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Atividade antibacteriana das enzimas Papaína e Bromelina livres e encapsuladas contra *Alicyclobacillus* spp.

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Tese apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de Doutora em Ciência de Alimentos.

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BIOGRAFIA

Márcia Maria dos Anjos Szczerepa nasceu em 20 de novembro de 1985 na cidade de Ponta Grossa, Paraná. Possui graduação em Tecnologia de Alimentos pela Universidade Tecnológica Federal do Paraná. Possui especialização em Higiene e Inspeção de Produtos de Origem Animal pelo Centro Universitário de Maringá e mestrado em Ciência de Alimentos pela Universidade Estadual de Maringá. Tem experiência nas áreas de Microbiologia e Controle de Qualidade, atuando principalmente nos seguintes temas: Controle de Qualidade de Produtos de Origem Animal, Análises Microbiológicas de Alimentos, Microbiologia Industrial e Bactérias Termoacidorresistentes.

Dedico...
Ao meu marido, pelo amor e apoio incondicional.

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OBRIGADA!

APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos.

1. Márcia Maria dos Anjos, Angela Aparecida da Silva, Isabela Carolini de Pascoli, Jane Martha Graton Mikcha, Miguel Machinski Jr, Rosane Marina Peralta, Benício Alves de Abreu Filho. **Antibacterial activity of papain and bromelain on *Alicyclobacillus* spp.** Revista International Journal of Food Microbiology. Artigo publicado.

2. Márcia Maria dos Anjos, Eliana Harue Endo, Fernanda Vitória Leimann, Odinei Hess Gonçalves, Benedito Prado Dias Filho, Benício Alves de Abreu Filho. **Microencapsulated Papain and Bromelain used as biopreservatives against *Alicyclobacillus* spp.** Revista Food Microbiology. Artigo em avaliação.

GENERAL ABSTRACT

INTRODUCTION. *Alicyclobacillus* spp. are Gram-positive, thermophilic, acidophilic, non-pathogenic micro-organisms that have the ability to form spores. They are present in soil and are often related to the deterioration of acidic products among them citrus juices and beverages, such as orange juice. Heat treatments, such as pasteurization, are used in the orange juice concentrate industry to inactivate pathogenic and spoilage micro-organisms. However, the spore of *Alicyclobacillus acidoterrestris* can survive such heat treatment, germinate, grow and thus cause the juice to deteriorate after reconstitution. Research is currently focused on the application of natural antimicrobial agents to foods as they provide a potential pathway for inhibiting a wide variety of microorganisms without risk to consumer health. Papain and Bromelain are two enzymes extracted from papaya (*Carica papaya*) and pineapple (*Ananas comosus*) respectively, where several studies characterize its antimicrobial effects, however these enzymes have not been evaluated against the genus *Alicyclobacillus* to date. Because they are two proteolytic enzymes, such compounds may undergo denaturation when subjected to the heat treatment employed, for example, in the pasteurization of orange juice. Microencapsulation is a technology that allows the fine coating of solid particles in order to overcome limitations in the use of ingredients, such as attenuating undesirable flavors, reducing volatility and reactivity as well as increasing the stability of these solid particles under adverse conditions. Spray drying is a viable method for the preparation of microparticles and the use of natural biopolymers as an encapsulating material makes their application possible in food products.

AIMS. To evaluate the antibacterial activity of the enzymes papain and bromelain against *Alicyclobacillus* spp., focusing on the *A. acidoterrestris* species and to microencapsulate these enzymes with alginate and chitosan to verify its effectiveness in the protection of the antibacterial activity after being submitted to the heat treatment.

MATERIAL AND METHODS. The determination of minimum inhibitory and bactericidal concentration (MIC and MBC) for papain and bromelain against *Alicyclobacillus* strains was performed. The time course of death of the microorganism was determined in the presence and absence of the enzymes and investigated the enzymatic activity as a mechanism of action on the microorganism through enzyme inhibitor and denaturation assays. The antibacterial activity of the enzymes was also evaluated in combination with nisin. The microencapsulation of the enzymes was performed by spray drying using alginate and chitosan as encapsulating material, after which the compounds were submitted to thermal treatment and after their inhibitory and bactericidal activity was evaluated against five different species of *Alicyclobacillus*. Microcapsules were characterized by Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC) and Infrared Spectroscopy (FT-IR).

RESULTS AND DISCUSSION. The results showed that for the *A. acidoterrestris* strain, the MIC of papain was 0.98 µg/mL and the MBC was 3.91

µg/mL, while the MIC of bromelain was 62.5 µg/mL and the MBC was 250 µg/mL. The concentration of 4XMIC for both the enzymes was sufficient to eliminate 4 logs of the micro-organism after 24 hours of incubation. Through the use of enzyme inhibitors specific for cysteine proteases, it was found that the antibacterial activity of papain and bromelain is not related to its proteolytic activity, but may be related to other activities, such as amidase and esterase. The synergistic activity of the enzymes revealed an fractional inhibitory concentration (FIC) level of 0.16. Combination with nisin revealed an FIC of 0.25 for papain and 0.19 for bromelain, indicating synergism between both compounds. Microencapsulation of the particles with alginate and chitosan was evidenced through the physical analysis. The results showed that papain microencapsulated with chitosan or alginate maintained low MIC values after submission to heat treatment, demonstrating its effectiveness and potential application as a biopreservative.

CONCLUSIONS. The application of the enzymes in reconstituted orange juice contaminated with *A. acidoterrestris* proved to be effective, since after 48 h of incubation at three different temperatures, the initial microbial population was eliminated. Therefore, papain and bromelain are enzymes with antibacterial action in *A. acidoterrestris*. The microencapsulation technique was effective in protecting the antibacterial activity of papain, characterizing this compound with great potential of application as a bioconservant in acidic products, being necessary more studies on stability and sensorial characteristics when applied to the food.

Key words: Papain, Bromelain, *Alicyclobacillus,* orange juice, polymers, biopreservative.

RESUMO GERAL

INTRODUÇÃO. *Alicyclobacillus* spp. são micro-organismos em forma de bacilos, Gram-positivos, térmófilos, acidófilos, não patogênicos e com a capacidade de formar esporos. Eles estão presentes no solo e são frequentemente relacionados a deterioração de produtos ácidos, entre eles bebidas e sucos cítricos, como o suco de laranja. Tratamentos térmicos são empregados na indústria de suco de laranja concentrado para inativar uma grande quantidade de micro-organismos patógenos e deteriorantes, no entanto, o esporo de *Alicyclobacillus acidoterrestris* pode sobreviver a estes tratamentos térmicos, germinar, crescer e assim deteriorar o suco após a sua reconstituição. Atualmente as pesquisas estão voltadas para a aplicação de agentes antimicrobianos naturais nos alimentos, pois proporcionam uma via potencial para inibir uma vasta variedade de micro-organismos, sem risco para a saúde dos consumidores. Papaína e Bromelina são duas enzimas extraídas, respectivamente, do mamão (*Carica papaya*) e do abacaxi (*Ananas comosus*), onde diversos estudos caracterizam os seus efeitos antimicrobianos, no entanto essas, até o o momento, não foram avaliadas frente ao gênero *Alicyclobacillus*. Por se tratar de duas enzimas proteolíticas, esses compostos podem sofrer desnaturação quando submetidos ao tratamento térmico empregado, por exemplo, na pasteurização do suco de laranja. A microencapsulação é uma tecnologia que permite o revestimento fino de partículas sólidas com o objetivo de solucionar limitações no emprego de ingredientes, como por exemplo, atenuar flavors indesejáveis, reduzir a volatilidade e reatividade assim como aumentar a estabilidade dessas partículas sólidas em condições adversas. A secagem por pulverização (spray dryer) é um método viável para a preparação de micropartículas e a utilização de biopolímeros naturais como material encapsulante torna viável sua aplicação em produtos alimentícios.

OBJETIVOS. Avaliar a atividade antibacteriana das enzimas papaína e bromelina em *Alicyclobacillus* spp., com foco na espécie *A. acidoterrestris* e microencapsular essas enzimas com alginato e quitosana para verificar a sua eficácia na proteção da atividade antibacteriana após submetidas ao tratamento térmico.

MATERIAIS E MÉTODOS. Foi realizada a determinação da concentração inibitória e bactericida mínima (CIM e CBM) para a papaína e bromelina frente as cepas de *Alicyclobacillus* spp. Foi determinado a curva do tempo de morte do micro-organismo na presença e ausência das enzimas e investigado a atividade enzimática como mecanismo de ação sobre o micro-organismo através de ensaios com inibidores enzimáticos e desnaturação. A atividade antibacteriana das enzimas foi também avaliada em combinação com nisina. A microencapsulação das enzimas foi realizada através de secagem por pulverização (*Spray dryer*) utilizando como material encapsulante o alginato e a quitosana, em seguida os compostos foram submetidos ao tratamento térmico e após sua atividade inibitória e bactericida foi avaliada frente a cinco espécies diferentes de *Alicyclobacillus*. As microcápsulas foram caracterizadas por

Microscopia Eletrônica de Varredura (MEV), Calorimetria de Varrimento Diferencial (DSC) e Espectroscopia de Infravermelho (FT-IR).

RESULTADOS E DISCUSSÃO. Os resultados mostraram que para a espécie *A. acidoterrestris*, a CIM da papaína foi de 0,98 µg/mL e a CBM foi de 3,91 µg/mL. Para a bromelina, a CIM foi de 62,5 µg/mL e a CBM foi de 250 µg/mL. A concentração de 4XCIM para ambas as enzimas foi suficiente para eliminar 4 logs do micro-organismo após 24 horas de incubação. Através da utilização de inibidores enzimáticos específicos para cisteína-proteases, verificou-se que a atividade antibacteriana da papaína e da bromelina não está relacionada com a sua atividade proteolítica, mas pode estar relacionada com outras atividades, tais como amidase e esterase. A atividade sinérgica entre as enzimas apresentou um índice de concentração inibitória fracional (FIC) de 0,16. A combinação com nisina apresentou um FIC de 0,25 para a papaína e 0,19 para a bromelina, ambos indicativos de sinergismo entre os compostos. O microencapsulamento das partículas com alginato e quitosana foi evidenciado através das análises físicas. Os resultados demonstraram que a papaína microencapsulada com quitosana ou alginato foi capaz de manter os valores de CIM baixos após a submissão ao tratamento térmico, demonstrando assim efetividade e potencial aplicação como bioconservante.

