions were then centrifuged (10,000 × g) to remove cells and recover the crude extract for purification.

Purification of bovicin HC5 was performed using a Sep-Pak C18 Plus Short Cartridge column as the stationary phase; a 0.1 % formic acid solution was used as the mobile phase and elution was carried out with 50 % acetonitrile. Then the purified

cell-free supernatant was lyophilized (Edwards; Super Modulyo, Livermore, CA, USA). Serial dilutions were made using sterilized distilled water.

The titer of ϵ -polylysine and bovicin HC5 were expressed in terms of microgram per milliliter ($\mu\text{g}/\text{mL}$).

3.3.1. Antimicrobial activities of the AMPs

The antimicrobial activity was analyzed by agar well diffusion assay using *A. acidoterrestris* DSMZ 2498 as the target bacteria as described by Ribeiro et al. (2022). Aliquots of 25 μL of each dilution of the AMPs were applied into wells that were perforated aseptically using sterile plastic straws with a diameter of 5 mm in AAM medium previously inoculated with *A. acidoterrestris* DSMZ 2498 (10^6 CFU/mL). The agar plates were then incubated at 4 °C overnight to allow the peptides to diffuse and then the plates were incubated at 45 °C for 24 h to allow the growth of the indicator organism. Antimicrobial activity was detected by measuring the zone of inhibition (including the diameter of the wells) that appeared after the incubation period.

3.4. Minimum inhibitory and bactericidal concentrations (MIC and MBC) of the AMPs

The antimicrobial efficacies of ϵ -polylysine and bovicin HC5 were tested by measuring their minimum inhibitory concentrations (MICs) against *A. acidoterrestris* DSMZ 2498 in AAM broth. The MICs were determined in 96-well microdilution plates, according to M7-A6 204 (CLSI, 2012). A series of two-fold dilutions of bovicin HC5 was prepared using sterilized distilled water, then 20 μL of these dilutions were dispensed in wells of a 96-well plate containing a mixture of 180 μL of AAM broth (pH 4) and approximately 10^6 CFU/mL of vegetative cells of *A. acidoterrestris* DSMZ 2498. Similarly, ϵ -polylysine was diluted and tested as described for bovicin HC5 treatment. Concentrations of bovicin HC5 and ϵ -polylysine suitable for testing their MIC were determined based on preliminary results obtained from its antimicrobial activity. Final concentrations of bovicin HC5 used were 0.31, 0.63, 1.25, 2.50, 5.00 and 10.00 $\mu\text{g}/\text{mL}$. For ϵ -polylysine, the following concentrations were used 3.91, 7.81, 15.63, 31.25, 62.50, and 125.00 $\mu\text{g}/\text{mL}$. The 96-well plates were incubated at 45 °C for 24 h and

bacterial growth was measured at 600 nm in a Spectronic 20D+ (Thermal Electron, Madison, WI, USA). The specific growth rate, lag phase, and OD values after 24 h incubation was determined (de Carvalho et al. 2008).

The same protocol was repeated to determine the minimum inhibitory concentrations (MICs) against *A. acidoterrestris* DSMZ 2498 spore cells. Prior to incubation at 45 °C for 24 h, the 96 well plates were heat shocked at 80 °C for 10 min to activate dormant endospores

After this period, the Minimum Bactericidal Concentration (MBC) was determined by sub-culturing 20 µL from each well with no growth on AAM agar and incubating at 45 °C for a further 24 h. The absence of growth was the parameter used to define if a given concentration was bactericidal against *A. acidoterrestris*. The tests were performed in technical triplicate (da Silva et al. 2020) and two biological replicates.

3.5. Combined effect of the AMPs against *A. acidoterrestris* DSMZ 2498 in AAM broth

The checkerboard method was used to evaluate the antibacterial effect of the combination of bovicin HC5 and ε-polylysine using 96-well plates to calculate the Fractional Inhibitory Concentration (FIC) index (Schelz et al. 2006). The test was performed using different combined concentrations, based on the MIC results of each antimicrobial peptide (da Silva et al. 2020).

The concentrations of each AMP used were 4X, 2X, 1X, 1/2X, 1/4X, and 1/8X of their MIC. Aliquots of 20 µL of each AMP were added to 160 µL of AAM broth containing approximately 10⁶ CFU/mL of *A. acidoterrestris* (vegetative cells) in the wells of a 96-well plate and the microplate was incubated at 45 °C for 24 h. Similarly, bacterial growth was monitored via changes in optical density (OD) at 600 nm in a Spectronic 20D+ (Thermal Electron, Madison, WI, USA) (de Carvalho et al. 2008).

The same protocol was repeated using endospores of *A. acidoterrestris* DSMZ as inoculum. Prior to incubation at 45 °C for 24 h, the 96-well plates were heat shocked at 80 °C for 10 min to activate dormant endospores.

The FIC indexes were calculated as $FIC_A + FIC_B$, where $FIC_A = MIC_A$ combined/ MIC_A alone and $FIC_B = MIC_B$ combined/ MIC_B alone. Results were interpreted as synergism ($FIC \leq 0.5$), addition ($0.5 < FIC \leq 1$), indifference ($1 < FIC \leq 4$) or antagonism ($FIC > 4$) (Schelz et al. 2006). The assay was performed in technical and biological duplicates.

3.6. Effect of the AMPs alone and in combination in orange juice (Time-kill assay)

Commercial shelf-stable orange juice was obtained from a local grocery store in Vicosa, MG, Brazil. The juice was analyzed for the absence of vegetative cells or spores according to Anjos et al. (2016). The soluble solid content and pH of the juice were determined. Aliquots of the orange juice were transferred into glass tubes, autoclaved, and cooled to room temperature. The concentration of AMPs used was determined by preliminary testing. The assay was performed by adding 0.5 mL of the serially diluted AMPs into 4.5 mL or 4 mL of orange juice containing approximately 10^6 CFU/mL of *A. acidoterrestris* (vegetative cells) to give the following final concentrations of bovicin HC5: 5, 10, 20, 40, 80 $\mu\text{g/mL}$. For ϵ -polylysine, the concentrations tested were 7.81, 15.63, 31.25, 62.5, 125.0, and 250.0 $\mu\text{g/mL}$. When the AMPs were tested in combination, the concentration used for bovicin HC5: ϵ -polylysine were 5.0:7.81, 10.0:15.63, 20.0:31.25, 40.0:62.5, 80.0:125.0 $\mu\text{g/mL}$. Tubes with orange juice and AMPs were incubated at 45 °C in a water bath for 0, 12, 24, 36, and 48 h. Then 100 μL was withdrawn from each tube at each time point, serially diluted, and spread on AAM agar for viable cell enumeration.

The same procedure was repeated with the endospores as inoculum. The assay was performed by adding 0.5 mL of the AMPs into 4.5 mL or 4.0 mL AAM broth containing approximately 10^6 CFU/mL of *A. acidoterrestris* to give final concentrations of 80, 160 $\mu\text{g/mL}$ bovicin HC5, 125, 250 $\mu\text{g/mL}$ of ϵ -polylysine and a combination of both AMPs 80:125, 160:250 $\mu\text{g/mL}$. Next, the tubes were exposed to thermal shock at 80 °C for 10 min at 0 h and incubated at 45 °C in a water bath for 0, 12, 24, 36, and 48 h of incubation. The thermal shock at 80 °C for 10 min was repeated after 24, and 48 h of incubation. Aliquots of 100 μL were withdrawn at each time point, serially diluted, and spread on AAM agar for enumeration of viable spores. The number of germinated spores was determined by the difference between the initial and the final spore count

at each time point (de Carvalho et al. 2008). All tests were performed in technical triplicate and biological duplicates.

In every batch, there were four side-by-side treatments: bovicin HC5, ϵ -polylysine, ϵ -polylysine + bovicin HC5, and control (i.e., no antimicrobial peptide added).

3.7. Determination of D-values for *A. acidoterrestris* DSMZ 2498 endospores in orange juice

The D-value of *A. acidoterrestris* endospores was determined at 95 °C, which is representative of the temperature used during the thermal processing of different fruit juices. Approximately 10^6 endospores/mL were incubated in glass tubes containing 5 mL of orange juice. The tubes were kept in a water bath at 95 °C and the thermal lag time (approximately 10 min) for the center of the tube to reach the desired temperature was considered. Then bovicin HC5 and ϵ -polylysine at concentrations 80 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$ respectively, and also in combination (80:125 $\mu\text{g/mL}$) were added. For the control (absence of antimicrobial peptides), dilutions (10^{-1} to 10^{-6}) of each time sampled were plated, while for the treatments containing the antimicrobial peptides, dilutions (10^{-1} to 10^{-6}) at times 0, 2, 4, 6, 8, 10, 12 min were plated. The plating was performed in triplicate using the microdroplet technique, and the plates were incubated at 45 °C for 24 - 48 h. The analysis was carried out in biological duplicates.

Survival curves were plotted using the number of germinated endospores surviving in the presence or absence of bovicin HC5 and/or ϵ -polylysine. The data were fitted using linear regression, according to the equation $\log N = \log N_0 - bt$, where N is the number of survivors, N_0 is the initial number of germinated endospores, t is the exposure time to the treatment temperature, and b is the slope of the survivor curve to reduce one logarithmic cycle. The D-value was calculated by plotting the log of the number of survivors versus time, where $D = 1/b$, is the inverse of the slope of the survival curve.