CONCLUSÕES. A aplicação das enzimas em suco de laranja reconstituído contaminado com *A. acidoterrestris* demonstrou ser eficaz, pois após 48 h de incubação em três diferentes temperaturas, já houve a eliminação da população microbiana inicial. Portanto, papaína e bromelina são enzimas com ação antibacteriana em *A. acidoterrestris*. A técnica de microencapsulamento foi eficaz na proteção da atividade antibacteriana da papaína, caracterizando esse composto com grande potencial de aplicação como bioconservante em produtos ácidos, sendo necessários maiores estudos sobre estabilidade e características sensoriais quando aplicado ao alimento.

Palavras-chave: Papaína, Bromelina*, Alicyclobacillus*, suco de laranja, polímeros, bioconservante.

ARTICLE 1

Antibacterial activity of Papain and Bromelain on *Alicyclobacillus* spp.

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ABSTRACT

Alicyclobacillus spp. are spore forming bacteria that are often related to the deterioration of acidic products such as beverages and citrus juices. After the process of industrial pasteurization, the spore produced by the bacteria can germinate and the microorganism can grow, causing sensory abnormalities in the product. Alternative biopreservatives, such as the antimicrobial compounds, are of considerable importance to the food industry. Papain and bromelain are proteolytic enzymes derived from papaya and pineapple, respectively. These enzymes are widely used in medicine and in the pharmaceutical and food industries, but while some studies have described their antibacterial action, no studies of the *Alicyclobacillus* spp. exist. The aim of this study was to analyze the antibacterial effect of papain and bromelain on *Alicyclobacillus* spp. through 1) determining minimum inhibitory and bactericidal concentration (MIC and MBC); 2) determining the death time curve of the micro-organism in the presence and absence of enzymes; 3) investigating the enzymatic mechanism on the microorganism. The antibacterial activity of enzymes in combination with nisin was also evaluated. The results showed that for the *A. acidoterrestris* strain, the MIC of papain was 0.98 µg/mL and the MBC was 3.91 µg/mL, while the MIC of bromelain was 62.5 µg/mL and the MBC was 250 µg/mL. The concentration of 4XMIC for both the enzymes was sufficient to eliminate 4 logs of the micro-organism after 24 hours of incubation. Through the use of enzyme inhibitors specific for cysteine proteases, it was found that the antibacterial activity of papain and bromelain is not related to its proteolytic activity, but may be related to other activities, such as amidse and esterase. The synergistic activity of the enzymes revealed an fractional inhibitory concentration (FIC) level of 0.16. Combination with nisin revealed an FIC of 0.25 for papain and 0.19 for bromelain, indicating synergism between both compounds. The application of enzymes in reconstituted orange juice contaminated with *A. acidoterrestris* was found to be effective, as after 48 h of incubation, at three different temperatures, the initial microbial

population was eliminated. This study showed that the enzymes papain and bromelain have an antibacterial effect on *A. acidoterrestris*.

KEY-WORDS: Papain, Bromelain*, Alicyclobacillus*, orange juice.

1. INTRODUCTION

Alicyclobacillus spp. are Gram-positive, thermophilic, acidophilic, non-pathogenic micro-organisms that have the ability to form spores. They are present in soil and are often related to the deterioration of acidic products such as citrus juices and beverages (Goto, 2003; Goto et al., 2002; Matsubara et al., 2002; Silva et al., 1999; Silva and Gibbs, 2001).

The ability of this micro-organism to survive high temperatures and low pH is attributed to two main factors: the composition of its membrane, where cyclic fatty acids are found, and its ability to form spores (Chang and Kang, 2004). Although this bacteria is not pathogenic, its ability to produce undesirable odors and flavors in products such as citrus juices, isotonic drinks, iced tea and tomato extracts is a major economic concern for the food processing industry (Chang and Kang, 2004; Walker and Phillips, 2005).

Several studies have identified a number of species isolated from industrialized natural citrus juices, such as *Alicyclobacillus acidocaldarius, Alicyclobacillus pomorum, Alicyclobacillus acidophilus* and *Alicyclobacillus herbarius*. However, *Alicyclobacillus acidoterrestris* is the most important for this type of deterioration, as it is capable of producing compounds such as 2-methoxyphenol (guaiacol) and 2.6-dibromophenol, which are the main compounds responsible for alterations to the odor and flavor of juice. Such changes have been described as an odor and a medicinal or antiseptic taste (Chang and Kang, 2004; Durak et al., 2010; Goto et al., 2002; Matsubara et al., 2002).

Heat treatments, such as pasteurization, are used in the orange juice concentrate industry to inactivate pathogenic and spoilage micro-organisms. However, the spore of *A. acidoterrestris* can survive such heat treatment, germinate, grow and thus cause the juice to deteriorate after reconstitution (Savaş Bahçeci et al., 2004). An alternative to heat treatment is the application of natural antimicrobial agents to food (Bevilacqua et al., 2008; Burt, 2004).

Nisin is an example of an antimicrobial compound used as a food preservative. It is a bacteriocin produced by *Lactococcus lactis* subsp. *Lactis,* which displays a wide variety of inhibitory effects on Gram-positive bacteria. Several studies have reported its effect as an inhibitor of the growth of *A. acidoterrestris* in acidic drinks (Bevilacqua et al., 2008; Peña et al., 2009; Ruiz et al., 2013; Yamazaki et al., 2000). Its use, however, is limited, due to the relatively high cost of extraction and purification and also because of the reduction in bactericidal activity in complex food substrates (Ross et al., 2003).

Papain is an important peptidase extracted from papaya (*Carica papaya*). It has a high proteolytic capacity, hydrolyzing large proteins into small peptides and amino acids. Some studies have described the antibacterial activity of papain and other papaya extracts against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Salmonella* Typhimurium*, Staphylococcus aureus* and *Proteus vulgaris* (Eshamah et al., 2014; Osato et al., 1993).

Da Silva et al. (2010), evaluated the toxic and mutagenic potential of papain and also identified its antioxidant activity. The result was negative for toxicity and mutagenicity tests, while the peptidase displayed protective activity against oxidative stress caused by H_2O_2 in strains of *Escherichia coli*.

Bromelain is a proteolytic enzyme derived from pineapple (*Ananas comosus*), and is a member of the *Bromeliaceae* family. Some studies have demonstrated that bromelain has an antimicrobial effect, as well as displaying helminthic activity against gastrointestinal nematodes (Rowan et al., 1990). Bromelain is considered to be a food supplement and is available to the general public in health food stores and pharmacies

in the US and Europe. It can be absorbed in the human intestine without degradation and without its biological activity decreasing. Several "*in vitro*" and "*in vivo*" studies have demonstrated that bromelain has low toxic and mutagenic potential (Castell et al., 1997; Chobotova et al., 2010; Pavan et al., 2012).

Papain and bromelain are widely used in the food, medicine, and pharmaceutical industries and in technical procedures for clinical and laboratory tests (Dutta and Bhattacharyya, 2013; Hale et al., 2005). Although some studies of the antibacterial effects of papain and bromelain on a number of species of bacteria have been performed, the action of these enzymes in bacteria of the genus *Alicyclobacillus* spp. has not been evaluated. The aim of this study was to evaluate the antibacterial activity of the proteolytic enzymes papain and bromelain in some species of *Alicyclobacillus* spp., with a focus on A*. acidoterrestris*.

2. MATERIALS AND METHODS

2.1 Microbial strains and enzymes

Alicyclobacillus spp. used were obtained from the Brazilian Collection of Microorganisms in the Environment and Industry (CBMAI), located at the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas – CPQBA / UNICAMP – São Paulo - Brazil, and were composed of the following reference species: *A. acidoterrestris* 0244^{T}; *A. hesperidium* 0246 ^{T}; *A. acidiphilus* 0247^{T}; *A. cycloheptanicus* 0297^{T} *A. acidocaldarius* 0298^{T}.

The enzyme papain (from *Carica papaya*, ≥ 3 U/mg, Enzyme Commission Number 3.4.22.2) and bromelain (from pineapple stem, 3 U/mg, Enzyme Commission Number 3.4.22.32) used in all experiments and the nisin (from *Lactococcus lactis*, 2.5%) used in the antimicrobial combinations were obtained commercially from Sigma-Aldrich® (Sigma Chemicals, St. Louis, MO). The papain was produced in Switzerland and the bromelain in Indonesia. To evaluate the homogeneity of commercial papain

and bromelain, the enzymes were submitted to dialysis using dialysis membranes with a 14,000 cut-off (Sigma-Aldrich, St. Louis, USA) against distilled water for 12 h at 4° C. The dialyzed enzymes were lyophilized and applied to denaturing electrophoresis in 10% polyacrylamide gel, SDS-PAGE (Laemmli, 1970). The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Papain and bromelain activities were evaluated using azocasein as substrate as described by Crawford (1987) in the absence or presence of one of two inhibitors: cysteine protease inhibitor, leupeptin (Sigma Chemicals, St. Louis, MO) at the concentration of 1 and 100 µg/mL, and a protease inhibitor cocktail, containing AEBSF, aprotinin, bestatin, E-64, EDTA (Sigma Chemicals, St. Louis, MO, code P2714) at the concentration of 1000 µg/mL

2.2 Determination of inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for each enzyme were determined in 96-well microdilution plates (TPP®, Switzerland), following the NCCLS M7-09 methodology (2012). For each enzyme serial dilutions in broth *Bacillus acidoterrestris* medium - BAT (Deinhard et al., 1987) were performed, giving concentrations ranging from 0.49 to 1000 µg/mL. Next, 5 µL of vegetative cells suspension was added after standardization with the McFarland Scale in 10^8 CFU/mL, followed by 1:10 dilution. The volume of the culture in each well was 100 µL and the initial inoculum level was 10^4 CFU/mL. Assays were performed individually for each species. The 96-well plate was incubated at 45° C for 24 h. Following this, wells turbidity was observed visually. The minimum inhibitory concentration was the lowest concentration resulting in growth inhibition as defined by visual observation. The minimum bactericidal concentration was determined by subculturing of 10 µL from each negative well to the surface of a BAT agar plate which was incubated at 45° C for 24 h. The assays were performed in triplicate.