3.8. Effects of AMPs on vegetative cells of *A. acidoterrestris* DSMZ 2498 as revealed by atomic force microscopy

The effects of combined AMPs on the morphology and cell structure of *A. acidoterrestris* DSMZ 2498 were determined using atomic force microscopy (AFM) (Ntegra Prima, NT-MDT Spectrum Instruments; Moscow, Russia). For sample preparation, the cultures of *A. acidoterrestris* DSMZ 2498 were activated in 50 mL of AAM broth, centrifuged ($1742 \times g$ for 10 min), and washed with phosphate buffer (pH 4.0, 50 mmol/L) three consecutive times. Subsequently, the pellet was suspended in 20 mL of phosphate buffer (pH 4.0, 50 mmol/L). Then 4.0 or 4.5 mL of the suspension were dispensed in sterilized bottles containing 0.5 mL of each AMP to give final concentrations of 1X and 2X MIC individual AMPs and combined AMPs. Suspension without both AMPs was used as control and incubated for up to 24 h at 45 °C in an electrostatic water bath. Aliquots of 1 mL each were withdrawn after 0, 8, and 24 h of incubation. The samples were centrifuged, the supernatant was discarded, and the cells were spread on glass slides (1 cm \times 1 cm) using pipette tips and a drop of sterilized distilled water. These procedures were performed aseptically.

The NT-MDT Nova (version 1.26.0.1443) software was used for image acquisition and topography measurements of the *A. acidoterrestris* DSMZ 2498 samples were performed using the intermittent mode to minimize the risk of deformation of the sample and to allow a greater lateral resolution as described by Ribeiro et al. (2022).

3.9. Statistical analysis

All incubations were carried out at least with two technical replicates and the results represent the mean \pm standard deviation of two independent experiments. The means were evaluated by one-way ANOVA and Tukey's post hoc analysis (Graphpad Prism Software, San Diego, California, USA) was used to determine the statistical difference between treatments. A P-value of 0.05 was used to declare significant differences (Hu et al. 2020).

4. RESULTS

4.1. Effect of AMPs on the growth of *A. acidoterrestris* DSMZ 2498

A. acidoterrestris DSMZ 2498 growth in AAM (pH 4.0) had an average maximum specific growth rate of approximately 0.26 h^{-1} when the initial inoculum was 10^6 CFU/mL of either vegetative cells or endospores. Cultures inoculated with vegetative cells had a lag phase of 1 h while a 4 h lag period was observed when endospores were used as inoculum. With the addition of different concentrations of bovicin HC5 (0.31, 0.63, 1.25, 2.50, 5.0, 10.0 $\mu\text{g/mL}$), the maximum specific growth rate of *A. acidoterrestris* DSMZ 2498 was reduced ($P < 0.05$) by approximately 54-77 % with concentrations of bovicin HC5 $\leq 1.25 \mu\text{g/mL}$ compared to the control without the addition of the AMP. However, no difference ($P > 0.05$) was observed between samples with concentrations of bovicin HC5 0.63 $\mu\text{g/mL}$ and 1.25 $\mu\text{g/mL}$. Moreover, the lag phase of *A. acidoterrestris* DSMZ 2498 in the presence of the different concentrations of bovicin HC5 was similar to the control when vegetative cells were used as inoculum. No growth was observed when higher concentrations $\geq 2.50 \mu\text{g/mL}$ of bovicin HC5 were tested (Figure 4A, Table 1). The optical densities ($\text{OD}_{600 \text{ nm}}$) of the culture (vegetative cells) in the presence of bovicin HC5 differ significantly when compared to the control ($P < 0.05$) after 24 h incubation (Figure 4B).

Using endospores as inoculum, there was no significant difference ($P > 0.05$) in the maximum specific growth rate of *A. acidoterrestris* DSMZ 2498 in media containing concentrations of bovicin HC5 ranging from 0.31– 2.50 $\mu\text{g/mL}$. The lag phase duration was similar across the concentrations tested when compared to the control except for concentration 2.50 $\mu\text{g/mL}$, which showed a long lag phase of 14 h. No growth was observed at bovicin HC5 concentrations $\geq 5.0 \mu\text{g/mL}$ (Figure 5A, Table 1). The $\text{OD}_{600 \text{ nm}}$ of the culture previously inoculated with endospores, with concentrations $\leq 1.25 \mu\text{g/mL}$ of bovicin HC5 did not differ ($P > 0.05$) while cultures with $\geq 2.50 \mu\text{g/mL}$ of bovicin HC5 were significantly different ($P < 0.05$) from control after 24 h of incubation (Figure 5B).

Table 1: Effect of different concentrations of bovicin HC5 against vegetative cells and germinated endospores of *A. acidoterrestis* DSMZ 2498 grown in AAM broth at 45 °C for 24 h.

	Concentration (µg/mL)	Vegetative cells		Germinated endospores	
		Specific growth rate- μ (h ⁻¹)	Lag phase- λ (h)	Specific growth rate- μ (h ⁻¹)	Lag phase- λ (h)
1	0.00	0.26±0.01 ^c	1	0.26±0.00 ^a	4
2	0.31	0.12±0.00 ^b	1	0.23±0.04 ^a	4
3	0.63	0.08±0.02 ^a	1	0.23±0.00 ^a	4
4	1.25	0.06±0.01 ^a	1	0.23±0.05 ^a	4
5	2.50	-	-	0.19±0.03 ^a	14
6	5.00	-	-	-	-
7	10.00	-	-	-	-

Values are mean ± SEM, - = no growth, inoculum size = 10⁶ CFU/mL

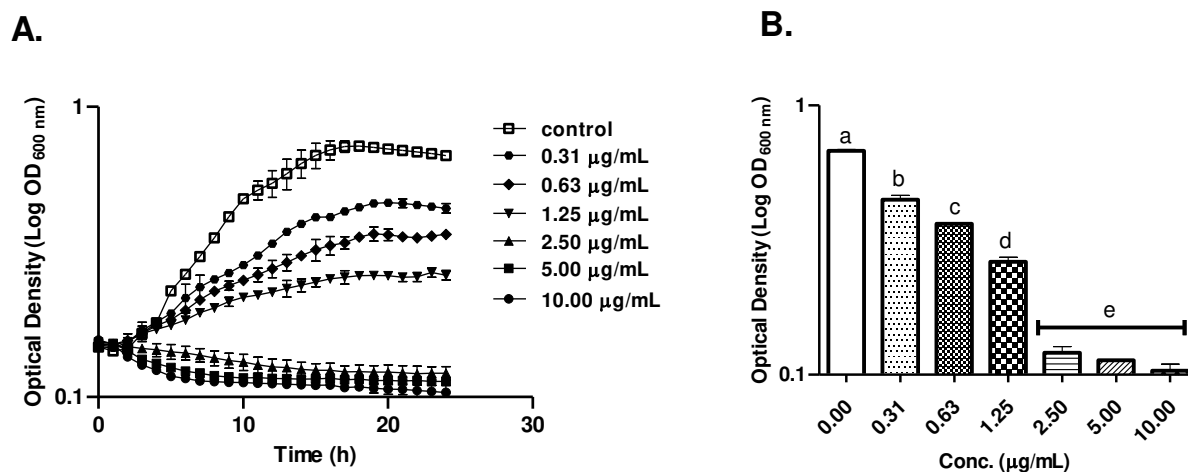


Figure 4: Effect of bovicin HC5 on vegetative cells of *A. acidoterrestis* DSMZ 2498 grown in AAM broth. (A) Growth kinetics of the vegetative cells of *A. acidoterrestis* DSMZ 2498 with different concentrations of bovicin HC5 during 24 h incubation and their (B) optical densities after 24 h incubation. Data were represented as mean ± SD. Bars bearing different letters are significantly different at $P < 0.05$, while bars bearing the same letter(s) are not significantly different ($P > 0.05$). Data were analyzed using one-way ANOVA followed by the Tukey post hoc test.

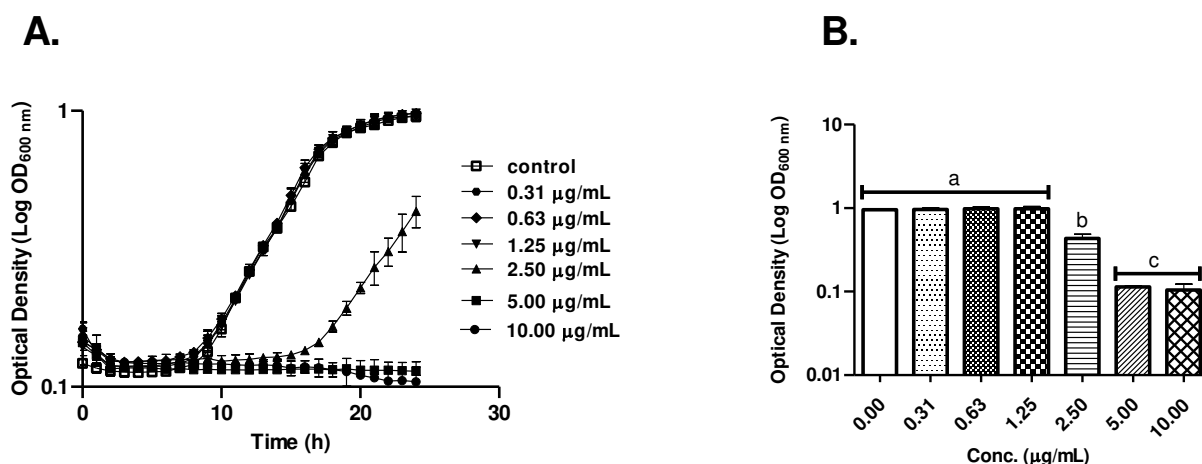


Figure 5: Effect of bovicin HC5 on activated endospores of *A. acidoterrestris* DSMZ 2498 grown in AAM broth. (A) Growth kinetics of germinated endospores of *A. acidoterrestris* DSMZ 2498 with different concentrations of bovicin HC5 during a 24 h incubation and their (B) optical densities after 24 h incubation. Data were represented as mean \pm SD. Bars bearing different letters are significantly different at $P < 0.05$, while bars bearing the same letter(s) are not significantly different ($P > 0.05$). Data were analyzed using one-way ANOVA followed by the Tukey post hoc test.