2.3 Death time curve

Prior to experimentation, the lower limit of bacterial detection was determined as previously described by the NCCLS (2012). The lower limit of bacterial detection was 50 CFU/ml. The death time curve test was performed for papain and bromelain in *A. acidoterrestris*. The enzymes were added to tubes containing 5 mL of BAT broth, in concentrations equivalent to 4 x MIC, 2 x MIC, 1 x MIC, 0.5 x MIC and 0.25 × MIC. A positive control, where the enzyme was not added to the BAT broth, was performed. Subsequently a standardized cell suspension was added to each tube, and all tubes were incubated at 45° C for 24 h. Aliquots of 20 µL were removed from each tube at 0, 3, 6, 9, 12 and 24 h of incubation, diluted in 0.9% saline and plated on surface in BAT agar. The plates were incubated at 45° C for 24 h. The assay was performed in triplicate.

2.4 Evaluation of enzymatic activity and antibacterial activity against *A. acidoterrestris* of papain and bromelain after different treatments

The effect of different treatments (pasteurization at 80º C for 10 min., boiling at 100º C for 5 minutes, dialysis against water for 24 h, reduction by addition of 1 mM β-mercaptoetanol, enzyme inhibition by addition of 1 µg/mL and 100 µg/mL leupeptin, and 1000 µg/mL protease inhibitor cocktail) on the enzymatic activity and antibacterial activity against *A. acidoterrestris* of bromelain and papain were evaluated. Enzyme preparations without any treatment were used as controls. All assays were performed in triplicate.

2.5 Antibacterial combinations

The checkerboard method for evaluating antibacterial combinations was performed using 96-well plates to obtain the fractional inhibitory concentration index (FIC) of bromelain in combination with papain (Schelz et al., 2006).Tests were also

conducted by combining nisin with both enzymes being studied. Assays were performed in different combination concentrations based on the MIC results of each compound, namely: 15.68 to 0.008 µg/mL for papain, 1000 to 0.49 µg/mL for bromelain and 125 to 1.95 µg/mL for nisin for both tests. After combination, a suspension of 5 x 10^4 CFU/mL of *A. acidoterrestris* was added to each well. The plates were incubated at 45° C for 24 h. The FIC indices were calculated as: FIC = FIC_A + FIC_B, where FIC_A = combined MIC_A / MIC_A only, and FIC_B = combined MIC_B / MIC_B only. The results were interpreted as synergism (FIC ≤ 0.5), addition (0.5 ≤ FIC ≤ 1), indifference (1 <FIC ≤ 4), or antagonism (FIC> 4). The experiment was performed in triplicate (Schelz et al., 2006).

2.6 Application of enzymes in reconstituted orange juice

Assays were performed using commercially obtained concentrated orange juice (±65°Brix), which was previously analyzed to ensure the absence of *Alicyclobacillus* spp. The juice was then reconstituted with sterile water at a concentration of approximately 11°Brix, pH 4.0 and placed in 24-well plates (TPP®, Switzerland). The enzymes were then added to the juice, and tests conducted with papain and bromelain, and a combination of the two enzymes. The three trials were performed at concentrations of 1xMIC, 4xMIC and 8xMIC. After the addition of enzymes the *A. acidoterrestris* was added of a 24 h broth culture in BAT, giving a final concentration in each well of 5 x 10^4 CFU/mL. The plates were then incubated, with tests performed with incubation at temperatures of 28° C, 35° C and 45° C. After 24 h, 48 h and 72 h each well of the suspension was diluted and plated in BAT agar and incubated at 45° C for 24 h, after the colonies was enumerated. All assays were performed in triplicate.

2.7 Statistical analysis

The mean values and standard deviations were calculated using Excel (Microsoft Corp, EUA). The data from the test of the application of enzymes to

reconstituted orange juice was analyzed by analysis of variance (ANOVA) using Assistat 7.7 beta (Campina Grande, Paraiba, Brazil). Tukey's test was used to compare the means of the treated groups. Statistical significance was set at $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of papain and bromelain for each strain are shown in Table 1. Papain was the most effective, with an inhibitory concentration varying from 0.98 µg/mL to 3.91 µg/mL for the five species of *Alicyclobacillus* evaluated. The same was true for bactericidal activity, where the concentrations varied among the strains from 3.91 µg/mL to 15.60 µg/mL, being the equivalent to approximately four times the MIC required to eliminate the evaluated strains.

Bromelain also demonstrated good inhibitory activity for the species, varying from 15.62 µg/mL to 62.50 µg/mL, except for the *A. acidocaldarius*, where the inhibitory concentration was 500 µg/mL. The MBC of bromelain for the strains varied from 62.50 µg/mL to 1000 µg/mL.

It is a relatively common practice to evaluate the antibacterial activity of fruit or plant extracts containing papain and bromelain. For example, Osato et al. (1993) evaluated the antibacterial action of a mixture of pulp and seeds of unripe papaya and found it active at the concentration of 500 µg/mL against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Proteus vulgaris, Salmonella* Typhimurium, *Klebsiella pneumoniae, Enterobacter cloacae* and *Bacillus subtilis.* Dutta and Bhattacharyya (2013) evaluated the antimicrobial activity of an aqueous extract of pineapple leaves which was active against strains of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The aqueous extract inhibited 70 to 95% of microbial growth and its MIC varied from 1.65 to 4.95 µg/mL. Considering that these studies

were conducted using crude extracts, a series of different small and large molecules can be responsible for the antibacterial activity. To the best of our knowledge, this is the first report where the antibacterial activity appears to be related to papain and bromelain activities. To confirm this possibility, firstly the commercial enzymes were submitted to dialysis to eliminate possible contaminants with small molecular weight. Subsequently, the enzymes were submitted to a SDS-PAGE, which revealed a single protein band in both cases (data not shown).

Table 2 shows the results of enzymatic activities and MIC and MBC values obtained for both enzymes after different treatments. After dialysis, no difference was observed in the enzyme activity, MIC and MBC. Also, no effect on the enzyme activity, MIC and MBC values were observed after reduction of enzymes with β-mercaptoethanol. When the enzymes were submitted to pasteurization and boiling, both enzyme and antibacterial activities were drastically reduced. The presence of an inhibitor specific for cysteine proteases (leupeptin) or a protease inhibitor cocktail, had a strong effect on the capability of the enzymes to hydrolyze the substrate azocasein, but barely affected the antibacterial activity. These data suggest that the antibacterial action of the enzymes on *A. acidoterrestris* is not due to their proteolytic activity, but that it depends on the native structure of these proteases. Both papain and bromelain are cysteine proteases and are strongly inhibited by specific inhibitors such as leupeptin and E64 (Schröder et al., 1993, Sawano et al., 2008). However, papain and bromelain have a fairly broad specificity. Papain, for example, has endopeptidase, amidase, and esterase activities (Kimmel and Smith, 1954). The three-dimensional structure of the papain-leupeptin complex has already been determined by X-ray crystallography and indicates that leupeptin contacts only the S-subsite of the papain active site and not the S'-subsite (Schröder et al., 1993). Considering the latter analysis and the results found in this paper, it seems reasonable to suppose that the antibacterial activity of papain is not due to its proteolytic activity, but that it can be related to other enzymatic actions, such as amidase and esterase activities. The

structure of bromelain has not been solved so far, but it is generally assumed that its three-dimensional structure is very similar to that of papain (Sawano et al., 2008). For this reason, it is possible to extend this interpretation also to bromelain.

The marked reduction in the antibacterial activity after pasteurization, with bacterial growth 64 times greater in the case of papain and eight times greater in the case of bromelain, suggests that for an industrial application of these enzymes against *Alicyclobacillus spp,* studies are required for improving its thermal stability. Other ways of using the enzymes against *Alicyclobacillus* spp include the use of other forms of sterilization such as filtration before the addition of enzyme to the final product.

3.2 Death time curve

The effects of bromelain and papain on the death time curves of *A. acidoterrestris* are shown in Figure 1. In Fig. 1A, it can be seen that the papain in a concentration of 4XMIC (3.91 µg/mL), after 12 h incubation at 45° C, caused a 2 logs reduction in the initial bacterial concentration, and that after 24 h of contact time all the starting concentration of the inoculum was inactivated until the limit of detection. When compared to the control, where the enzyme is not added, *A. acidoterrestris* concentration, from 4 initial logs to approximately 8 logs after 24 h. It can be seen that after 12 h, all the tested concentrations of papain were sufficient to inactivate more than 1 log, and after 24 h the concentration of 4 x MIC inactivated to the limit of detection.

In Figure 1B, where bromelain was added, the situation was somewhat different. After 12 h incubation at 45° C, all concentrations tested were able to reduce the initial concentration by at least 1 log, however, the concentration of the inoculum was only maintained or reduced after 24 h of incubation at the concentrations 1xMIC (62.50 µg/mL) and 2xMIC (125 µg/mL) only. After 12h the lower bromelain concentrations showed an exponential growth of inoculum, until 24 hours of incubation. After 24 h of incubation the 4xMIC (250 µg/mL) concentration, as in the case of papain, was able to

eliminate all the initial concentration of inoculum, demonstrating its bactericidal activity after 24 h.

3.4 Antibacterial combinations

Figure 2 shows the isobolograms of the synergistic interactivity between the enzyme and nisin in *A. acidoterrestris*. The first isobologram (A) shows the interactivity between papain and bromelain, where the FIC index was 0.16, characterized as synergism between these substances. As isobologram (B) shows, there was also synergism between papain and nisin, with an FIC index was 0.25. The same occurred between nisin and bromelain (isobologram C), where the FIC index was 0.19.

Interactivity between nisin and other compounds have been evaluated by Ruiz et al., (2013), who found synergistic interaction between nisin and an extract of the *Piper aduncum* plant (*Piperaceae* family), using a chloroform fraction, and Razavi Rohani et al., (2011) who also identified the synergistic activity of nisin with essential oil of garlic (*Allium sativum*) against *Listeria monocytogenes*.

3.5 Application of enzymes in reconstituted orange juice

Table 3 shows the results of experiments using reconstituted orange juice as a growth medium for *A. acidoterrestris*. Through this test it is possible to evaluate the application of the antibacterial activity of the enzymes in the processing of orange juice. Three incubation temperatures were evaluated: 28° C, 35° C and 45° C. A temperature of 45° C is optimal for the growth of *A. acidoterrestris,* and lower temperatures were evaluated taking into account the ambient temperature surrounding the juice after reconstitution and bottling. The incubation times used to evaluate the efficacy of the enzymes were 24 h, 48 h and 72 h.

It was observed that the control, to which no antibacterial substance was added, showed considerable growth (above 5 logs) at all evaluated temperatures. At 24 h and 48 h of incubation, there was no significant difference between the temperatures,

however, at 72 h of incubation at 28° C a decline in population was observed, representing the early stage of cell death.