With the addition of ϵ -polylysine to the vegetative cell culture ($\sim 10^6$ CFU/mL) of *A. acidoterrestris* DSMZ 2498, the maximum specific growth rate and lag phase of the culture with 3.91 $\mu\text{g/mL}$ ϵ -polylysine were not affected ($P > 0.05$) compared to control. However, when higher concentrations ≥ 7.81 $\mu\text{g/mL}$ of ϵ -polylysine were added, no growth was observed ($P < 0.05$) (Figure 6A, Table 2). The OD_{600 nm} of the vegetative cell culture in the presence of ϵ -polylysine differed significantly from the control ($P < 0.05$) (Figure 6B).

When endospores were used as inocula, the maximum specific growth rate of *A. acidoterrestris* DSMZ 2498 was reduced ($P < 0.05$) by approximately 50 % with 3.91 $\mu\text{g/mL}$ concentration of ϵ -polylysine compared to control and a lag phase of 18 h was observed. No growth was observed when concentrations ≥ 7.81 $\mu\text{g/mL}$ of ϵ -polylysine were added (Figure 7A, Table 2). The OD_{600 nm} measurements of the growth of the culture previously inoculated with endospores in the presence of ϵ -polylysine differed significantly ($P < 0.05$) from the control after 24 h of incubation (Figure 7B).

Table 2: Effect of different concentrations of ϵ -polylysine against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 grown in AAM broth at 45 °C for 24 h.

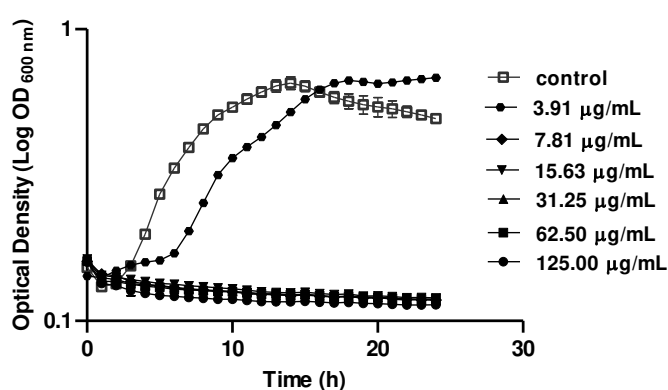
	Concentration ($\mu\text{g/mL}$)	Vegetative cells		Germinated endospores	
		Specific growth rate- μ (h^{-1})	Lag phase- λ (h)	Specific growth rate- μ (h^{-1})	Lag phase- λ (h)
1	0.00	0.25 ± 0.02^a	1	0.26 ± 0.00^a	4
2	3.91	0.22 ± 0.01^a	1	0.13 ± 0.01^b	18
3	7.81	-	-	-	-
4	15.63	-	-	-	-
5	31.25	-	-	-	-
6	62.50	-	-	-	-
7	125.00	-	-	-	-

Values are mean \pm SEM ,

- = no growth

inoculum size = 10^6 CFU/mL

A.



B.

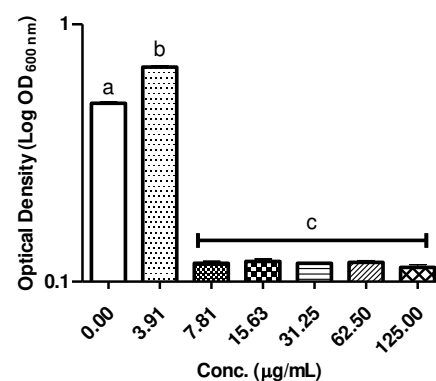


Figure 6: Effect of ϵ -polylysine on vegetative cells of *A. acidoterrestris* DSMZ 2498 grown in AAM broth. (A) Growth kinetics of the vegetative cells of *A. acidoterrestris* DSMZ 2498 with different concentrations of ϵ -polylysine during 24 h incubation and their (B) optical densities after 24 h incubation. Data were represented as mean \pm SD. Bars bearing different letters are significantly different at $P < 0.05$, while bars bearing the same letter(s) are not significantly different ($P > 0.05$). Data were analyzed using one-way ANOVA followed by the Tukey post hoc test.

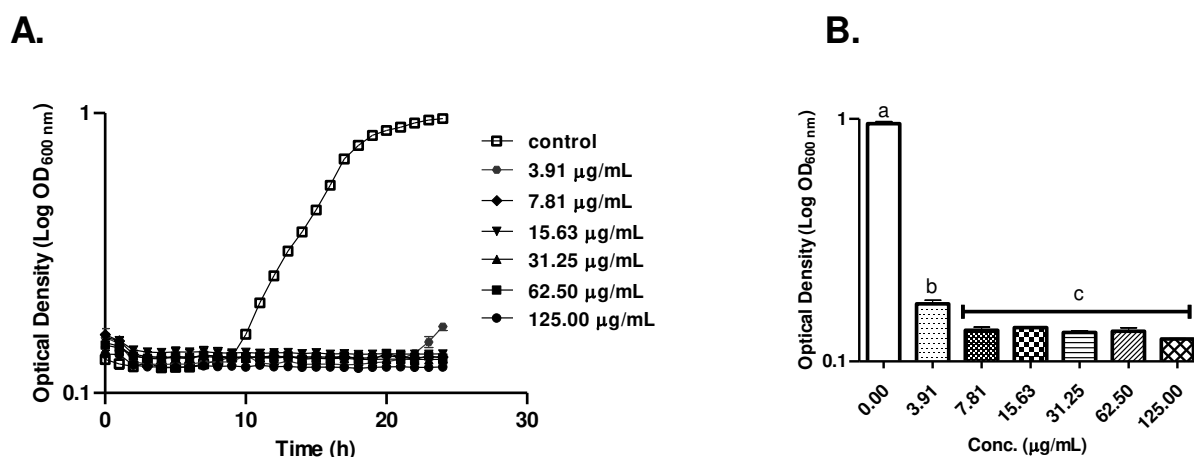


Figure 7: Effect of ϵ -polylysine on activated endospores of *A. acidoterrestris* DSMZ 2498 grown in AAM broth. (A) Growth kinetics of germinated endospores of *A. acidoterrestris* DSMZ 2498 with different concentrations of ϵ -polylysine during a 24 h incubation and their (B) optical densities after 24 h incubation. Data were represented as mean \pm SD. Bars bearing different letters are significantly different at $P < 0.05$, while bars bearing the same letter(s) are not significantly different ($P > 0.05$). Data were analyzed using one-way ANOVA followed by the Tukey post hoc test.

4.2. Evaluation of MIC and MBC for bovicin HC5 and ϵ -polylysine

The MICs for bovicin HC5 against vegetative cells and spores of *A. acidoterrestris* DSMZ 2498 were 2.50 $\mu\text{g/mL}$ and 5.00 $\mu\text{g/mL}$, respectively. For ϵ -polylysine, the same MIC of 7.81 $\mu\text{g/mL}$ was obtained against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 (Table 2).

For bovicin HC5, the MBCs were 5.00 $\mu\text{g/mL}$ against both vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 while for ϵ -polylysine, the MBC values could not be determined as ϵ -polylysine showed bacteriostatic effect against vegetative cells and endospores in all concentrations (7.81 – 125.00 $\mu\text{g/mL}$) tested in this study (Table 2).

Table 3: Minimum Inhibitory and Bactericidal Concentration of bovicin HC5 and ϵ -polylysine against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 grown in AAM.

Inoculum	Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	bovicin HC5 ($\mu\text{g/mL}$)	ϵ -polylysine ($\mu\text{g/mL}$)	bovicin HC5 ($\mu\text{g/mL}$)	ϵ -polylysine* ($\mu\text{g/mL}$)
Vegetative cells	2.50	7.81	5.00	-
Germinated endospores	5.00	7.81	5.00	-

* Bacteriostatic effect only

4.3. Fractional Inhibitory Concentration (FIC) index of the Combined AMPs

The FIC index of the combined AMPs (CAMPs) against vegetative cells of *A. acidoterrestris* DSMZ 2498 was based on the MIC values of each molecule when used in combination. When bovicin HC5 was combined with ϵ -polylysine the MIC was 1.25 $\mu\text{g/mL}$ compared to a MIC of 2.50 $\mu\text{g/mL}$ when used alone, which resulted in an FIC of 0.5 $\mu\text{g/mL}$. Similarly, the FIC of ϵ -polylysine was also 0.5 $\mu\text{g/mL}$, which was calculated from a MIC 3.91 $\mu\text{g/mL}$ for the combination of ϵ -polylysine + bovicin HC5 and a MIC of 7.81 $\mu\text{g/mL}$ when used alone. The FIC index of the combined AMPs was 1.0 which represents an additive effect (Figure 8A) based on the criteria defined by Schelz et al. (2006) who established the following effects for association of antimicrobials: synergism ($\text{FIC} \leq 0.5$), addition ($0.5 < \text{FIC} \leq 1$), indifferent ($1 < \text{FIC} \leq 4$), or antagonism ($\text{FIC} > 4$) (Schelz et al. 2006).