All the concentrations of papain tested were significantly different from control, except for the concentration of 1xMIC (0.98 µg/mL). The concentrations of 4xMIC (3.92 µg/mL) and 8xMIC (7.84 µg/mL) showed values that were significantly different from the values found for 1xMIC and at 28° C, these concentrations were sufficient to reduce the population to the limit of detection after 72 h. At 35° C with 8xMIC there was no significant difference from the values found for 4xMIC. At 45° C, after 48 hours of incubation, the concentration of 8xMIC inactivated the entire population of *Alicyclobacillus*.

All the concentrations of bromelain measured differed from the control. At a temperature of 28° C and 35° C, the concentration of 8xMIC of bromelain (500 µg /ml) was more effective than the concentration of 8xMIC of papain, and was able to eliminate all cells of *A. acidoterrestris* after incubation for 48h.

The combination of the two antimicrobial enzymes was also evaluated. A concentration obtained by testing antimicrobial combinations of 1xMIC (0.12 µg/mL of papain with 15.62 µg/mL of bromelain) produced a significant reduction compared to the control at temperatures of 28° C and 35° C only in the time periods evaluated. The concentrations of 4xMIC and 8xMIC showed a significant difference from the control at all temperatures evaluated, and the concentration of 4xMIC (0.48 µg/mL of papain with 62.48 µg/mL of bromelain) was able to eliminate *Alicyclobacillus* after 72 h of incubation at all studied temperatures. The concentration of 8xMIC (0.96 µg/mL of papain with 124.96 µg/mL of bromelain) eliminated the population after 48 h for all the tested temperatures.

4 CONCLUSION

The enzymes papain and bromelain showed effective inhibitory and bactericidal activity at low concentrations, against the species of *Alicyclobacillus* that were

evaluated, except for *A. acidocaldarius*. The low values of MIC and MBC found for *A. acidoterrestris*, which is the most related to the deterioration of citrus juices, is a very important factor for the industry. For both enzymes, the concentration 4XMIC was sufficient to eliminate 4 logs of this micro-organism until the limit of detection after 24 hours of incubation at 45° C. It was found that the antibacterial activity of the enzymes on *A. acidoterrestris* is not related to their proteolytic action, but can be related to its amidase and esterase activities. Synergistic interaction between both enzymes was proven, as well as the interaction of both enzymes with nisin, a fact relevant to the industry, as the use of combinations of compounds may be favorable due to factors such as the cost and availability of these compounds. The tests applied in reconstituted orange juice demonstrated efficacy and the potential for use of the enzymes in the final product due the fact that it could eliminate the initial population of *A. acidoterrestris* at different temperatures. The application of papain and bromelain in reconstituted orange juice as preservatives against *A. acidoterrestris* remains a possibility for the future and requires larger studies of the stability and sensory characteristics of the juice added to these enzymes.

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CAPTIONS:

Table 1

Table 1 – Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Papain and Bromelain for *Alicyclobacillus* spp.

Table 2

Table 2 – Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) of Papain and Bromelain for *A. acidoterrestris* following treatment applied to evaluate enzymatic action.

Table 3

Table 3 – Growth of *A. acidoterrestris* in reconstituted orange juice with the addition of the enzymes Papain, Bromelain and a combination of both in accordance with the methodology of antimicrobial combinations. Comparison of temperature (28 °C, 35 °C and 45 °C) and time (24 h, 48 h and 72 h) of incubation. Initial inoculum concentration (time 0 h): 4 logs.

Figure 1

Figure 1 – Death time curve of *A. acidoterrestris* for: A) Papain (MIC 0.98 µg/mL) and B) Bromelain (MIC 62.50 µg/mL). (- -) Limit of detection; (♦) Control; (■) 0.25xMIC; (▲) 0.5xMIC; (x) 1xMIC; (∗) 2xMIC and (●) 4xMIC.

Figure 2

Figure 2- Isobolograms exhibiting synergistic interactivity in *A. acidoterrestris* between: A) Papain and Bromelain (FIC=0.16); B) Papain and Nisin (FIC=0.25) and C) Bromelain and Nisin (FIC=019). (•) The lines result in highly concave curves, characterizing synergism between the compounds (FIC index <0.5).

Table 1

Species	Papain		Bromelain	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
A. acidoterrestris 0244^{T}	0.98	3.91	62.50	250
A. hesperidium 0246 ^{T}	1.95	7.81	31.25	125
A. acidiphilus 0247 ^{T}	1.95	7.81	62.50	125
A. cycloheptanicus 0297^{T}	1.95	7.81	15.62	62.50
A. acidocaldarius 0298^{T}	3.91	15.62	500	1000

Table 2

Treatments	Papain				Bromelain	
	Proteolytic activity (%)	**MIC (µg/mL)**	**MBC µg/mL)**	**Proteolytic activity (%)**	**MIC (µg/mL)**	**MBC (µg/mL)**
No treatment	100	0.98	3.91	100	62.50	250
Dialysis	97	0.98	3.91	99	62.50	250
β-mercaptoetanol	95	1.95	3.91	98	62.50	250
Pasteurization (80 °C, 10 min)	10	62.50	1000	8	500	1000
Heat denaturation (100 °C, 5 min)	0	250	500	0	500	>1000
Leupeptin	3	1.95	3.91	95	62.50	250
Protease inhibitor cocktail	5	1.95	3.91	96	62.50	250

Table 3

Concentration	28 °C			35 °C			45 °C		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control	5.77± 0.51^{Ba}	7.81 ± 0.30^{Aa}	6.28 ± 0.53^{Ba}	6.88 ± 0.26^{Ba}	8.05 ± 0.03^{Aa}	8.39 ± 0.48^{Aa}	7.55 ± 0.45^{Ba}	8.75 ± 0.39^{Aa}	8.52 ± 0.55^{Aa}
Papain 1xMIC	4.41± 0.49^{Ba}	5.76 ± 0.36^{Ab}	4.45 ± 0.55^{Bb}	6.58 ± 0.41^{Aa}	5.08 ± 0.06^{Bb}	5.49 ± 0.68^{Bb}	6.57 ± 0.65^{Aa}	7.02 ± 0.39^{Ab}	7.38 ± 0.52^{Aa}
Papain 4xMIC	2.30± 0.40^{Abc}	0.97 ± 0.04^{Bde}	0.00 ± 0.00^{Cd}	1.66 ± 0.44^{Bc}	3.38 ± 0.12^{Ac}	1.88 ± 0.59^{Bd}	2.35 ± 0.35^{Abc}	2.34 ± 0.23^{Ad}	2.39 ± 0.26^{Ac}
Papain 8xMIC	1.17± 0.11^{Abc}	0.87 ± 0.09^{Abe}	0.00 ± 0.00^{Bd}	0.87 ± 0.18^{Bc}	2.78 ± 0.25^{Ac}	1.44 ± 0.59^{Bd}	1.92 ± 0.43^{Abcd}	0.00 ± 0.00^{Be}	0.00 ± 0.00^{Bd}
Bromelain 1xMIC	2.07± 0.89^{Abc}	2.59 ± 0.78^{Ac}	2.43 ± 0.58^{Ac}	2.29 ± 0.39^{Cbc}	4.17 ± 0.78^{Abc}	3.27 ± 0.22^{Bc}	3.20 ± 0.80^{Bb}	4.08 ± 0.46^{Abc}	4.29 ± 0.54^{Ab}
Bromelain 4xMIC	1.93± 0.89^{Abc}	0.91 ± 0.09^{Be}	0.00 ± 0.00^{Cd}	1.61 ± 0.50^{Ac}	0.89 ± 0.15^{Ad}	0.00 ± 0.00^{Be}	1.83 ± 0.42^{Abcd}	0.77 ± 0.31^{Be}	0.00 ± 0.00^{Bd}
Bromelain 8xMIC	1.53 ± 0.71^{Abc}	0.00 ± 0.00^{Be}	0.00 ± 0.00^{Bd}	0.77 ± 0.31^{Ac}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ae}	0.44 ± 0.14^{Ad}	0.00 ± 0.00^{Ae}	0.00 ± 0.00^{Ad}
Combination 1xMIC	2.75 ± 0.48^{Ab}	2.40 ± 0.53^{Acd}	2.18 ± 0.12^{Ac}	3.58 ± 0.27^{Bb}	5.55 ± 0.63^{Ab}	5.64 ± 0.73^{Ab}	6.20 ± 0.70^{Ba}	7.68 ± 0.58^{Aab}	7.57 ± 0.24^{Aa}
Combination 4xMIC	1.72 ± 0.64^{Ab}	0.93 ± 0.09^{Ae}	0.00 ± 0.00^{Bd}	1.89 ± 0.39^{Ac}	0.82 ± 0.23^{Bd}	0.00 ± 0.00^{Be}	2.74 ± 0.52^{Ab}	2.44 ± 0.29^{Ad}	2.42 ± 0.28^{Ac}
Combination 8xMIC	1.10 ± 0.13^{Ac}	0.00 ± 0.00^{Be}	0.00 ± 0.00^{Bd}	0.82 ± 0.23^{Ac}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ae}	0.91 ± 0.60^{Acd}	0.00 ± 0.00^{Be}	0.00 ± 0.00^{Bd}

Values with different upper case letters in the same line are significantly different ($p < 0.05$). Values with different lower case letters in the same column are significantly different ($p < 0.05$).