The FIC index of the combined AMPs against endospores of *A. acidoterrestris* DSMZ 2498 was also based on the MIC values of each when used in combination. When bovicin HC5 was combined with ϵ -polylysine the MIC was 2.50 $\mu\text{g/mL}$ compared to a MIC of 5.00 $\mu\text{g/mL}$ when used alone, which resulted in an FIC of 0.5 $\mu\text{g/mL}$. Similarly, the FIC of ϵ -polylysine was also 0.5 $\mu\text{g/mL}$, which was calculated from a MIC of 3.91 $\mu\text{g/mL}$ for the combination of ϵ -polylysine + bovicin HC5 and a MIC of 7.81

$\mu\text{g/mL}$ when used alone. The FIC index of the combined AMPs was 1.0 which represents an additive effect (Figure 8B).

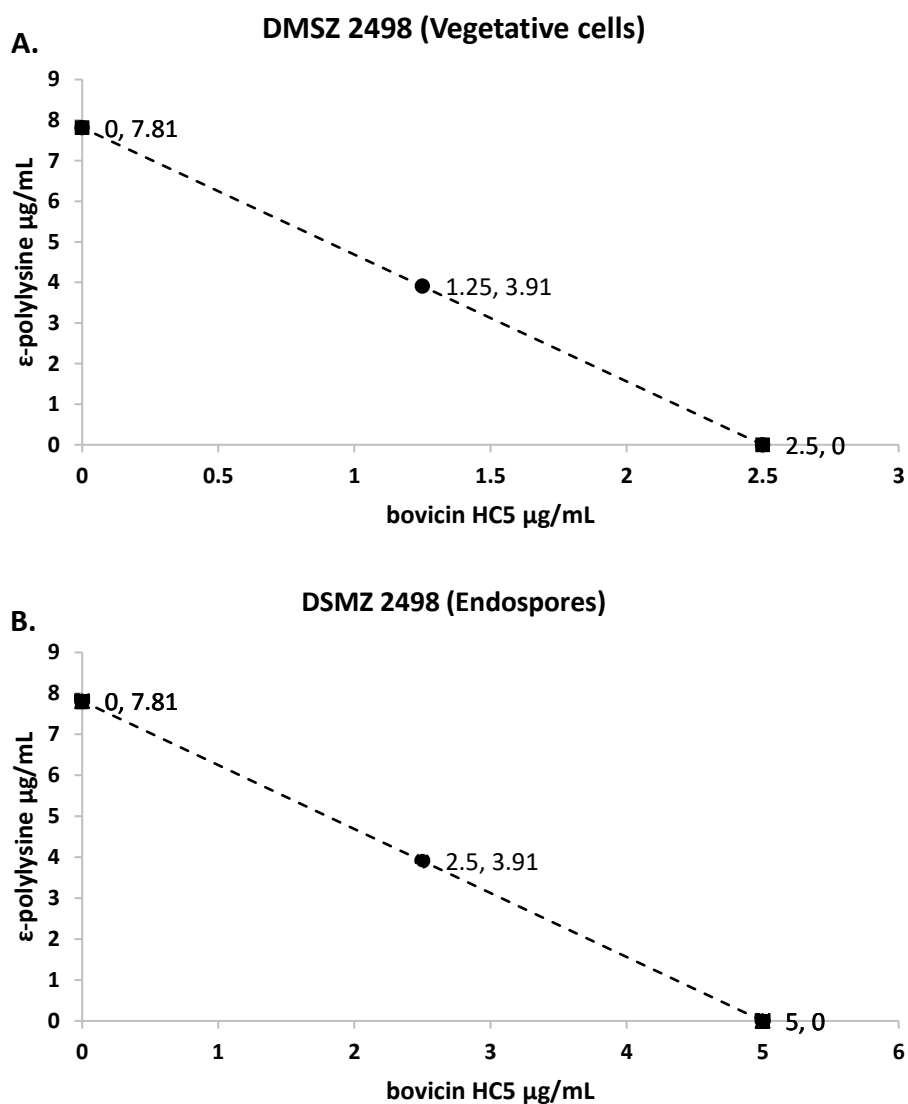


Figure 8: Isobologram of checkerboard assay showing additive effect of bovicin HC5 and ϵ -polylysine against vegetative cells (A) and germinated endospores (B) of *A. acidoterrestris* DSMZ 2498. Both combinations showed an FIC index = 1.0 which is a characteristic of an additive effect between the two AMPs.

4.5. Effect of AMPs on the survival of *A. acidoterrestris* in orange juice

The time-kill assay was carried out in sterilized 100 % orange juice with a pH 3.68 and TSS 11 °Bx as determined. Other properties of the juice as seen on label were sugar content 17 g, carbohydrate 21 g, protein 1.4 g, and vitamin C 45 mg (Table 4).

When the juice was inoculated with ~ 6 log CFU/mL of vegetative cells of *A. acidoterrestris* DSMZ 2498 in the absence of AMPs, the number of viable cells was on average, constant after 48 h of incubation at 45 °C. In the presence of 5.0 µg/mL and 10.0 µg/mL of bovicin HC5, viable cell counts did not differ from control after 24 h but significantly decreased after 36 h, and then increased slightly after 48 h of treatment (Figure 9A). The sample treated with 20.0 µg/mL of bovicin HC5 showed no reduction in cell counts after 24 h when compared to the control, but viable cells reduced below the detection limit after 36 h of incubation while those treated with 80.0 µg/mL of bovicin HC5 showed a greater and constant reduction of viable cell count at all sampling points (12, 24, 36, 48 h). Additionally, the area under the time-kill curve (AUC) for all treatments was reduced ($P < 0.05$) compared to the control (Figure 9B).

Addition of ϵ -polylysine to orange juice caused a reduction in viable cell count (4.81 – 4.90 log CFU/mL) compared to that of the control (5.54 log CFU/mL) (Figure 9C) with ϵ -polylysine at 250.0 µg/mL showing the highest effect in decreasing cell viability. The AUC with different concentrations of ϵ -polylysine was significantly reduced compared to the control ($P < 0.05$). The exception was samples treated with 31.25 µg/mL and 62.50 µg/mL of ϵ -polylysine where no significant difference ($P > 0.05$) in viability was observed after 48 h of treatment (Figure 9D).

After the effect of the combined AMPs was analyzed against the vegetative cells of *A. acidoterrestris*, a reduction ($P < 0.05$) in the viable cell count at all treatment intervals (12, 24, 36, 48 h) was observed compared to the control (Figure 9D). Combined concentrations of bovicin HC5 and ϵ -polylysine (40.0:62.5 µg/mL and 80.0:125.0 µg/mL, respectively) reduced viable count below the detection limit after 36 h of incubation. Also, AUC with all concentrations was significantly different ($P < 0.05$) from the control after 48 h (Figure 9E).

Table 4: Characteristics of the orange fruit juice sample utilized for this study

Properties	Value
Product	Campo Largo - Laranja Integral
Juice percentage	100 %*
pH	3.68
Total Soluble Solids	11° Brix
Sugar content	17 g*
Carbohydrates	21 g*
Proteins	1.4 g*
Vitamin C	45 mg*

*as seen on the label

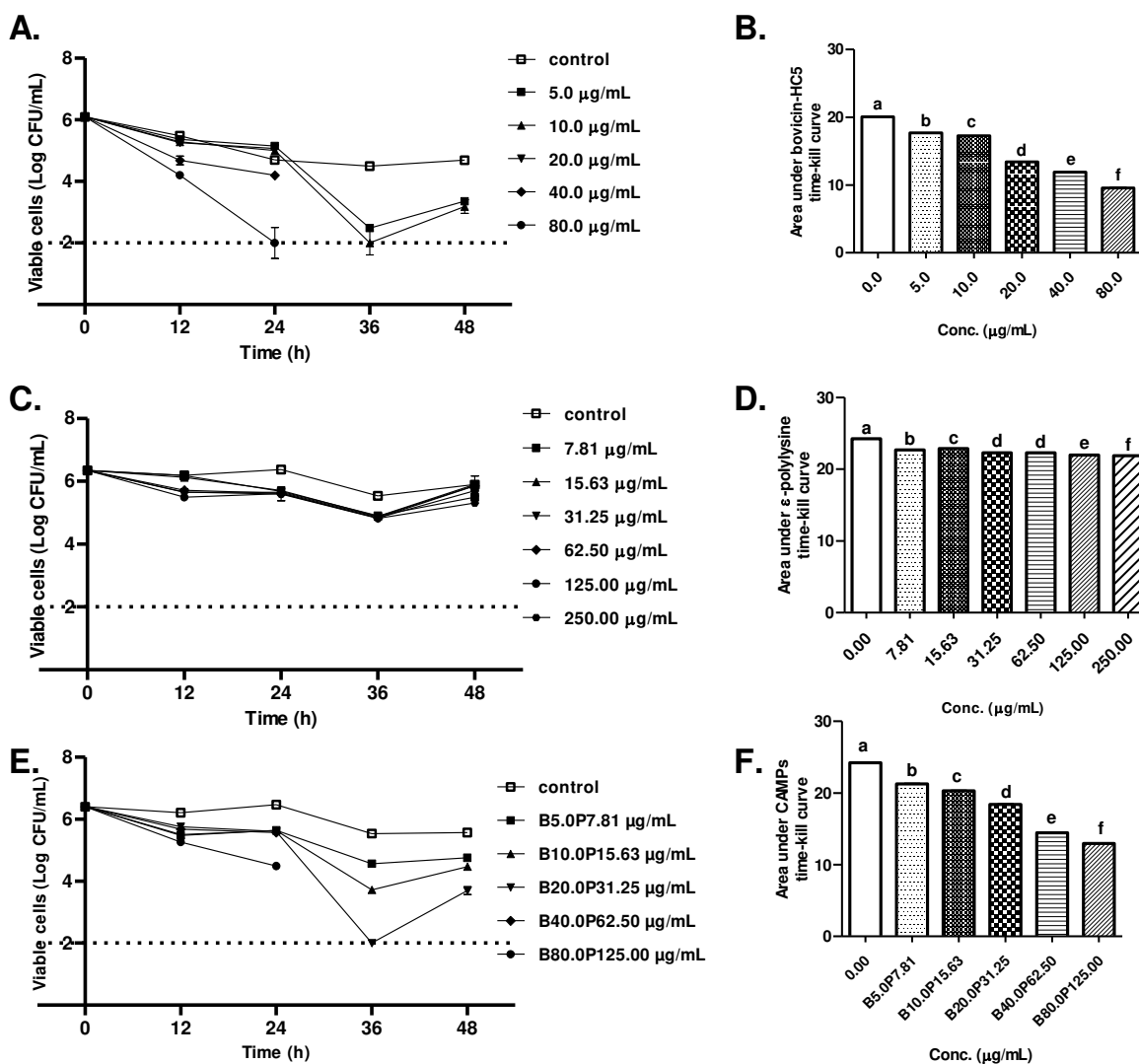


Figure 9: Time-kill curve and AUC of *A. acidoterrestris* DSMZ 2498 (vegetative cells) treated with bovicin HC5 alone (A, B), ϵ -polylysine alone (C, D), and AMPs in combination (E, F) with B, P representing bovicin HC5 and ϵ -polylysine respectively. Data are presented as mean \pm SEM, $n = 3$. Bars showing different letters differ ($P < 0.05$) from the control. Data were analyzed using one-way ANOVA followed by Tukey post hoc test. Dotted lines represent the detection limit of the assay.

When the juice was inoculated with ~ 6 log CFU/mL of endospores of *A. acidoterrestris* DSMZ 2498 in the absence of AMPs, the number of viable spores reduced by approximately one log cycle. In the presence of 80.0 and 160.0 $\mu\text{g/mL}$ of bovicin HC5, the viable spores reduced below the detection limit after 36 h of incubation (Figure 10A). Also, the AUC with both treatments was significantly reduced ($P < 0.05$) compared to the control (Figure 10B).

With the addition of ϵ -polylysine to orange juice, a reduction in viable spore count (3.84 - 3.60 log CFU/mL) compared to that of control (4.30 log CFU/mL) was observed (Figure 10C). The AUC with different concentrations of ϵ -polylysine was significantly different from control ($P < 0.05$) (Figure 10D).

When the effect of the combined AMPs was analyzed against the activated endospores of *A. acidoterrestris*, a reduction ($P < 0.05$) in the viable spore count at all treatment intervals was observed compared to that of the control. Also, all concentrations reduced the viable spore count below the detection limit after 36 h of treatment (Figure 10E). Additionally, the AUC with all concentrations reduced ($P < 0.05$) compared to that of control (Figure 10F).

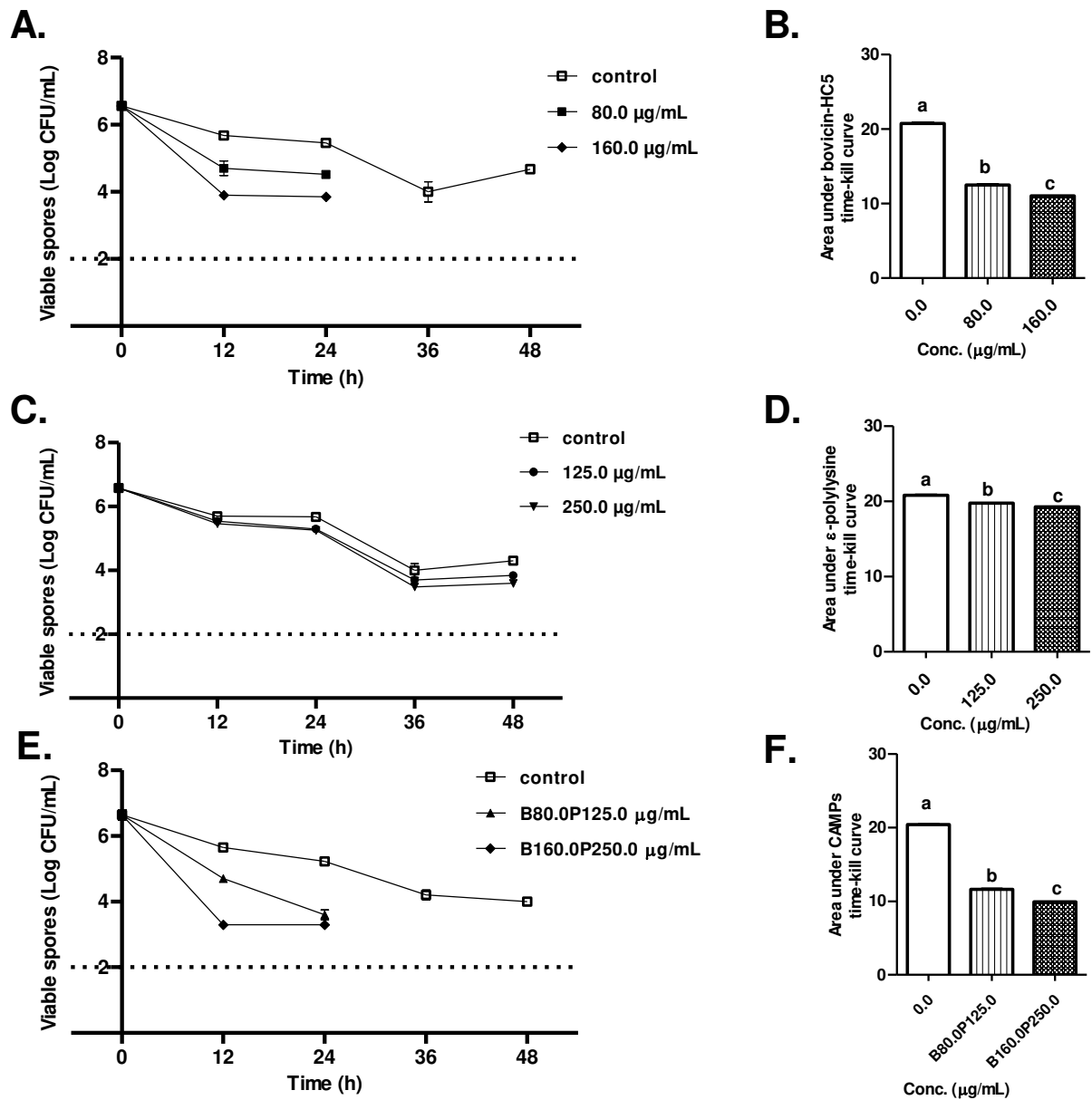


Figure 10: Time-kill curve and AUC of *A. acidoterrestris* DSMZ 2498 germinated endospores in orange juice and treated with bovicin HC5 alone (A,B), ε-polylysine alone (C,D), and AMPs in combination (E,F) with B, P representing bovicin HC5 and ε-polylysine respectively. . Data are presented as mean ± SEM, n = 3. Bars showing different letters are significantly different (P < 0.05) from control. Data were analyzed using one-way ANOVA followed by Tukey post hoc test. Dotted lines represent the detection limit of the assay.

4.6. Effect of bovicin HC5 and ϵ -polylysine on the thermal resistance of *A. acidoterrestris* endospores in orange juice

The average D-value at 95 °C ($D_{95^{\circ}\text{C}}$) of *A. acidoterrestris* DSMZ 2498 endospores in orange juice without antimicrobial peptides was 7.68 min (Table 5). The addition of bovicin HC5 (80 $\mu\text{g}/\text{mL}$) decreased ($P < 0.05$) the $D_{95^{\circ}\text{C}}$ value to 2.19 min, which represented a 71.48 % reduction in the number of viable spores compared to the control (Table 5, Figure 11A). In contrast, when 125 $\mu\text{g}/\text{mL}$ of ϵ -polylysine was added to orange juice inoculated with endospores of *A. acidoterrestris*, the number of viable spores did not differ ($P > 0.05$) from the control after 12 min heating time (Table 5, Figure 11B).