Figure 1

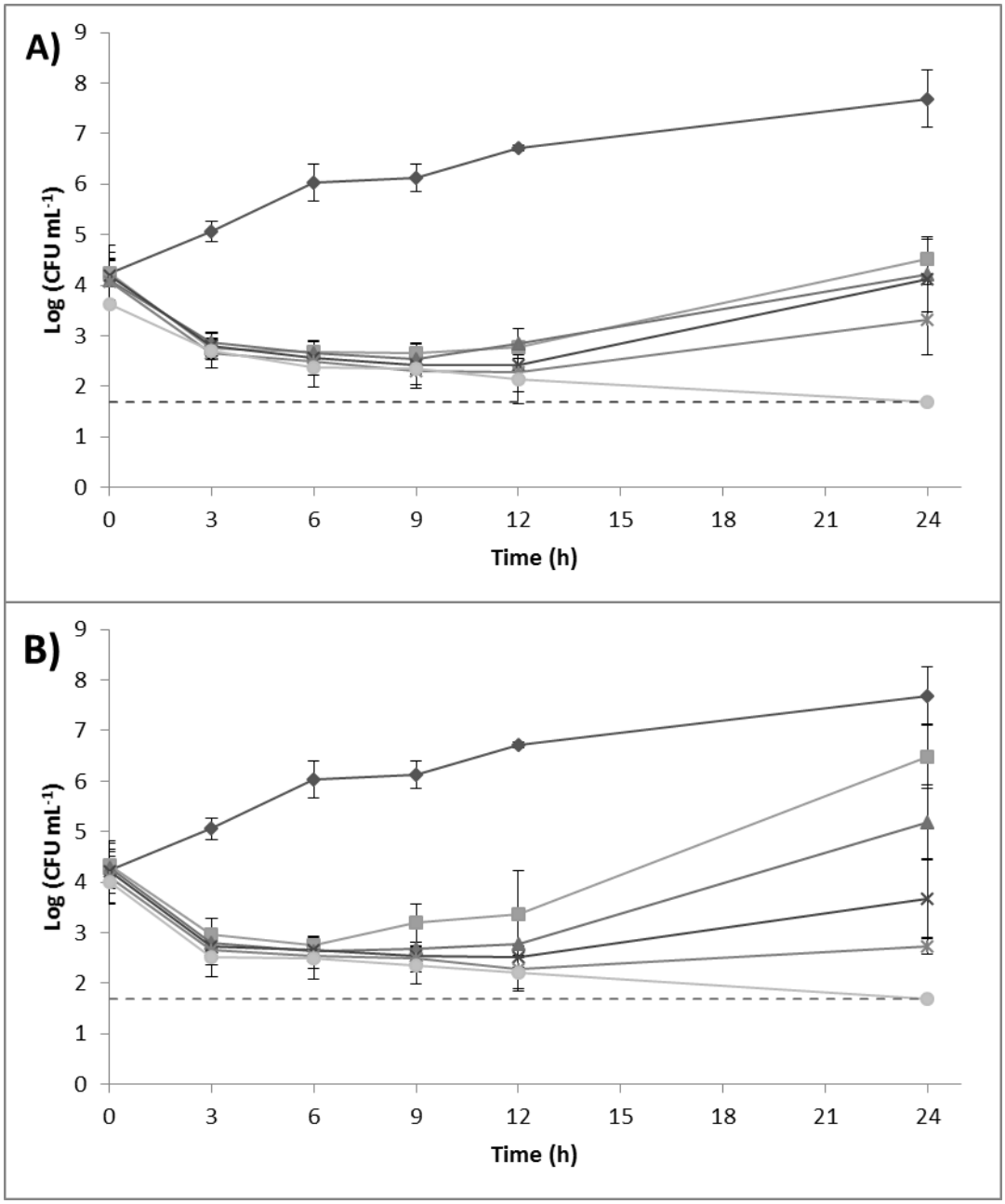
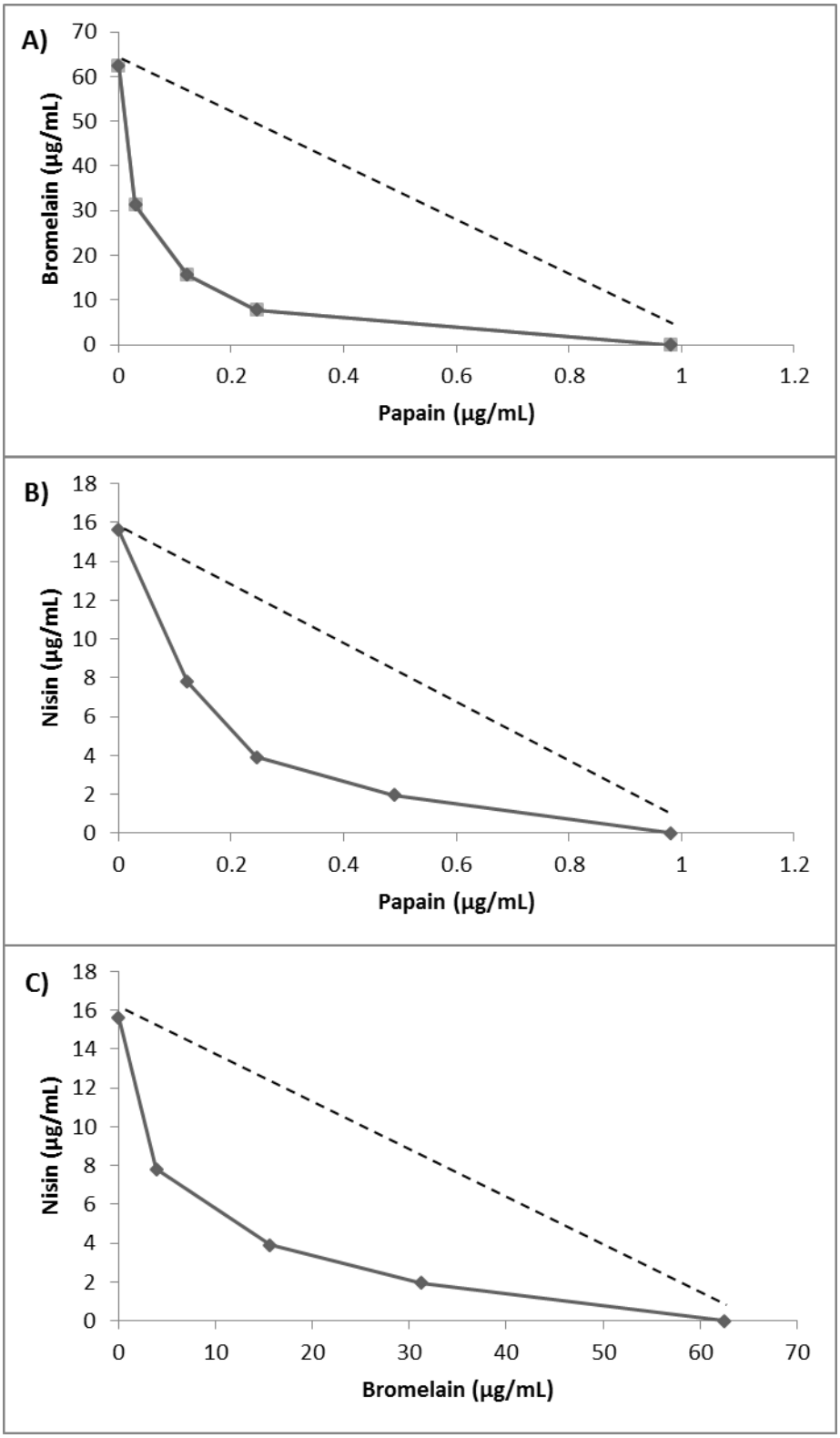


Figure 2


A)
Bromelain (µg/mL)
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
Papain (µg/mL)
B)
Nisin (µg/mL)
18
16
14
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1
1.2
Papain (µg/mL)
C)
Nisin (µg/mL)
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
Bromelain (µg/mL)

ARTICLE 2

Microencapsulated Papain and Bromelain used as biopreservatives against *Alicyclobacillus* spp.

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ABSTRACT

The *Alicyclobacillus* genus consists of thermoacidophilic bacteria that spoil foods with low pH, such as citrus juices, changing their odor and taste. The thermal processes used with such products fail to eliminate these spore-forming bacteria. Different methods for their control have been studied, including the use of proteases such as papain and bromelain, which have demonstrated effective antibacterial action against this genus. However, their application is limited as such action is drastically diminished when the enzymes are subjected to high temperatures. The objective of the present study was to microencapsulate these enzymes with alginate and chitosan and evaluate their action following thermal processes. Microencapsulation was performed by spray drying and the compounds were subjected to high temperatures. Their inhibitory and bactericidal activity against five different species of *Alicyclobacillus* was then evaluated. The microcapsules were characterized by Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC) and Infrared Spectroscopy (FT-IR). The microencapsulation of the particles was evidenced. The results showed that papain microencapsulated with chitosan or alginate maintained low minimum inhibitory concentration values after submission to heat treatment, demonstrating its effectiveness and potential application as a biopreservative.

KEY-WORDS: bromelain, papain, alginate, chitosan, biopreservative.

3. INTRODUCTION

Alicyclobacillus spp. are gram-positive, thermophilic, acidophilic, non-pathogenic bacilli with the ability to form spores. They are present in the soil and are often related to the deterioration of acidic products such as citrus drinks and juices (Silva et al.,1999; Silva and Gibbs, 2001; Goto et al., 2002; Matsubara et al., 2002; Goto, 2003).

The ability of this bacterium to survive at high temperatures and low pH is attributed to two main factors: the composition of its membrane, where cyclic fatty acids are found, and its ability to form spores. Although it is not pathogenic, its ability to produce undesirable odors and flavors in products such as citrus juices, isotonic drinks, iced teas and tomato extracts represents a major economic problem for the food industry (Chang and Kang, 2004; Walker and Phillips, 2005).

Alicyclobacillus acidoterrestris is the most important species associated with this type of deterioration. It is capable of producing compounds such as 2-methoxyphenol (guaiacol) and 2,6-dibromophenol, which are the main compounds responsible for changes in the odor and taste of juice. These changes are described as a medicinal or antiseptic odor and taste (Chang and Kang, 2004; Durak et al., 2010; Goto et al., 2002; Matsubara et al., 2002).

Thermal treatments are employed in the concentrated orange juice industry to deactivate many pathogenic and deteriorating microorganisms. *A. acidoterrestris* spores, however, can survive these thermal treatments, germinate, grow and deteriorate juice after reconstitution (Savaş Bahçeci et al., 2004).

Preservation methods other than thermal treatments, such as the application of natural antimicrobial agents in food, are therefore desirable. These provide a potential strategy for inhibiting a wide variety of microorganisms without risk to the health of consumers (Bevilacqua et al., 2008; Burt, 2004).

As described by Anjos et. al. (2016), the enzymes papain and bromelain have demonstrated efficacy as antibacterial agents against strains of *A. acidoterrestris* inoculated in orange juice, and are therefore potential preservative agents for this product. The addition of these enzymes to juice after reconstitution and pasteurization eliminated four logs of the microorganism after 48 hours of contact with the product.

Papain is an important peptidase extracted from papaya (*Carica papaya*). It has a high proteolytic capacity and hydrolyzes large proteins in small peptides and amino acids. Studies have demonstrated the antibacterial activity of papain and other papaya extracts against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Salmonella* Typhimurium*, Staphylococcus aureus* and *Proteus vulgaris* (Eshamah et al., 2014; Osato et al., 1993).