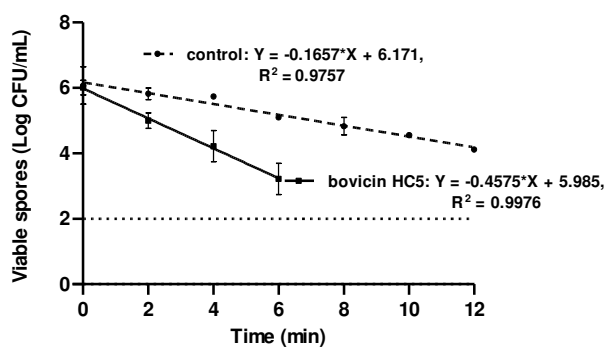
Orange juice that was added with bovicin HC5 and ϵ -polylysine at 80:125 $\mu\text{g}/\text{mL}$, respectively also showed a significant reduction in the number of viable spores below the detection limit ($P < 0.05$) with a $D_{95^{\circ}\text{C}}$ of 2.43 min (Table 5). Therefore, addition of AMPs to orange juice decreased the $D_{95^{\circ}\text{C}}$ of *A. acidoterrestris* DSMZ 2498 endospores by 68.36 % which is similar to the effect observed when bovicin HC5 alone was added to orange juice (Table 5, Figure 11C).

Table 5: Effect of bovicin HC5, ϵ -polylysine, or both AMPs on the D-value at 95 °C of *A. acidoterrestris* DMSZ 2498 endospores in orange juice.

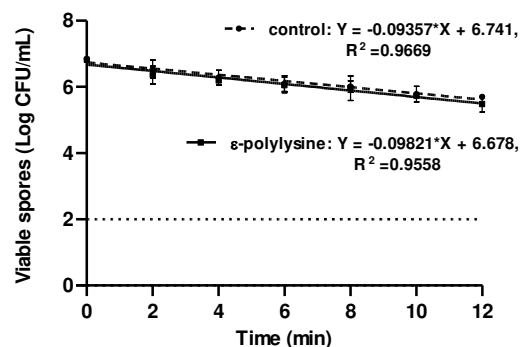
	AMPs	Concentration ($\mu\text{g}/\text{mL}$)	D-value (min)*	Reduction (%)
1	Control	-	7.68 \pm 1.51 ^a	
2	Bovicin HC5	80	2.19 \pm 0.35 ^b	71.48
3	ϵ -Polylysine	125	7.31 \pm 1.41 ^a	4.78
4	CAMPs	80:125	2.43 \pm 0.23 ^c	68.36

*Values are mean \pm standard error of mean ($n = 3$). Significant correlation between the D-values was analyzed using linear regression. Values with different letters differ ($P < 0.05$) from the control

A.



B.



C.

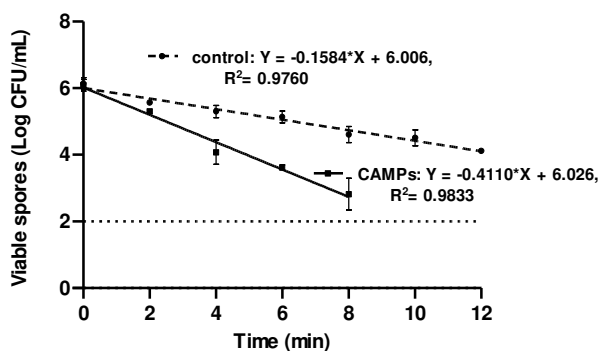


Figure 11: Survival curve of *A. acidoterrestris* DSMZ 2498 endospores in orange juice. The orange juice was treated with bovicin HC5 at 80 $\mu\text{g}/\text{mL}$ (A), ϵ -polylysine at 125 $\mu\text{g}/\text{mL}$ (B), or a combination of both AMPs (B80:P125 $\mu\text{g}/\text{mL}$) (C). The incubation was carried out at a temperature of 95 $^\circ\text{C}$ for 12 min. The dotted line represents the detection limit.

4.7. Morphological characteristics of *A. acidoterrestris* DSMZ 2498 treated with AMPs

The changes in *A. acidoterrestris* cell morphology caused by treatment with AMPs were evaluated by atomic force microscopy (AFM). Untreated vegetative cells of *A. acidoterrestris* DSMZ 2498 exhibited regular, smooth, and fully rounded rod shape even after 24 h of incubation in phosphate buffer (pH 4) at 45 °C. However, upon treatment with the antimicrobial peptides, distinct changes in the cell morphology were observed (Figures 12, 13, 14).

With the addition of 2.5 and 5.0 µg/mL (1X and 2X MIC) of bovicin HC5, rough areas, and deformation of the cell surface were observed with incubation time. Cultures that were treated with 5.0 µg/mL of bovicin HC5 showed greater deformation of the cell structure with wrinkles observed after 8 h of incubation and the presence of deep dents in the center of the cells after 24 h of incubation (Figure 12). When 7.81 and 15.63 µg/mL (1X and 2X MIC) of ε-polylysine were used as treatment against the vegetative cells of *A. acidoterrestris* DSMZ 2498, irregular cell surface and shape deformation were observed after all incubation times (Figure 13).

Additionally, when 1X and 2X MIC of combined bovicin HC5 and ε-polylysine were used as treatment (1.25:3.91 µg/mL and 2.50:7.81 µg/mL, respectively), increased cell surface roughness and presence of extrusions were observed after each incubation time point (Figure 14).

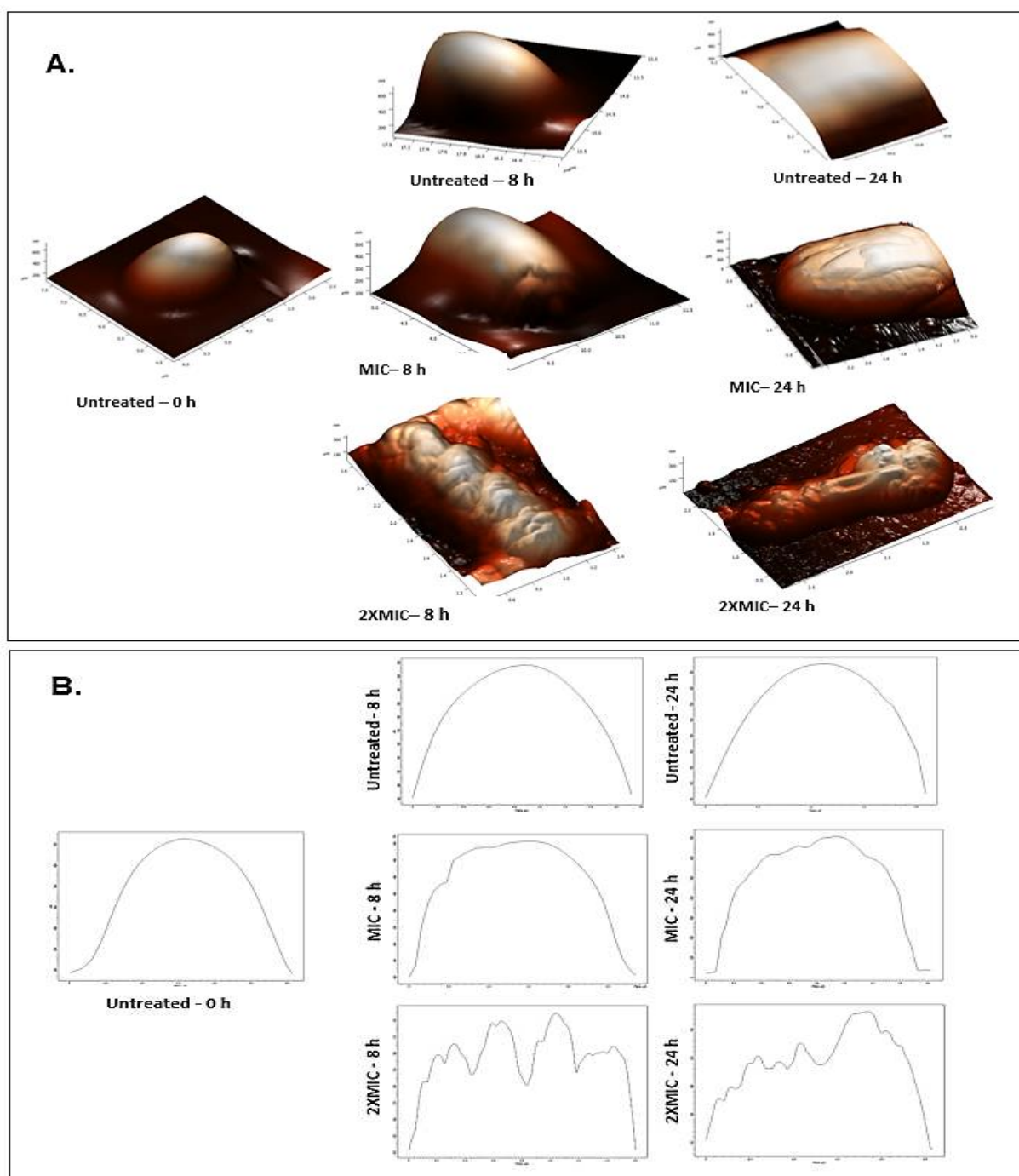


Figure 12: Effect of bovicin HC5 on vegetative cells of *A. acidoterrestris* DMSZ 2498. Three-dimensional topographical images (Panel A) and cross-sectional measurements (Panel B) of vegetative cells of *A. acidoterrestris* DMSZ 2498 obtained by AFM (NT-MDT) after 0, 8 h, 24 h of incubation. The cell suspensions were incubated with MIC - 2.5 $\mu\text{g}/\text{mL}$ and 2XMIC - 5.0 $\mu\text{g}/\text{mL}$ of bovicin HC5 in phosphate buffer (pH 4.0) at 45 $^{\circ}\text{C}$. The images were observed using a scanning area of 1 to 4 μm .