Bromelain, which is also a proteolytic enzyme, is derived from pineapple (*Ananas comosus*), which is also a member of the *Bromeliaceae* family. Studies have demonstrated its antimicrobial effect, as well as its anthelmintic activity against gastrointestinal nematodes and its anti-*Candida* activity (Rowan et al., 1990).

Papain and bromelain are widely used in the food, medicine and pharmaceutical industries and in clinical-laboratory analysis technical procedures (Dutta and Bhattacharyya, 2013; Hale et al., 2005). Although the antibacterial action of papain and bromelain against *A. acidoterrestris* in orange juice is effective, the addition of these compounds as preservatives must occur after the pasteurization of the juice, as the heat treatment interferes with the structure of the enzyme and decreases its antibacterial effect.

Spray drying is an important and widely applied technique in the pharmaceutical and food industries. It is a viable method for the preparation of microparticles, being a single step process, and is an alternative to microencapsulation methods that use various steps and organic solvents. Atomization occurs through compressed air, which breaks up the liquid fed to the nozzle into small droplets, and the solvent in these droplets is rapidly evaporated by the high temperatures. The technique can be applied to heat sensitive substances and hydrophilic and hydrophobic polymers can be used. It can also be easily adapted to industrial scale production (Guiunchedi et. al., 2002).

Natural biopolymers are potentially effective encapsulating agents due to their biodegradability and biocompatibility. Chitosan is a natural cationic polysaccharide obtained from the N-deacetylation of chitin, while alginate is a natural anionic polysaccharide of the

linear copolymers α-L-guluronate and β-D-mannuronate. Both are suitable for use in food applications (Coppi et. al., 2002).

The objective of the present study was to microencapsulate papain and bromelain enzymes with alginate and chitosan and to verify the effectiveness of this technique in maintaining antibacterial activity against *Alicyclobacillus* spp. after heat treatment.

4. MATERIALS AND METHODS

4.1 Microbial lineages, enzymes and polymers

The lineages of the species of *Alicyclobacillus* spp. used were obtained from the Coleção Brasileira de Micro-organismos de Ambiente e Indústria (the Brazilian Environmental and Industrial Microorganisms Collection) (CBMAI), situated in the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (the Pluridisciplinary Center for Chemical, Biological and Agricultural Research) (CPQBA/UNICAMP), São Paulo, Brazil. The following reference species were studied: *A. acidoterrestris* CBMAI 0244^T; *A. hesperidum* CBMAI 0298^T; *A. acidophilus* CBMAI 0247^T; *A. cycloheptanicus* CBMAI 0297^T; and *A. acidocaldarius* CBMAI 0299^T.

The papain and bromelain enzymes, as well as the alginate and chitosan polymers, were obtained commercially from Sigma-Aldrich® (Sigma Chemicals, St. Louis, MO). The papain was produced in Switzerland and the bromelain was produced in Indonesia.

4.2 Microencapsulation of enzymes

The alginate and chitosan microparticles containing the papain and bromelain enzymes were produced by the spray-drying technique. First, the alginate (Sigma-Aldrich, viscosity of 250 cps at 2 %) was dissolved in distilled water under constant stirring at a concentration of 2 % w/v. The enzyme (papain or bromelain) was dispersed in an aqueous alginate solution to give an enzyme-polymer ratio of 1:2. The final suspension was spray-dried in a LM-MSD 1.0 Mini model spray dryer with a 0.7 mm nozzle, under the following conditions: air inlet temperature 100 °C, air outlet temperature 50-60 °C and a spray flow rate of 1.0 L/h. The microparticles of the enzymes prepared with chitosan (Sigma-Aldrich, low molar mass, viscosity 20,000 cps) were obtained from the dissolution of chitosan in 1% acetic acid at a concentration of 2 % w/v, under stirring. The enzymes were dispersed in chitosan acetic acid solution, resulting in an enzyme-polymer ratio of 1:2. The atomization conditions were the same as those described for alginate.

The efficiency of the process was calculated by dosing the proteins of the particles before and after the microencapsulation in a UV-Vis spectrophotometer at 280 nm, and the microparticles obtained were dissolved in sterile distilled water at pH 4.0 and filtered in 0.45 µm membrane before analysis.

4.3 Characterization of microparticles

4.3.1 Scanning Electron Microscopy (SEM)

For morphological analysis, the microparticles were fixed on stubs, coated with gold and observed in a Shimadzu SS-550 scanning electron microscope. The particles were sized using the SizeMeter 1.1 (2001) software package and the polydispersion index (PDI) was calculated using the following equation:

$$PDI = \frac{SD^2}{D^2}$$

where: PDI = polydispersion index; D = mean diameter of the particles and SD = standard deviation of the mean diameter of the particles.

4.3.2 Differential scanning calorimetry (DSC)

Thermal analysis of the microparticles was performed using Differential Scanning Calorimetry (DSC) (Shimadzu DSC50). Prior to analysis, the samples were kept in a desiccator for three days. Approximately 5 mg of the powder was used for analysis, with the sample sealed in an aluminum vessel, and an empty vessel used as a reference. The heating rate used was 20°C min^{-1} under a nitrogen atmosphere.

4.3.3 Infrared Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR, Shimadzu IR AFFINITY-1, from 400-4000 cm^{-1}, 1 cm^{-1} resolution) was used to investigate the interactions between the polymers (alginate and chitosan) and the enzymes. The particles were dispersed in spectroscopic grade potassium bromide (KBr). The pellet was then formed by compressing the sample at 150 MPa and the FT-IR spectra were collected in transmission mode.

4.4 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of microencapsulated enzymes after heat treatment

The minimum inhibitory concentration (MIC) for enzymes, polymers, enzymes after heat treatment and for microencapsulated enzymes was determined by microdilution in 96-

well plates (TPP®, Switzerland), in accordance with the methodology of the National Committee for Clinical Laboratory Standards (CLSI, 2012). Initially, each microencapsulated enzyme underwent heat treatment at 80 °C for 10 minutes, simulating the pasteurization process used in the production of orange juice. Serial dilutions in BAT broth were carried out for each enzyme, obtaining concentrations ranging from 0.49 to 1000 µg/mL. Next, 5 µL of the microorganism suspension was added after standardization with the McFarland Scale at 10^8 CFU/mL, followed by 1:10 dilution. The tests were carried out individually for each species. The 96-well plate was incubated at 45°C for 24 h. After this period, the inhibitory concentration, defined as the lowest concentration that resulted in the inhibition of bacterial growth, was read. The minimum bactericidal concentration was determined from the subculture of 10 µL of each negative well on the surface of a BAT agar plate incubated at 45°C for 24 h. The assays were performed in triplicate.

3 RESULTS AND DISCUSSION

3.1 Obtaining and characterization of the microparticles

Spray drying is an attractive technique for preparing microparticles because it is a rapid particle formation process, which involves only the preparation of a solution of the compound with the polymer, resulting in a homogeneous distribution of those particles. The encapsulation efficiency was determined by the ratio of the actual enzyme content to the theoretical content. Assuming that all the enzyme present in the solution that was atomized was trapped in microparticles, the theoretical enzyme content was 33.3% (w / w) for both types of microparticles. The protein dosage showed that the actual content of papain in the alginate microparticles was 22.9%, which corresponds to an encapsulation efficiency of 68.76% and for the chitosan microparticles the actual papain content was 26, 18% and the encapsulation efficiency was 78.62%. The actual content of bromelain in the alginate microparticles was 23.22% and for the chitosan microparticles was 20.80%, corresponding to a process efficiency of 69.73% and 62.46%, respectively.

Scanning electron microscopy of the particles identified their uneven distribution and irregular structure, and found that the compounds were larger prior to microencapsulation (Figure 1). The particles produced after the microencapsulation were smaller and spherical, highlighting changes in their morphological characteristics. The mean microparticle sizes obtained were 3.73 (±1.43) µm for papain and alginate and 3.3 (±1.65) µm for papain and chitosan, with PDI of 0.15 and 0.23, respectively. For the bromelain microparticles, the size of the particles was 2.35 (± 1.26) µm with alginate and 2.8 (± 1.33) µm with chitosan, with

PDI of 0.29 and 0.22, respectively. All the microparticles obtained had a PDI above 0.1, which therefore characterizes all polydisperse particles.

Thermal analysis of the microparticles (Figure 2) revealed that the endothermic peaks correlated with the loss of water associated with hydrophilic groups of polymers, whereas the exothermic peaks resulted from the degradation of polyelectrolytes from the capsules due to dehydration and depolymerization reactions, most probably the partial decarboxylation of the protonated carboxylic groups and the oxidation reactions of the polyelectrolytes (Soares, 2004, Zhuriaan and Shokrolahi, 2004; Mimmo et al., 2005).

In general, the endothermic and exothermic peaks of the capsules were maintained, at similar temperatures, in the microencapsulated particles, especially in the enzyme particles combined with alginate.

Bromelain exhibited an endothermic peak at about 159°C, which disappeared after microencapsulation with alginate (Figure 2A). This may indicate that there was interaction between the polymer and the enzyme.

In the DSC results for the papain microparticles, an endothermic peak for the papain of around 215°C was observed. This was not present in the particles microencapsulated either with alginate (Figure 2C) or with chitosan (Figure 2D), which may indicate an interaction.

Chitosan exhibited an exothermic peak at about 320°C. However, when the microencapsulation process was applied, this peak occurred at a temperature below 300°C (Figures 2B and 2D), which may characterize an interaction with the encapsulating material. Vasconcellos et al. (2011) observed this same characteristic when microencapsulating papain with chitosan using cross-linking agents for application in biomedicine.

Figure 3 shows the results for the FT-IR analysis of the samples. Bromelain (Figures 3A and 3B) exhibited a band at around 1544 cm^{-1} which relates to the presence of C-N, as described by Soares et. Al. (2012). This band was not present in the capsules and appeared in the physical mixtures with both alginate and chitosan. It appeared in the compound microencapsulated with chitosan, but not the compound microencapsulated with alginate, thus agreeing with the results of the DSC analysis, where there was no evidence of interaction in the microencapsulation of bromelain with chitosan. Another band related to the presence of C-N appeared in the bromelain at 1265 cm^{-1}. It was present in the compound microencapsulated with chitosan, reaffirming that there may be no interaction between these two compounds.

Papain (Figures 3C and 3D) exhibited bands at around 1543 cm^{-1} and 1240 cm^{-1}, which refer to the amide II region and are attributed to C-N elongation (Moreno-Cortez et. al, 2015). The band at 1543 cm^{-1} was not present in the alginate or in the microencapsulated particle, but did appear in the physical mixture of these compounds, characterizing

interaction. The same occurred with the band at 1240 cm^{-1}, which was not present in the chitosan or the microencapsulated sample, but appeared in the physical mixture.

3.2 Determination of antibacterial activity

In Tables 1 and 2 are presented the minimum inhibitory concentration and the minimun bactericidal concentration, respectively, for the papain (with and without heat treatment), alginate, chitosan and for the papain with alginate and papain with chitosan after being submitted to heat treatment, against the five strains of *Alicyclobacillus* evaluated. It was observed that the papain exhibited excellent inhibitory activity against the evaluated strains, as described by Anjos et al. (2016). Although the polymers (alginate and chitosan) had some inhibitory effect, the MIC values for papain were much greater.

Gartika et. al. (2014) described the antibacterial activity of papain against *Streptococcus mutans*, the main bacterium responsible for the development of tooth decay. The inhibitory concentration found was 7.5%, whereas the bactericidal concentration was 15%. These values are considered relatively high in comparison with the antibacterial activity of papain against the strains of *Alicyclobacillus*, suggesting a different interaction between papain and this genus of bacteria.

After being subjected to heat treatment of 80°C for 10 minutes, simulating the pasteurization of orange juice, papain loses its inhibitory activity, as the MIC values increase. This suggests that its inhibitory activity may be related to the three-dimensional structure of the enzyme, which is lost after being subjected to heat. In the case of *A. acidoterrestris*, which is the species most significantly associated with the deterioration of orange juice, the MIC value is over 60 times greater. It is therefore necessary to increase the concentration of papain added to the juice, or to add the enzyme after the heat treatment, which may be unfeasible in industrial processing.

No effective antibacterial activity was observed after heat treatment and microencapsulation with alginate, with the MIC value increasing for all strains except for *A. acidoterrestris*. With this strain the MIC value remained low, being four times greater than when an enzyme not subjected to heat treatment was used. With papain microencapsulated with chitosan, however, the results demonstrated effectiveness in the microencapsulated form, with the MIC value remaining low after heat treatment for all strains evaluated.

The fact that microencapsulation of papain with alginate has an effect only on the A. acidoterrestris species can be explained by the different fatty acid composition of the plasma membrane between Alicyclobacillus species. Different species may present different concentrations of omega-cyclic fatty acids which are a characteristic of the genus, as reported by Chang and Kang (2004).

The results for the microencapsulation of bromelain with alginate and chitosan are set out in Tables 3 and 4. As with papain, bromelain loses its inhibitory power when submitted to the thermal processing stimulating the pasteurization of the juice, which makes the use of these two enzymes as biopreservatives non-viable. However, the microencapsulation of bromelain with alginate or chitosan did not exhibit satisfactory results for all the *Alicyclobacillus* species evaluated.

As with papain, most studies of the antibacterial activity of bromelain are aimed at the medical field, as this compound has immunomodulatory, anti-thrombotic and anti-inflammatory effects, among other properties (Maurer, 2001). Studies related to the microencapsulation of these compounds and their application in the food industry as antibacterial agents remain limited.

Praveen et. al. (2014) evaluated the antibacterial activity of bromelain against aerobic (*Enterococcus faecalis* and *Streptococcus mutans*) and anaerobic (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*) periodontal pathogens and identified a MIC ranging from 2 mg/mL to 31,25 mg/mL among the strains evaluated. The inhibition of *Alicyclobacillus* strains in the present study was much higher, but the microencapsulation process resulted in unsatisfactory inhibition values.

The microencapsulation of papain with alginate, papain with chitosan and bromelain with alginate was characterized by physical analysis. The results of the microparticles obtained against the strains of *Alicyclobacillus* revealed that the microencapsulation process with chitosan was effective for papain, guaranteeing its inhibitory and bactericidal activity after undergoing heat treatment and demonstrating its potential application as a biopreservative for food.

4 CONCLUSION

The enzymes papain and bromelain evaluated in this work exhibited significant antimicrobial potential against strains of *Alicyclobacillus*. The microencapsulation of papain with chitosan is an alternative for their application as biopreservatives in acidic products that are submitted to pasteurization, such as orange juice.

Microencapsulation of papain with both alginate and chitosan was evidenced through physical analysis. For bromelain, only microencapsulation with alginate was evidenced. The results of the papain microencapsulated with chitosan demonstrated that the use of the microencapsulation technology was effective because after heat treatment the MIC value remained low for all the strains evaluated, which characterizes this process as an alternative for the use of this compound as a biopreservative. However, further studies on stability and sensory changes after the application of this compound in food products are necessary.

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CAPTIONS:

TABLE 1: Minimum Inhibitory Concentration (MIC) for Papain, the polymers and the microencapsualted compounds against *Alicyclobacillus* spp.

TABLE 2: Minimum Bactericidal Concentration (MBC) for Papain, the polymers and the microencapsualted compounds against *Alicyclobacillus* spp.

TABLE 3: Minimum Inhibitory Concentration (MIC) for Bromelain, the polymers and the microencapsualted compounds against *Alicyclobacillus* spp.

TABLE 4: Minimum Bactericidal Concentration (MBC) for Bromelain, the polymers and the microencapsualted compounds against *Alicyclobacillus* spp.

FIGURE 1: Scanning Electron Microscopy of the compounds used in the study, as well as the microencapsualted compounds. A) Papain (Mag. x200); B) Bromelain (Mag. x200); C) Alginate (Mag. x200); D) Chitosan (Mag. x400); E) Papain with Alginate (Mag. x1000); F) Papain with Chitosan (Mag. x1000); G) Bromelain with Alginate (Mag. x1000); H) Bromelain with Chitosan (Mag. x1000).

FIGURE 2: DSC curve of particles. A) Comparison between Bromelain and Alginate, B) Comparison between Bromelain and Chitosan; C) Comparison between Papain and Alginate com Alginate and D) Comparison between Papain and Chitosan.

FIGURE 3: Espectro FT-IR of particles. A) Comparison between Bromelain and Alginate, B) Comparison between Bromelain and Chitosan, C) Comparison between Papain and Alginate and D) Comparison between Papain and Chitosan.

TABLE 1

Strain	Papain (µg/mL)	Alginate (µg/mL)	Chitosan (µg/mL)	Papain 80°C - 10 min. (µg/mL)	Papain with Alginate (µg/mL)	Papain with Chitosan (µg/mL)
0244	0.98	500	62.5	62.5	3.91	1.95
0298	1.95	500	500	31.25	62.5	7.81
0247	1.95	500	100	62.5	125	3.91
0297	1.95	500	500	62.5	500	1.95
0299	3,91	500	1000	125	1000	15.62

TABLE 2

Strain	Papain (µg/mL)	Alginate (µg/mL)	Chitosan (µg/mL)	Papain 80°C - 10 min. (µg/mL)	Papain with Alginate (µg/mL)	Papain with Chitosan (µg/mL)
0244	3.91	1000	1000	1000	62.5	7.81
0298	7.81	500	1000	31.25	125	31.25
0247	7.81	1000	1000	62.5	250	3.91
0297	7.81	1000	1000	1000	>1000	7.81
0299	15.60	500	1000	500	1000	15.62

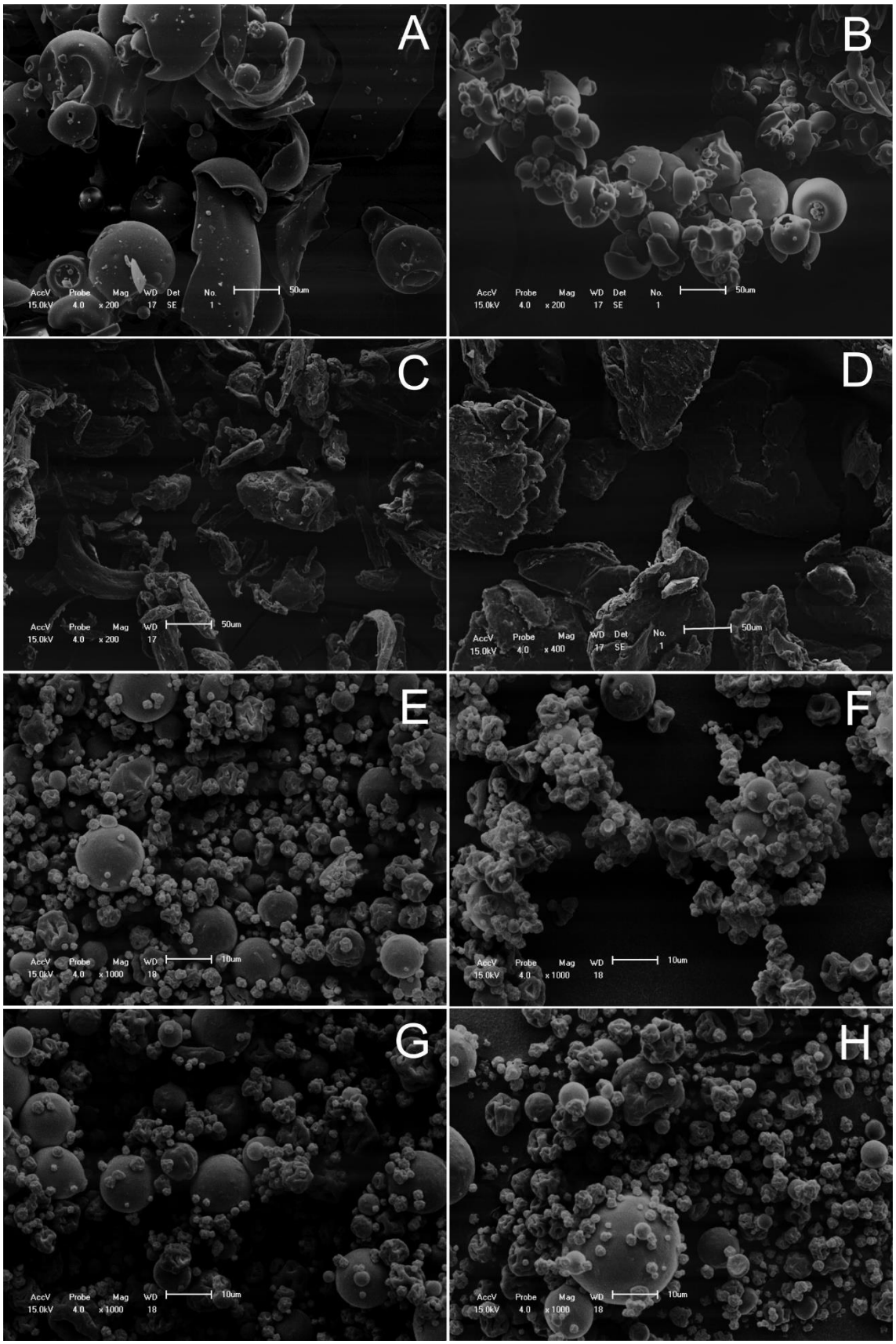
TABLE 3

Strain	Bromelain (µg/mL)	Alginate (µg/mL)	Chitosan (µg/mL)	Bromelain 80°C - 10 min. (µg/mL)	Bromelain with Alginate (µg/mL)	Bromelain with Chitosan (µg/mL)
0244	62.5	500	62.5	500	1000	500
0298	72.92	500	500	1000	1000	1000
0247	62.5	500	100	1000	1000	1000
0297	15.62	500	500	1000	1000	1000
0299	500	500	1000	1000	1000	1000