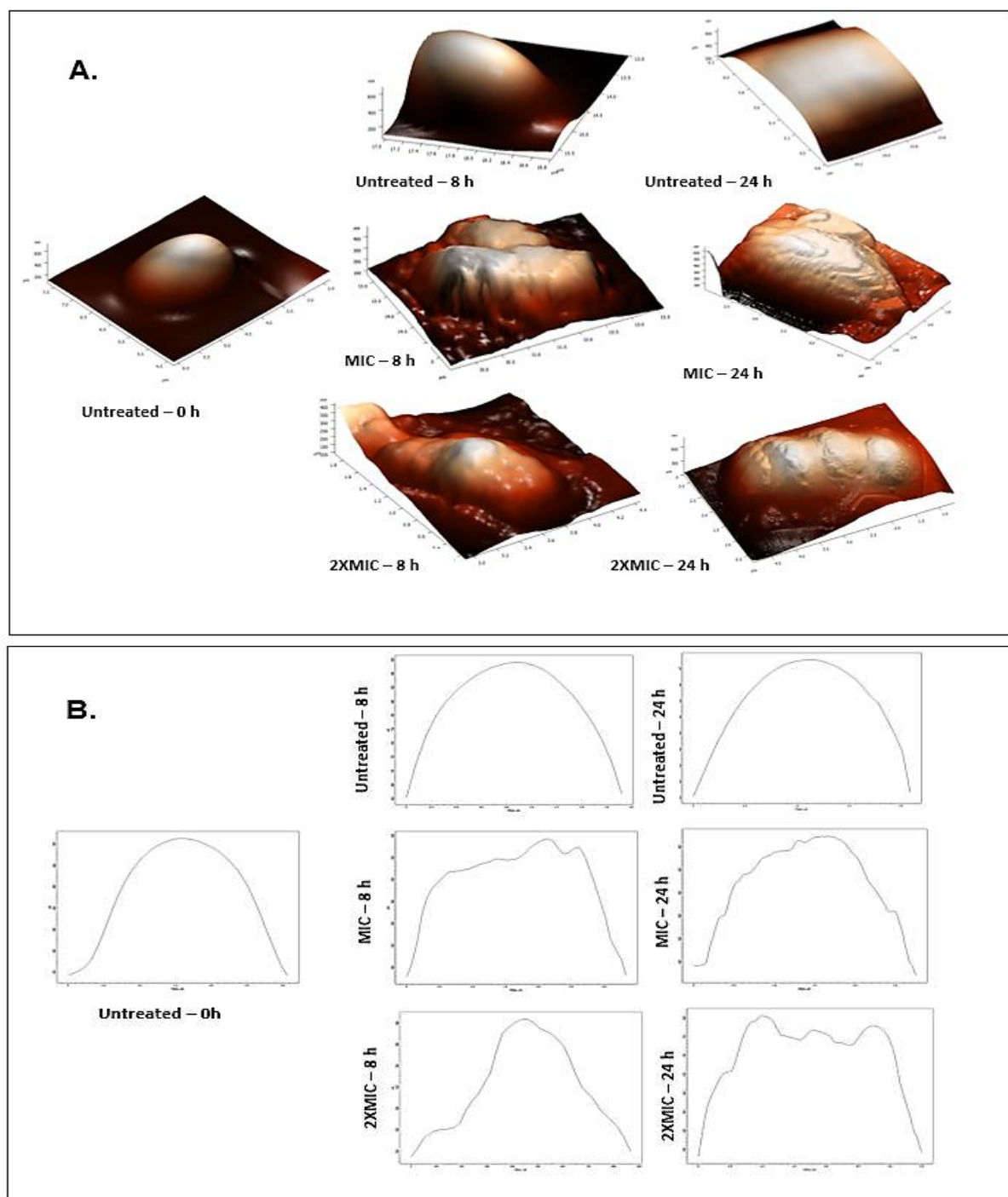


Figure 13: Effect of ϵ -polylysine on vegetative cells of *A. acidoterrestris* DMSZ 2498. Three-dimensional topographical images (Panel A) and cross-sectional measurements (Panel B) of vegetative cells of *A. acidoterrestris* DMSZ 2498 obtained by AFM (NT-MDT) after 0, 8 h, 24 h of incubation. The cell suspensions were incubated with MIC - 7.81 $\mu\text{g}/\text{mL}$ and 2XMIC - 15.63 $\mu\text{g}/\text{mL}$ of bovicin HC5 in phosphate buffer (pH 4.0) at 45 $^{\circ}\text{C}$. The images were observed using a scanning area of 1 to 4 μm .

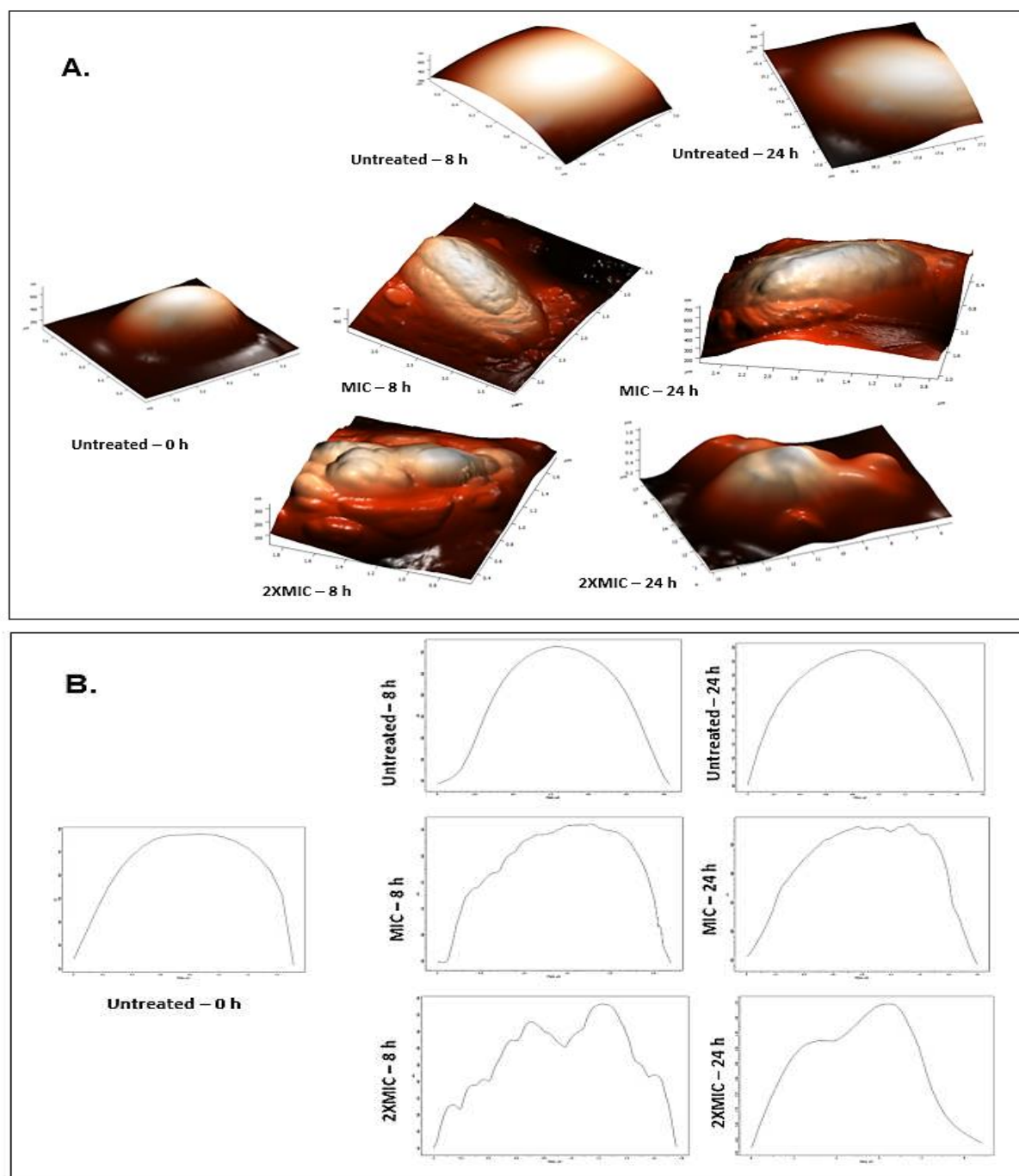


Figure 14: Effect of combination of bovicin HC5 and ϵ -polylysine on vegetative cells of *A. acidoterrestris* DMSZ 2498. Three-dimensional topographical images (Panel A) and cross-sectional measurements (Panel B) of vegetative cells of *A. acidoterrestris* DMSZ 2498 obtained by AFM (NT-MDT) after 0, 8 h, 24 h of incubation. The cell suspensions were incubated with MIC - 1.25:3.91 $\mu\text{g}/\text{mL}$ and 2XMIC - 2.50:7.81 $\mu\text{g}/\text{mL}$ of the combination of bovicin HC5 and ϵ -polylysine in phosphate buffer (pH 4.0) at 45 $^{\circ}\text{C}$. The images were observed using a scanning area of 1 to 4 μm .