TABLE 4

Strain	Bromelain (µg/mL)	Alginate (µg/mL)	Chitosan (µg/mL)	Bromelain 80°C - 10 min. (µg/mL)	Bromelain with Alginate (µg/mL)	Bromelain with Chitosan (µg/mL)
0244	250	500	62.5	1000	1000	1000
0298	125	500	500	>1000	>1000	1000
0247	125	500	100	>1000	>1000	1000
0297	62.5	500	500	>1000	>1000	1000
0299	1000	500	1000	>1000	1000	1000

FIGURE 1


A
AccV Probe Mag WD Det No.
15.0kV 4.0 x200 17 SE 1
50um
B
AccV Probe Mag WD Det No.
15.0kV 4.0 x200 17 SE 1
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C
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15.0kV 4.0 x200 17
50um
D
AccV Probe Mag WD Det No.
15.0kV 4.0 x400 17 SE 1
50um
E
AccV Probe Mag WD
15.0kV 4.0 x1000 18
10um
F
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15.0kV 4.0 x1000 18
10um
G
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H
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15.0kV 4.0 x1000 18
10um

FIGURE 2

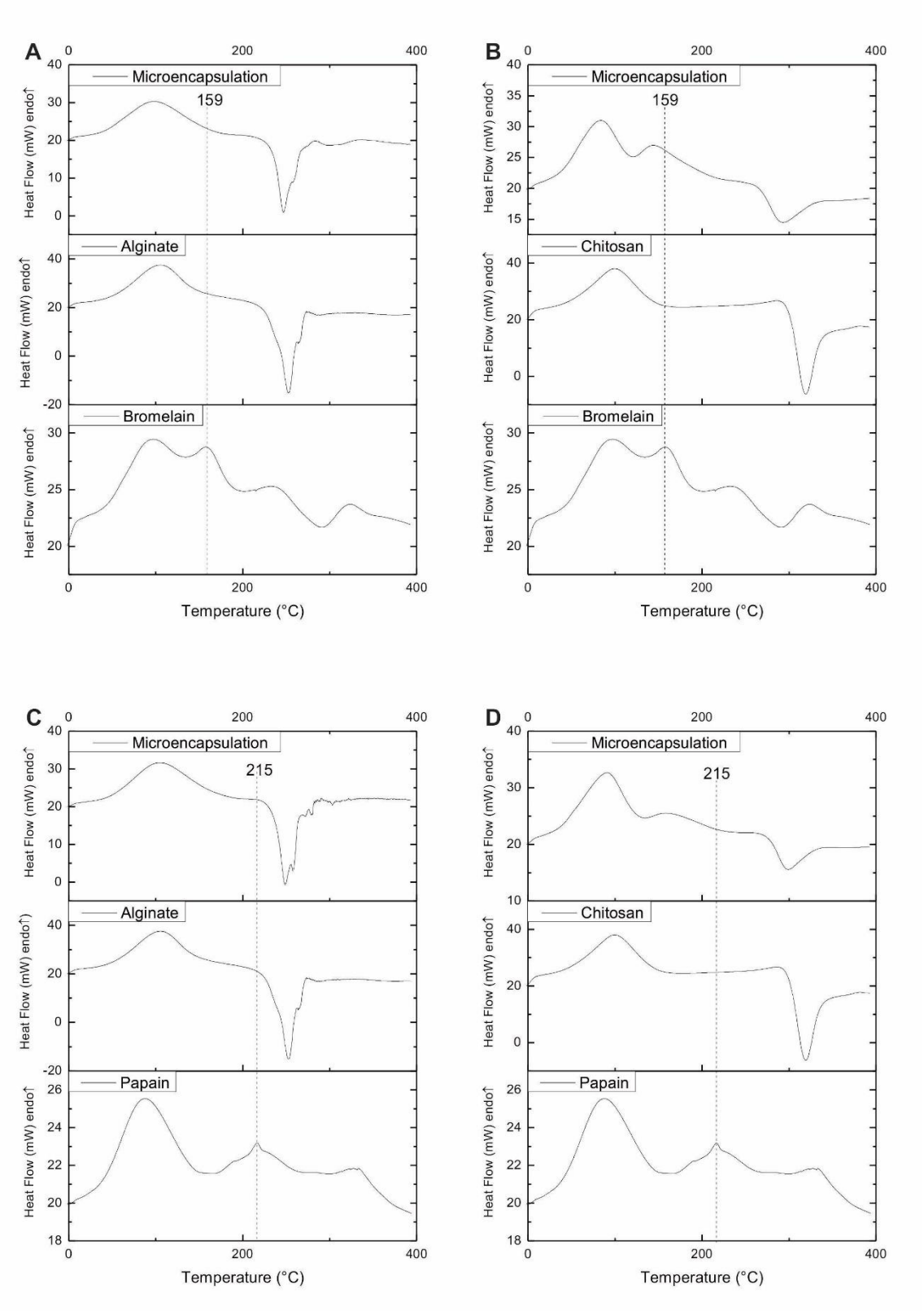
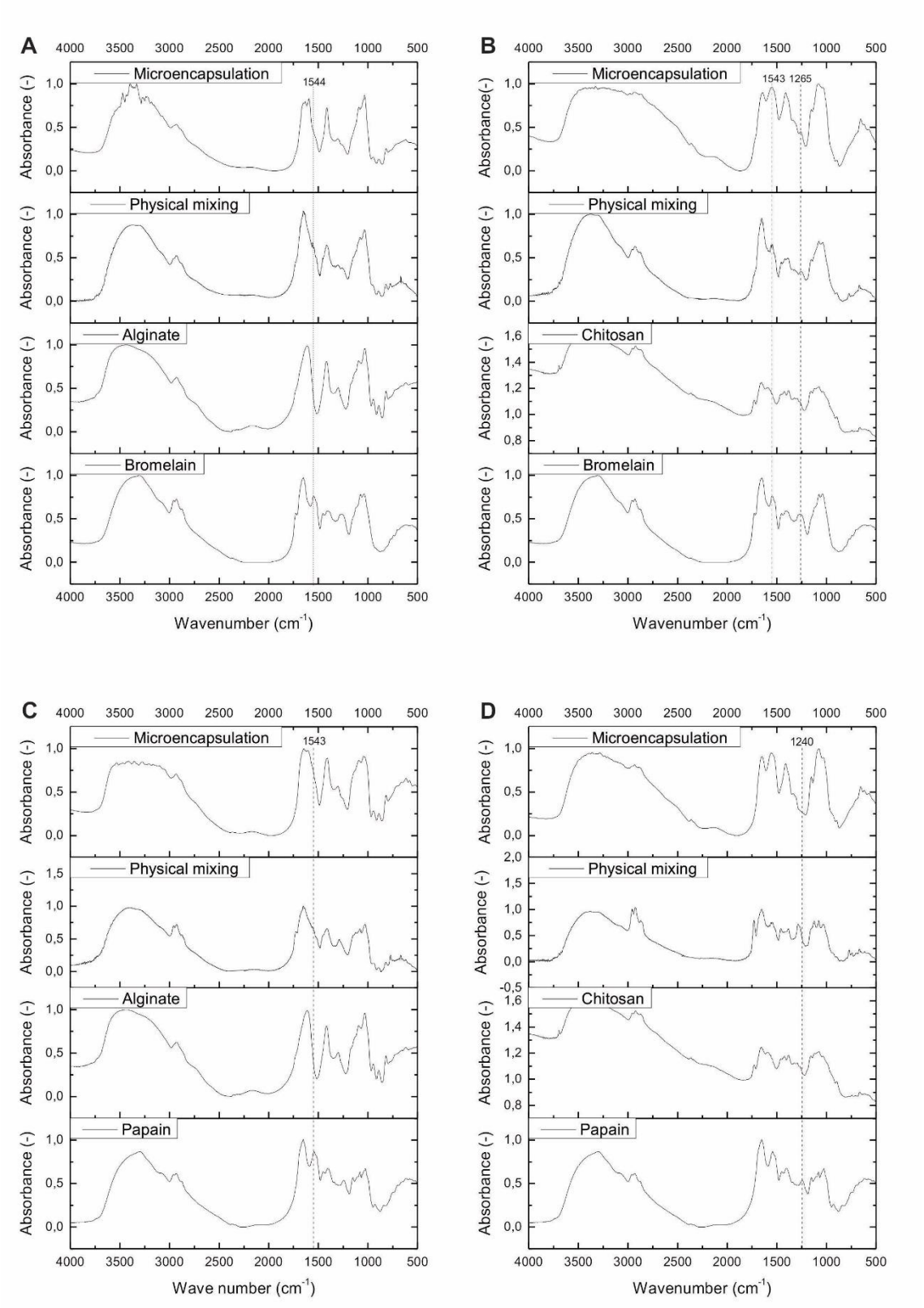

A
Microencapsulation
159
Alginate
Bromelain
B
Microencapsulation
159
Chitosan
Bromelain
C
Microencapsulation
215
Alginate
Papain
D
Microencapsulation
215
Chitosan
Papain
Heat Flow (mW) endo↑
Temperature (°C)

FIGURE 3


A
Microencapsulation
1544
Physical mixing
Alginate
Bromelain
Absorbance (-)
Wavenumber (cm-1)
B
Microencapsulation
1543 1265
Physical mixing
Chitosan
Bromelain
Absorbance(-)
Absorbance (-)
Wavenumber (cm-1)
C
Microencapsulation
1543
Physical mixing
Alginate
Papain
Absorbance (-)
Wave number (cm-1)
D
Microencapsulation
1240
Physical mixing
Chitosan
Papain
Absorbance (-)
Wavenumber (cm-1)