4.8. Discussion

Currently, *Alicyclobacillus* species are considered a serious and unresolved problem in the food industry due to their ability to produce heat-resistant endospores and survive the typical pasteurization process (Sokołowska et al. 2020). *A. acidoterrestris* in particular, has been responsible for several spoilage incidents associated with unpleasant changes in the odor and taste of fruit juices due to the production of spoilage compounds such as guaiacol (Pornpukdeewattana et al. 2019). Given that traditional food processing methods have limited efficacy to prevent spoilage of acidic beverages by *A. acidoterrestris*, it is of most importance to study alternative methods to improve the safety of these products (Barbosa et al. 2017).

In this study, the effect of antimicrobial peptides on the growth of vegetative cells and germination of endospores *A. acidoterrestris* were investigated in AAM broth. Based on the results obtained, bovicin HC5 and ϵ -polylysine showed effective antimicrobial effects and caused varying reductions in the specific growth rate and lag phase of *A. acidoterrestris*. When different inoculum sources (vegetative cells and endospores) were exposed to 2.50 $\mu\text{g}/\text{mL}$ of bovicin HC5, there was a noticeable variation in the lag phase of *A. acidoterrestris*. This suggests that the bovicin HC5 may interfere with spore germination, though not sufficient to completely inhibit growth in comparison to that of the vegetative cells at the same concentration. According to Gut et al. (2011), one possible explanation is that, like nisin, binding of bovicin HC5 to lipid II (achieved with the above-mentioned concentration) can inhibit the vegetative cells but is inadequate to effectively inhibit spore outgrowth. Similar to other lantibiotics, bovicin HC5 exhibits a higher affinity for lipid II than anionic lipid membranes (Zheng et al. 2022). This may help to explain why the specific growth rate of *A. acidoterrestris* germinated endospores with bovicin HC5 concentrations $\leq 2.50 \mu\text{g}/\text{mL}$ was higher than that of vegetative cells. More bovicin HC5 has likely been bound to lipid II on the spore membrane, rendering it unavailable to bind to the vegetative cells that emerged from the germinated spores. However, further studies will be required to fully understand the underlying mechanism.

The strong poly-cationic property of ϵ -polylysine, which supports its capacity to adhere to anionic surfaces, thus increasing cell membrane permeability

and causing inhibition of enzyme activity, may be responsible for its inhibitory action (Tan et al. 2019). When both inocula (vegetative cells and endospores) of *A. acidoterrestris* were used in the presence of 3.91 µg/mL ε-polylysine, variations in the specific growth rate and lag phase of *A. acidoterrestris* were observed. This may be due to the fact that during spore germination the spore membrane becomes permeable (Egan et al. 2016), which further aids the membrane permeability of ε-polylysine and facilitates its penetration into the germinated spore.

Current research demonstrated several benefits of antimicrobial peptides over chemical preservatives, suggesting that these molecules could enhance the storage life of food products by preventing the growth of food-spoilage microorganisms. Hence, by combining two antimicrobial peptides, the efficacy of antimicrobial peptides could be increased, resulting in additional hurdle against food spoilage (Rai et al. 2016). In this study, the combined effect of bovicin HC5 and ε-polylysine against vegetative cells and germinated endospores of *A. acidoterrestris* DSMZ 2498 proved to be additive (FIC index = 1) in AAM broth at pH 4, which implies that the combined effect is the sum of the effect of the two antimicrobial peptides working independently. Liu et al. (2022) reported that although bacteriocins alone have effective antimicrobial properties, their use in combination with other physical, chemical, or antimicrobials hurdles promotes additive/synergistic effects. For a synergistic effect, it is suggested that mechanistic insights associated with the combined effects of the antimicrobials in different substrates will be helpful to achieve optimal results (Liu et al. 2022).

Furthermore, the time-kill assays demonstrated significant inhibitory effects on the growth of vegetative cells and spore germination of *A. acidoterrestris* after exposure to both AMPs (individually and in combination) in concentrated orange juice. Both AMPs replicated their individual bacteriostatic or bactericidal activities in orange juice as observed in the laboratory media but at higher concentrations. These results suggest the interaction of this bacteriocin with components of the fruit juice matrix (Todorov et al. 2022). However, bovicin HC5 showed a more pronounced antibacterial effect compared to that of ε-polylysine.

To implement effective strategies for fruit juice preservation, evaluation of the heat resistance in spoilage-causing microorganisms is important (Ribeiro et al. 2022). The heat resistance of *A. acidoterrestris* inoculated in orange juice obtained in this

study was represented by a mean D-value of 7.68 min at 95 °C. It is important to note that the D-value of the endospores of *A. acidoterrestris* is affected by varying factors such as growth conditions or characteristics of the study design, including food matrix used (i.e, type of fruit juice), media or fruit juice pH, TSS content of fruit juices, temperature, quality of the culture medium, inactivation method, bacterial strain tested, among others (Sokołowska et al. 2020). For different combinations of these factors, Huertas et al. (2021) reported a D_{95 °C} value of 5.24 min of *A. acidoterrestris* in orange juice. Comparably, these results confirm the high heat resistance of *A. acidoterrestris* endospores and the ineffectiveness of pasteurization with a temperature range of 80 – 100 °C for less than 30 s, commonly used in fruit juices processing (Ağçam et al. 2018).

The efficacy of bovicin HC5 (individually and in combination) to significantly reduce the heat resistance of endospores of *A. acidoterrestris* can be attributed to its high stability under pasteurization temperatures (Ribeiro et al. 2022). Although its sporicidal effect has not been completely elucidated, its interaction with endospores could be similar to what has been reported for nisin (Egan et al. 2016). The effect of bovicin HC5 like nisin on endospores might occur after germination has been initiated. When endospores germinate, lantibiotics such bovicin HC5 could bind to the lipid II in the cell membrane thus inhibiting the outgrowth of endospores and reducing viability (Egan et al. 2016).

The reduction in effect of ϵ -polylysine when added to orange juice at pH 3.68 at a temperature of 95 °C could be attributed to its amine groups which are strongly cationic at pH values < 9.0 allowing it to interact with anionic components within the food matrix, forming insoluble precipitates which further affects its ability to interact with the bacterial cell membrane (Chang et al. 2014). Also, this result reveals the need to investigate other varying factors that affect the activity of ϵ -polylysine under food processing conditions.

Normal cell morphology and an integral cell membrane are vital for bacterial growth and maintenance of vital metabolic functions (Fan et al. 2021). Previous studies demonstrated that some antimicrobial peptides like enterocin AS-48, bificin C6165, bacteriocin RC20975, nisin, and bacteriocin AMA-K could induce structural changes in *A. acidoterrestris* cells (Grande et al. 2005; Todorov et al. 2011; Pei et al. 2014, 2017). In the current study, both ϵ -polylysine and bovicin HC5 as well as their combinations

at MIC concentration and 2XMIC induced drastic changes in the cell morphology of *A. acidoterrestris*. The structural changes observed on the vegetative cells of *A. acidoterrestris* treated with bovicin HC5 provides further evidence of its dual mechanism of action through inhibition of cell wall biosynthesis and formation of pores in the cell membrane as also reported by Ribeiro et al. (2022). Hence, the hindering of cell wall biosynthesis by binding to lipid II may result in cell death (Ribeiro et al. 2022). Additionally, the pore-like lesions observed provide evidence of depolarization of the membrane potential and cellular leakage, which eventually renders the bacterial cell non-viable as described by Egan et al. (2016).

The effects of ϵ -polylysine are similar to that reported by Tan et al. (2019) against *S. aureus*. The authors demonstrated that ϵ -polylysine induced structural change of the peptidoglycan in the cell wall and affected the integrity of the cytoplasmic membrane in treated cells.

The effect of the combined MICs of ϵ -polylysine and bovicin HC5 which were a combination of 50 % lower concentrations of the MICs of the individual AMPs revealed structural changes similar to those obtained with the MICs of the AMPs and this result was replicated with 2XMIC as shown in Figure 14. These results correlate with the Loewe additivity theory, the effect of antimicrobials in combination is determined not by the sum of their normalized effects, but rather by the sum of their normalized dosages, such that their combined effect is the same across all combinations that have the same total normalized dosage (Russ and Kishony 2018), which justifies the linear isobole obtained in this study.

5. CONCLUSION

Bovicin HC5 and ϵ -polylysine show antibacterial activity at low concentrations against vegetative cells and endospores of *A. acidoterrestris*, significantly reducing viable vegetative cells and germinated endospores over time in culture media.

Bovicin HC5 demonstrates bactericidal against *A. acidoterrestris* and caused a significant decrease in the thermal resistance of the endospores of *A. acidoterrestris* in orange juice.

ϵ -Polylysine is bacteriostatic against *A. acidoterrestris* in orange juice.

The combination of bovicin HC5 and ϵ -polylysine at MIC or 2X MIC shows additive effect against *A. acidoterrestris*.

Overall, the findings in this study indicate that bovicin HC5 and ϵ -polylysine could be used as natural preservatives in hurdle technologies to control *A. acidoterrestris* and improve the microbiological stability and safety of fruit juices.

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